POLISH ACADEMY OF SCIENCES
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THE 24-HOUR RHYTHM OF ACTIVITY IN THE COMMON BUZZARD (*BUTEO BUTEO* L.) AND IN THE ROUGH-LEGGED BUZZARD (*BUTEO LAGOPUS* L.) IN DIFFERENT CONDITIONS OF LIGHTING

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Received 2 February, 1955

Besides the animals which live in stable ecological conditions and do not exhibit any periodicity (Demel 1949), all others show a distinct rhythm of activity (Katabuchow 1940). Many papers have been written on this theme. Research workers have tried to establish whether this rhythm is innate or caused by external conditions such as light, temperature and humidity (Kowalski 1949).

The lighting no doubt exerts a great influence upon the activity of animals. It is true that the need for food is the direct stimulus of their activity, and the necessity for rest causes their quiescence, but the distribution of these periods in the course of the day depends to a considerable extent on the changes in lighting.

The research on the 24-hour rhythm of activity in animals was initiated by S l o n a k e r (1907). His experimental animal was a rat placed in a cylinder rolling on a horizontal axis. S l o n a k e r established its activity to be greater at night.

Further studies on this problem were undertaken by S z y m a ń s k i (1914). He carried out experiments in conditions approximating to the natural and studied numerous animals belong-

ing to different classes. He has established that all animals may be divided into one phase animals which have one period of rest and one of activity within 24 hours, and many-phase animals which in the course of a day and night show several short alternating periods of quiescence and activity. The occurrence of one or more phases depends on the susceptibility of senses. Visual types are usually active all day long, olfactory types, being rather independent of light, show a many-phase rhythm. S z y m a ń s k i used a canary as his experimental animal among others. It was kept in permanent darkness for 73 days and preserved its 24-hour rhythm almost without change.

Interesting results were obtained by H. W a g n e r (1930) in his experiments on birds. His aim was to study not only the 24-hour rhythm, but also the annual rhythm during the spring and autumn migrations. He used for his experiments the following species of small song birds which travel by night:

Sylvia communis Lath. (Whitethroat) — 19 specimens
Erithacus rubecula L. (Robin) — 10 specimens
Erithacus cyanecula Wolf. — (Bluethroat) 2 specimens
Turdus philomelos Brehm — (Song thrush) 10 specimens
Turdus merula L. (Blackbird) — 8 specimens
Turdus musicus L. (Redwing) — 9 specimens

The results of Wagner's work may be summarized as follows:

1. The factors releasing the annual rhythm are unknown, although feeding with thyroid preparation may in some species bring about the migration unrest in periods of quiescence.

2. Beyond the period of migration the 24-hour cycle falls into the diurnal phase in which the bird is active, and the nocturnal phase in which it rests.

3. In the birds investigated during their migrations, unrest was noticed to appear in dark phases in which they are accustomed to sleep when not migrating.

4. If permanent darkness be applied the 24-hour rhythm is preserved for some time, then it lessens and disappears.

5. In case of applying permanent darkness the migrational unrest lasts 9—13 days.

6. By the application of 12-hour changes of illumination the bird's 24-hour rhythm may be disturbed, and a 12-hour rhythm forced upon it.

D r o s t (1934) carried out research on the activity of birds

during their spring and autumn migrations. He established greater nocturnal activity to exist at that period in which the birds beyond the period of migrations lead a diurnal life.

A s c h o f f (1953) drew the attention to the spreading of activity during the day in the bullfinch (*Pyrrhula pyrrhula* L.). He noticed that there occur in this bird two peaks of activity, one in the morning, the other at dusk. Both these maxima are shifted in the course of the year depending on the sunrise and the sunset. From the above the inference may be drawn that the evening maximum is provoked by the approach of dusk. In laboratories research on the activity is carried out by the so-called artificial day. Light is switched on and extinguished all of a sudden so that no dusk occurs. In such conditions the evening activity may be lacking. It occurs when the period of illumination is shorter than the proper length of the day at that time of the year. The evening unrest seems to be extinguished by the darkness falling all of a sudden. However, if the period of illumination is long enough, then in spite of the lack of dusk the maximum of the evening activity is quite distinct.

Further on Aschoff studied the behaviour of the bullfinch in conditions of perpetual lighting. He noticed that very strong light caused a quick abolishment of the 24-hour rhythm. When, however, less strong light was applied the periodicity of activity lasted for a long time differing in so much as both maxima appeared ever and ever earlier, so that e. g. the morning peak of activity occurred after 6—9 days at midnight. Constant darkness brought about a reverse phenomenon, that is the peak of activity occurred later and later.

All these experiments carried out on birds kept in captivity showed that light exerts a great influence upon their activity. The problem seemed interesting, how do birds behave which live in natural conditions and are not subject to rhythmic changes in light and darkness. Such conditions prevail in polar regions where for a certain time in winter the sun does not rise at all, and in summer instead it shines permanently over the horizon. The question arises whether the birds preserve the 24-hour rhythm in perpetual light of the polar summer, or acquire another rhythm of their activity, or lose the periodicity altogether.

Observations on the activity of different genera of birds at the periods of „bright nights" were undertaken by several research workers.

P a l m g r e n (1935) carried out observations on birds between 15th June and 10th July near the polar circle and also farther north from the 68° to 70° of northern latitude. Their quiescent period lasted from 6 p. m. to 11 p. m., during the rest of the day they were active. This proved that they preserved their 24-hour rhythm of activity with a rest period much shortened.

Similar results were obatined by M a r s c h a l (1938) in his observations at the Spitsbergen. He also established the existence in birds of a period of several hours' rest beginning at about 1 a. m. It falls at the time of the worst conditions of temperature, light and humidity. Marshall noticed also that at any sudden change in atmospheric conditions the birds also pass into a state of quiescence at other periods of the day.

F r a n z (1949) studied the rhythm of activity in birds in a place called Alakurti in Finland at 67° of northern latitude. In autumn and early spring the rhythm of activity is similar to that exhibited by birds in central geographical latitudes and depends on the course of the height of the sun. Late in spring, instead, and in summer during the bright northern nights certain differences are marked. The birds retire to rest about 8 p. m. and awake about midnight and sometimes before that time. Thus the period of activity is longer there than in central latitudes.

The present work deals with the activity in buzzards. In the experiments two specimens of the common buzzard (*Buteo buteo* L.) were used, marked Nos. 1 and 2, and one specimen of the rough-legged buzzard (*Buteo lagopus* L.).

This quantity was thought sufficient for the following reasons: 1) The results obtained were so pronounced and similar in general outlines for all the specimens that a repetition of experiments with a larger number of birds would have brought nothing new to the whole problem. 2) For technical reasons this quantity was also judged sufficient, as it was very difficult to acquire a larger number of buzzards tamed to an extent suitable for experiments of this sort. Besides, it would have been impossible to find accomodation for a larger number of birds, as well as to build more cages suitable for recording the activity of birds, and to install expensive meters to register their movements.

The experiments were divided into 8 series in which activity of the birds was studied at a normal 24-hour rhythm, constant illu-

mination, reversed rhythm, 16-hour rhythm, constant illumination, 36-hour rhythm, constant illumination and constant darkness.

The work was carried out in the Laboratory of Animal Psychology and Ethology of the Jagellonian University in Cracow, partly owing to a scientific scholarship of the Polish Academy of Sciences.

Thanks are due to Professor R. J. Wojtusiak for suggesting the theme and all his directions imparted in the course of the work.

THE METHOD

The research was carried out in a room isolated from daylight and illuminated by a 100-watt lamp in order to safeguard the animal against changes in the intensity of lighting. The lamp was placed at a distance of 1 m from the cage, but the distance between the lamp and the rod on which the bird used to perch amounted to about 2 m. The dimensions of the cage were: 1.55 × 1.15 × 0.80. In the room there was a thermometer. As the experiments lasted from November 1951 till April 1952, and from January till June 1953 the oscillations of temperature were rather great, 12°C to 23°C. The 24-hour amplitude, however, did not exceed 4°C.

The apparatus for recording the animal's movements (built according to a device of Prof. Dr R. J. Wojtusiak by Eng. W. Węglarski) was provided with 12 electromagnetic meters which were automatically put in operation by turns every hour. Each of them recorded the bird's movements within the given hour. In the apparatus there were also two dials with hands. One of them indicated the hour, the other the meter which was in operation at the given hour.

In the first two series of experiments the only moving thing was the perching rod. In the following series a moving floor was also applied. Both the floor and the perching rod were hung on springs. When the bird moved there occurred a stretching out of the springs and the blades placed on the rod and on the floor came into contact with the electric wire leading to the recording apparatus. When the electric current ran in the wire the cipher on the meter changed. The meter was read twice daily, at 8 a. m. and at 8 p. m.

When the 16-hour rhythm was applied there arose the need for changing the light during the night. The apparatus for an automatic switching off and on the light consisted of a clock and a toothed wheel connected with it, both connected by means of wiring with the lamp which illuminated the dark room. The dial of the clock was divided into 24 sectors denoting the hours. There was no hand, but the whole dial turned round. In order to switch on or off the light at the given hour special little tags were set in at the corresponding ciphers of the dial. As the little tags protruded above the dial they checked the corresponding tooth when the given cipher of the dial passed the toothed wheel and turned it by a certain angle, switching the light on or off.

The animals were fed with horse meat twice or three times a day at varying periods in order not to provoke an artificial amount of activity at

the time of feeding, which might have occurred if the hours of feeding were stable.

Each portion of food at one time amounted to about 100 g. This amount was quite sufficient, as the birds often left over a part of their food uneaten. During the whole period of experiments the birds were healthy and not fattened. After the experiments have been finished the birds were transferred to the Zoological Garden of Krakow.

RESULTS OF EXPERIMENTS

A normal 24-hour rhythm

The experiments were carried out for 17 days with the common buzzard specimen No. 1, for 5 days with specimen No. 2, and for 7 days with the rough-legged buzzard. Twelve hours of light and twelve hours of darkness occurred alternately. The light was switched on at 8 a. m. and extinguished at 8 p. m. The behaviour of all the birds of this series was alike. As soon as the room was illuminated their activity increased suddenly and distinctly. In the whole course of the period of illumination it was rather high although it varied in particular hours, these oscillations being quite considerable. However, the increase or reduction of activity was not connected with any definite part of the day. After the light has been extinguished the movement-activity of the birds decreased instantly and became very feeble during the night hours. Figs. 1, 2 and 3 represent the diagram of the mean 24-hour activity for each of the three specimens.

During this series of experiments with specimen No. 1 the meter No. 6 got often damaged and did not record the animal's movements. In order to have the data of the hours 6—7 a. m. and 6—7 p. m. arithmetic means of the numbers of movements in the preceding and the following hours were taken.

Perpetual lighting

In the experiments of this series each bird was subject to the influence of constant lighting that means its room was illuminated all the time long.

Specimen No. 1 kept its 24-hour rhythm during the first day after constant illumination had been applied, but its period of activity was shifted towards the night hours. It exhibited activity between midday and 10 p.m. (Fig. 4). During the following days

the bird passed from a 2-phase rhythm to that of many phases. In the course of 24 hours there occurred several periods of activity with intervening quiescent periods during which the buzzard was quite inactive (Fig. 5a, b). The number of periods of activity varied from 2 to 6. In the further course of experiments the many-phase rhythm disappeared, too, and there followed a more or less evenly spread activity over the 24 hours. This was accompanied by a general fall of the bird's movement-activity, that means the number of movements in each hour decreased (Fig. 6).

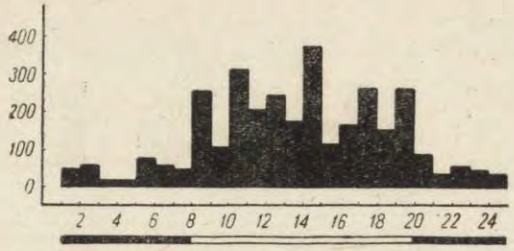

Fig. 1. Activity of the common buzzard (*Buteo buteo* L.) No 1 at a normal rhythm of lighting (12 hours of light and 12 hours of darkness). The black sector of the stripe under each diagram denotes the hours of darkness, the bright sector of the stripe means the hours of lighting

The buzzards No. 2 kept his 24-hour rhythm in perpetual light for four days (Fig. 7). On the fifth day there appeared the tendency of passing to a many-phase rhythm although the period of the animal's activity still occurred rather during the day hours, (Fig. 8). About the end of this series of experiments the activity became spread over the 24 hours, and at the same time it lessened considerably (Fig. 9).

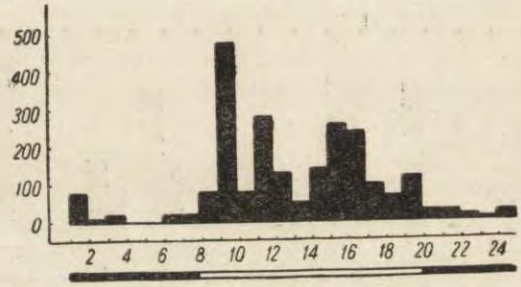

Fig. 2. Activity of the common buzzard (*Buteo buteo* L.) No 2 at a normal rhythm of lighting

The rough-legged buzzard exposed to constant illumination did not show

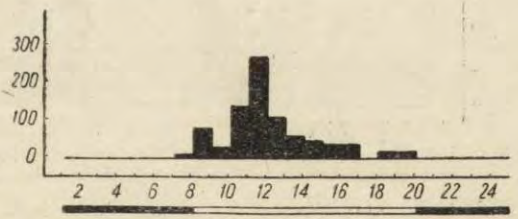

Fig. 3. Activity of the rough-legged buzzard (*Buteo lagopus* L.) at a normal rhythm of lighting

any 24-hour rhythm. From the very first day the bird passed to a many-phase rhythm which was maintained to the end of the series with the amount of activity gradually decreasing (Fig. 10a, b, c).

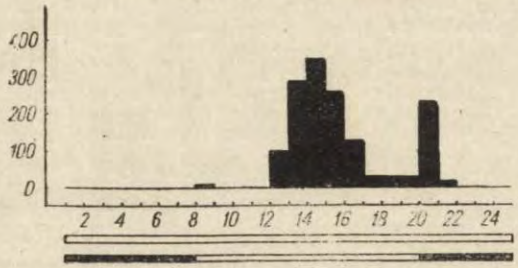

Fig. 4. Activity of the common buzzard (*Buteo buteo* L.) No 1 on the first day after application of constant lighting. The upper stripe under the diagram denotes lighting, the lower one means the changes of light and darkness in the normal rhythm

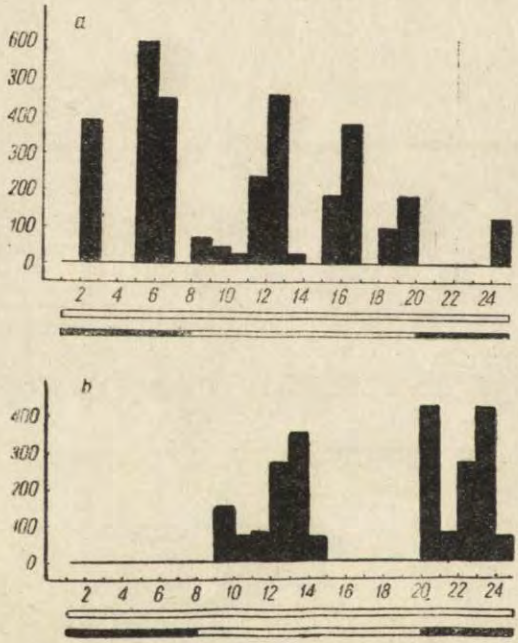

Fig. 5. The change from one-phase into many-phase rhythm of activity in the common buzzard (*Buteo buteo* L.) No 1 on: a — the fifth, b — the eighth day of constant lighting

A reversed 24-hour rhythm

In this series certain improvements in the apparatus were introduced at the study of the activity of the buzzard No. 1. In the preceding series only the rod on which the bird perched was mobile, now a moving floor was also applied. This enable the noting of almost all movements of the bird. In consequence the number of the bird's movements recorded in every hour was much higher than heretofore. This increase, however, should not be explained by a real growth of activity at this period. This change refers only to the specimen No. 1, because experiments with the two other buzzards were from the very beginning carried out through all the series by means of improved apparatus.

The experiments at a reversed 24-hour rhyth were performed on two

specimens only, namely on Nos. 1 and 2. In both animals a pronounced dependence of the 24-hour rhythm of activity on light was noticed. Immediately after the room had been illuminated the activity of the animals increased and lasted for the whole period of lighting. After the light had been extinguished the movement-activity lessend and was very feeble in the phase of darkness (Fig. 11 and 12).

In this series the periods of illumination occurred at night, and those of darkness during the day, being thus reverse to the normal 24-hour rhythm. In spite of that the birds became instantly accustomed to this change without showing any disturbances in the rhythm.

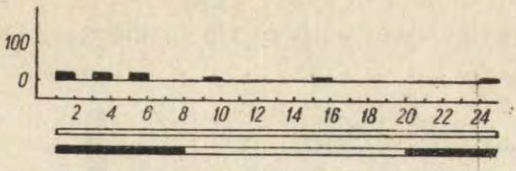

Fig. 6. Disappearance of the many-phase rhythm of activity in the common buzzard (*Buteo buteo* L.) No 1 after several days of constant lighting

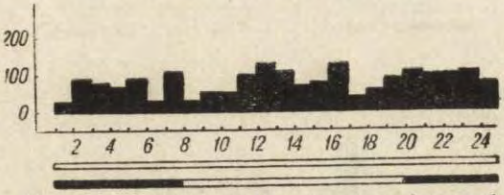

Fig. 7. Activity of the common buzzard (*Buteo buteo* L.) No 2 on the fourth day of constant lighting

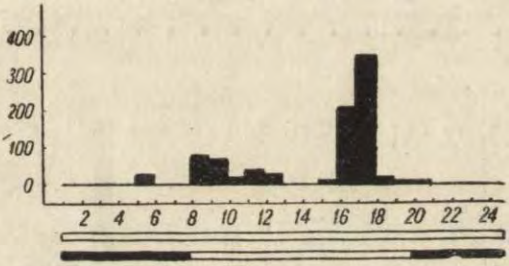

Fig. 8. Activity of the common buzzard (*Buteo buteo* L.) No 2 on the fifth day of constant lighting

A 16-hour rhythm

These experiments consisted in creating an artificial 16-hour day for the bird by applying of alternate 8 hours of illumination and 8 hours of darkness. The light was alternately, switched on and extinguished at 8 p.m.,

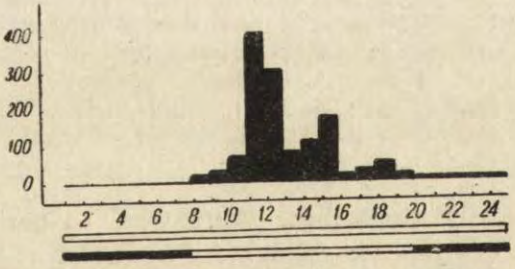

Fig. 9. Disappearance of the many-phase rhythm of activity in the common buzzard (*Buteo buteo* L.) No 2 after several days of constant lighting

4 a.m. and 12 a.m. Thus, two 24-hour periods covered three 16-hour periods. For each specimen the experiments lasted for ten 24-hour periods which comprised *thirteen* 16-hour periods.

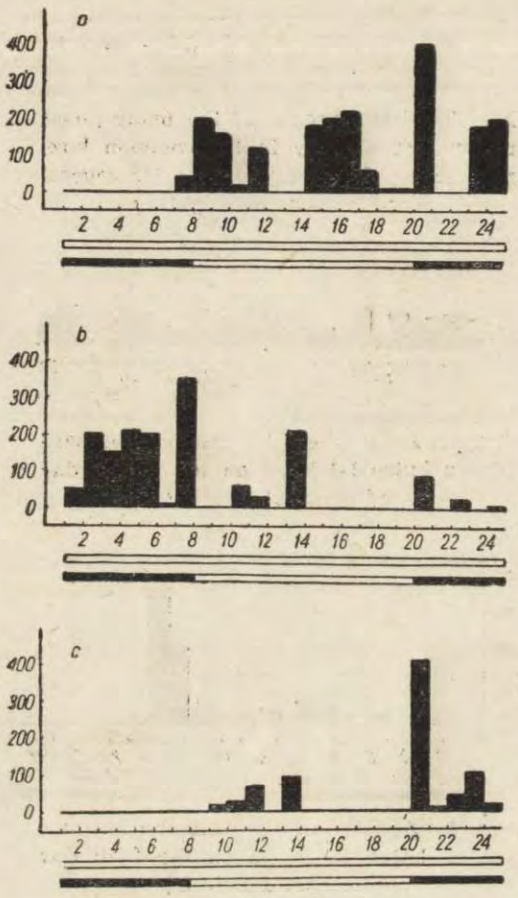

Fig. 10. Changes in the rhythm of activity in the rough-legged buzzard (*Buteo lagopus* L.) under the influence of constant lighting on: a — the first, b — the sixth, and c — the eighth day of constant lighting

From the very beginning the birds became accustomed to the changed rhythm of illumination. Their activity grew when the light was switched on, and lessened when extinguished, independent of the fact whether the period of illumination fell on the day or on the night. The animals behaved all alike, as it appears from Figs. 13a, b, 14a, b and 15a, b.

Perpetual lighting

In order to test whether the 16-hour rhythm would be preserved in stable conditions perpetual lighting was applied for several days. However, in no one of the three animals investigated any traces of the 16-hour rhythm have been noticed. The activity of the specimen No. 1 was spread all over the 24 hours and it was greatly diminished. There were no periods of perfect quiescence. A many-phase rhythm did not occur at all (Fig. 16 a, b).

The two other animals that is the common buzzard No. 2 and the roughlegged buzzard passed immediately to a many-phase rhythm, and kept it to the end of this series (Fig. 17 a, b and 18 a, b).

A 36-hour rhythm

The experiments consisted in applying a prolonged 36-hour period. The room was illuminated for 18 hours and dark for the other 18 hours. Two 36-hour periods covered three 24-hour periods. The changes in lighting and darkness occurred at 8 p.m., 2 p.m., 8 a.m., and 2 a. m. The experiments on each of the birds lasted for nine 24-hour periods which comprised six 36-hour periods.

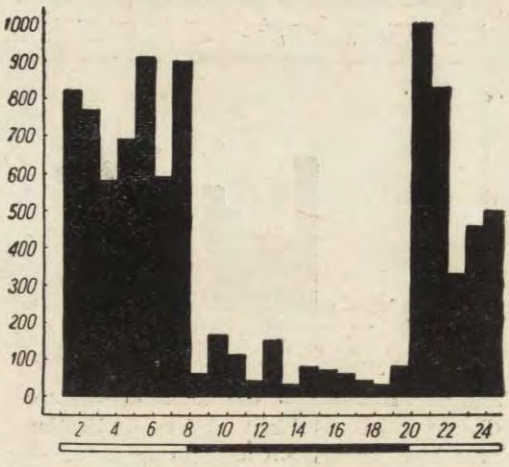

Fig. 11. Activity of the common buzzard (*Buteo buteo* L.) No 1 at a reversed rhythm of lighting

Much like in the preceding series the dependence of the buzzard on light was marked here also. The activity of the bird was great in phases of illumination, and small in darkness. In the period of darkness the activity of the birds was very low but evenly spread over all hours of this phase. During the period of illumination, instead, there occurred very great differences in their activity at the particular hours, so that this period of diurnal movement-activity was as if divided into several phases. This phenomenon of many-phase periodicity during the illuminated phases of the 36-hour rhythm occurred in each of the three specimens (Fig. 19, 20 and 21).

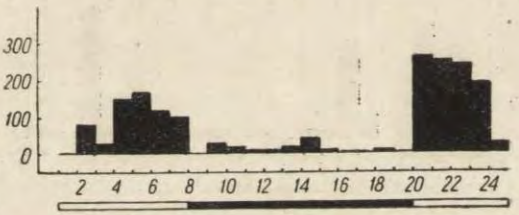

Fig. 12. Activity of the common buzzard (*Buteo buteo* L.) No 2 at a reversed rhythm of lighting

Perpetual lighting

While the experiments of this series were carried out the room was constantly illuminated all the time. The 36-hour rhythm was not preserved in stable conditions. It was replaced by a many-phase

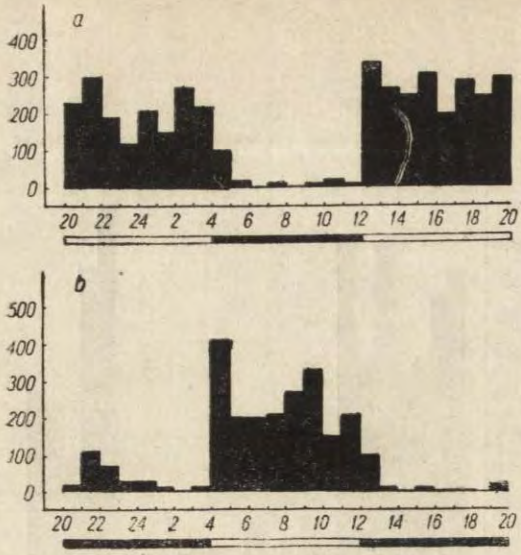

Fig. 13. Activity of the common buzzard (*Buteo buteo* L.) No 1 at the application of a 16-hour rhythm of lighting on: a — the third day of experiments, b — the fourth day of experiments

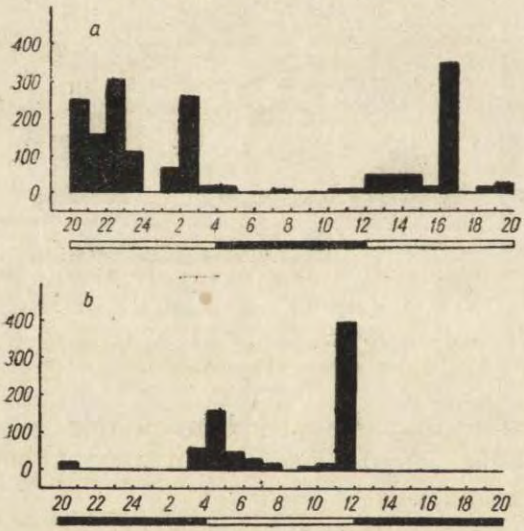

Fig. 14. Activity of the common buzzard (*Buteo buteo* L.) No 2 at the application of a 16-hour rhythm of lighting on: a — the third day of experiments, b — the fourth day of experiments

rhythm. The specimen of the common buzzard No. 2 and that of the rough-legged buzzard kept it to the end of the experiments. The periods of activity became gradually shorter and shorter, and the number of movements in each hour smaller and smaller (Fig. 22a, b and 23). The activity of the common buzzard No. 1 after having passed through a many-phase period became spread over all hours of the constant lighting period and preserved a comparatively great highness (Fig. 24 a, b).

Perpetual darkness

This series was carried out only with the specimen No. 1 of the common buzzard. It proved impossible to apply perpetual darkness for a longer time because the lack of light affected the bird badly and it fell ill at the first attempt of this sort. Therefore darkness was applied for two 24-hour periods, next succeed a normal 24-hour periods followed after several days by two

24-hour periods of darkness. In this way there occurred six 24-hour periods of perpetual darkness. At that time the buzzard was fed several times in the course of the day and for these short intervals the light was switched on. The recording apparatus was not in operation in order that it should not record the movements the high number of which was provoked by the change in lighting conditions.

Fig. 25 represents the diagram of mean activity of all the six 24-hour periods. It may be seen that the movement-activity of the buzzard was on the whole very low in perpetual darkness with the exception of the period from 6 a.m. to 2 p.m. in which a certain increase of the activity occurred. This was a slight marking of the normal 24-hour rhythm.

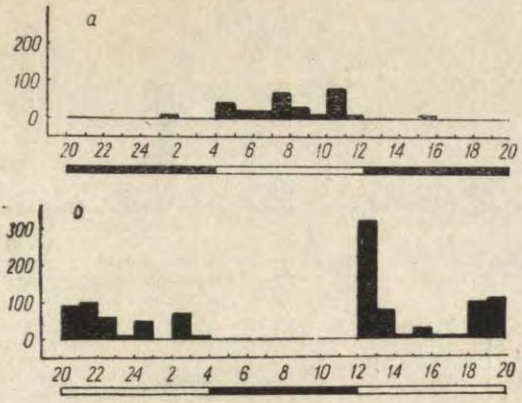

Fig. 15. Activity of the rough-legged buzzard (*Buteo lagopus* L.) at the application of a 16-hour rhythm of lighting on: a — the second day of experiments, b — the third day of experiments

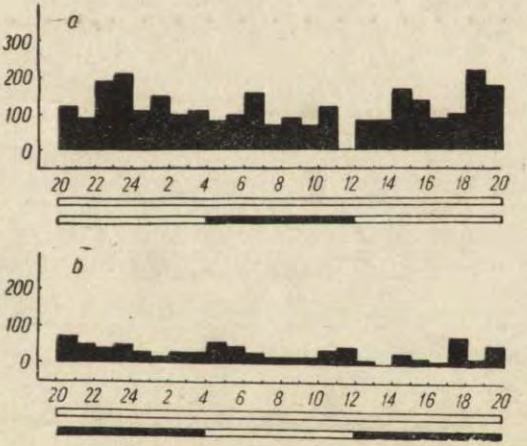

Fig. 16. Activity of the common buzzard (*Buteo buteo* L.) No 1 under the influence of constant lighting applied to test whether the 16-hour rhythm would be preserved in stable conditions: a — the first day of experiments, b — the fifth day of experiments. The upper stripe under the diagram denotes constant lighting, the lower one the changes of light and darkness in the 16-hour rhythm

DISCUSSION OF RESULTS

From all the series of our experiments it appears clearly that the rhythm of the 24-hour period depends in the

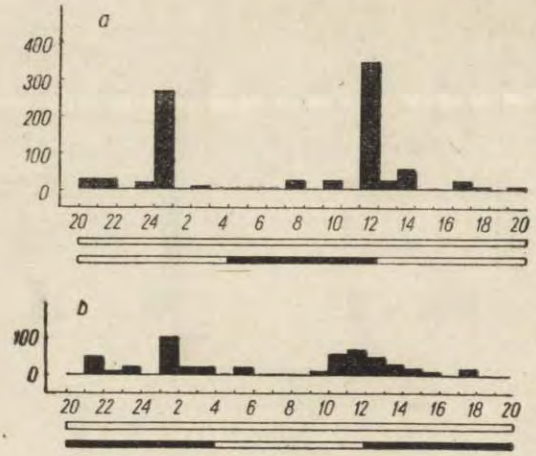

Fig. 17. Changes in the rhythm of activity in the common buzzard (*Buteo buteo* L.) No 2 under the influence of constant lighting applied to test whether the 16-hour rhythm would be preserved in stable conditions; a — the first day of experiments, b — the sixth day of experiments

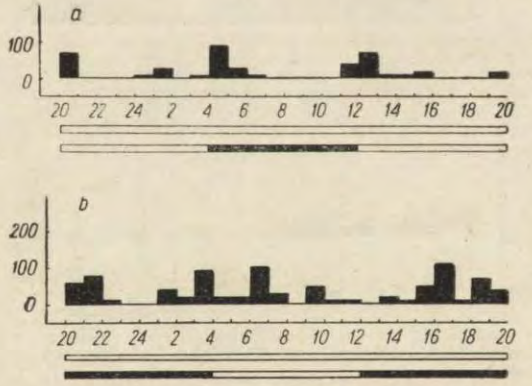

Fig. 18. Changes in the rhythm of activity of the rough-legged buzzard (*Buteo lagopus* L.) under the influence of constant lighting applied to test whether the 16-hour rhythm would be preserved in stable conditions; a — the first day of experiments, b — sixth day of experiments

buzzard fully on the rhythmic changes of light and darkness. With the moment the room is illuminated the animal's activity grows, and it lessens immediately when darkness follows. This was to be expected when a normal 24-hour rhythm was applied, that is 12 hours of light and 12 hours of darkness, because this is the rhythm most closely resembling that occurring in natural conditions. However, other series of experiments also showed the same dependence.

At a reversed rhythm the activity of the buzzard grew during the illumination phase although this occurred at night.

Perpetual lighting applied for several 24-hour periods caused a gradual change in the rhythm of activity. The many-phase rhythm occurred first but disappeared after several days, and the movement-activity of the animal became more or less equally vivid in all hours. This was accompanied by a general lessening of the activity.

When perpetual dark-

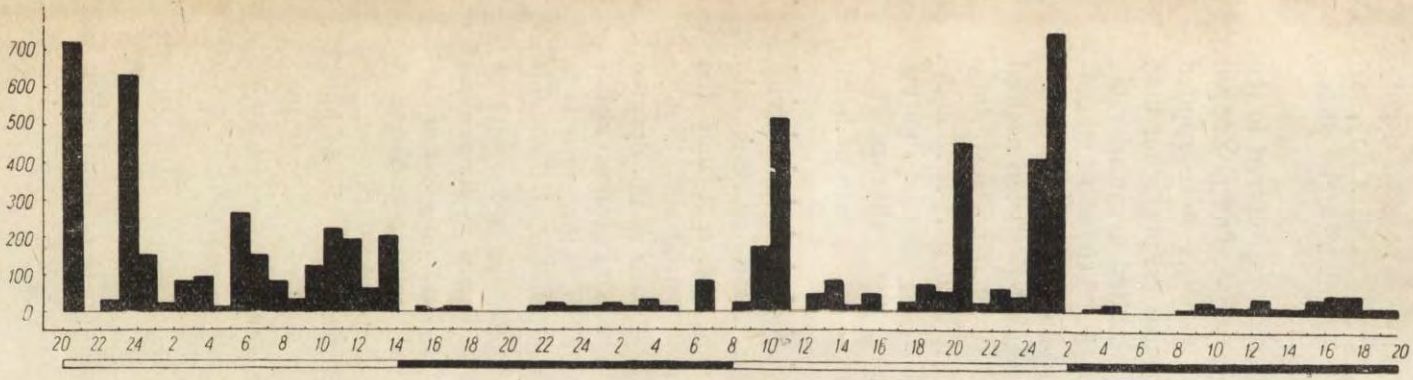

Fig. 19. Activity of the common buzzard (*Buteo buteo* L.) No 1 at a 36-hour rhythm of lighting

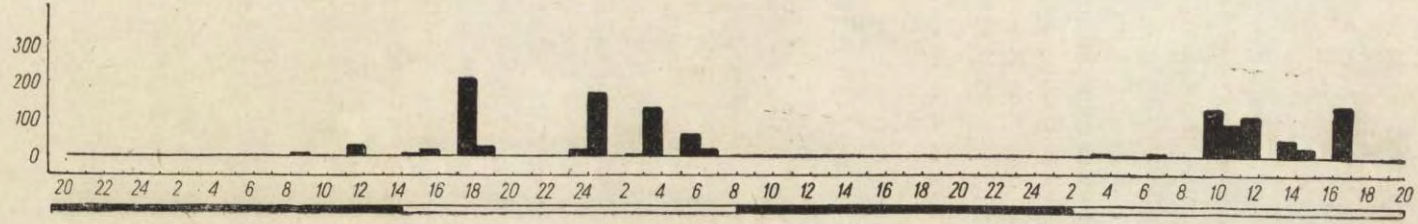

Fig. 20. Activity of the common buzzard (*Buteo buteo* L.) No 2 at a 36-hour rhythm of lighting

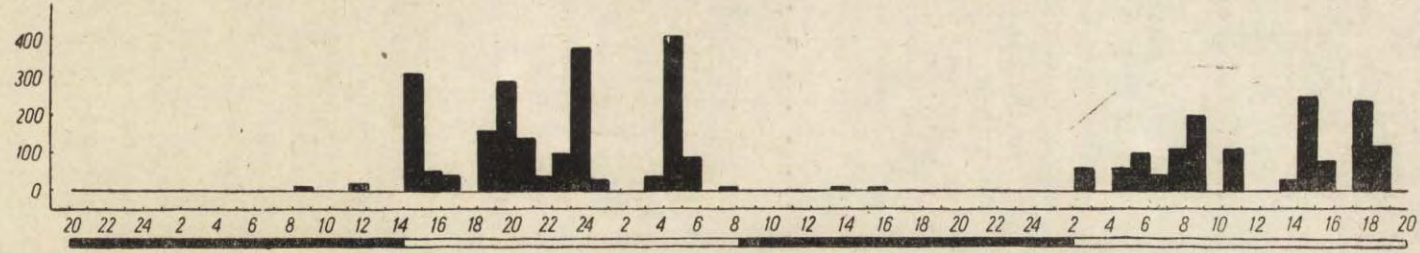

Fig. 21. Activity of the rough-legged buzzard (*Buteo lagopus* L.) at a 36-hour rhythm of lighting

ness was applied movement-activity of the buzzard diminished in the particular hours and the normal 24-hour rhythm was marked slighty.

Thus, as far as the rhythm is concerned the buzzard is a typical one-phase animal having one period of activity in the light phase, and one of quiescent phase when darkness reigns.

All the series of experiments described heretofore refered to the changes of illumination within the 24-hour rhythm. The prolonged 36-hour period and the shortened one of 16 hours were applied in order to establish whether the bird may acquire a rythm other than that of 24 hours. In this case the buzzard exhibited a considerable plasticity of its rhythm accomodating it to the changes in illumination immediately and without any disturbances.

In the experiments with the 36-hour rhythm it has been noticed that there exist several periods of activity divided by rest; they are analogical to the many-phase periodicity when perpetual lighting is applied. The assumption offers itself that perpetual lighting is not essential to the occurrence of the many-phase rhythm. It is enough to choose a rhythm with a sufficiently long phase of light, in this case that of 18 hours.

The results obtained in the present experiments on the activity of the buzzard differ in certain points from the conclusions submitted by other authors. This concerns chiefly the fact that the birds preserve their 24-hour rhythm in stable conditions. S z y m a ń s k i (1914) and H. W a g n e r (1930) are of the opinion that birds kept in perpetual darkness for a longer time exhibit a 24-hour rhythm of activity. The present author has failed to establish it in the buzzard. The experiments of this series, however, must be accepted with certain reservations, as they were carried out with interruptions caused by the bad influence which was exercised upon the birds by perpetual darkness. But in none of the six periods a pronounced 24-hour rhythm was noticed although it should have occurred from the first day of perpetual darkness.

Perpetual lighting also brings about abolishment of the 24-hour periodicity in the buzzard. Of the three birds studied only one preserved the 24-hour rhythm of activity for 4 days. In the other two birds it either did not occur at all, or did only on the first day of perpetual lighting and not quite distinctly either.

According to A s c h o f f (1953) the 24-hour rhythm disappears quickly if the light is too strong. But if a light of lesser, well-matched strength be applied the 24-hour rhythm may be maintained

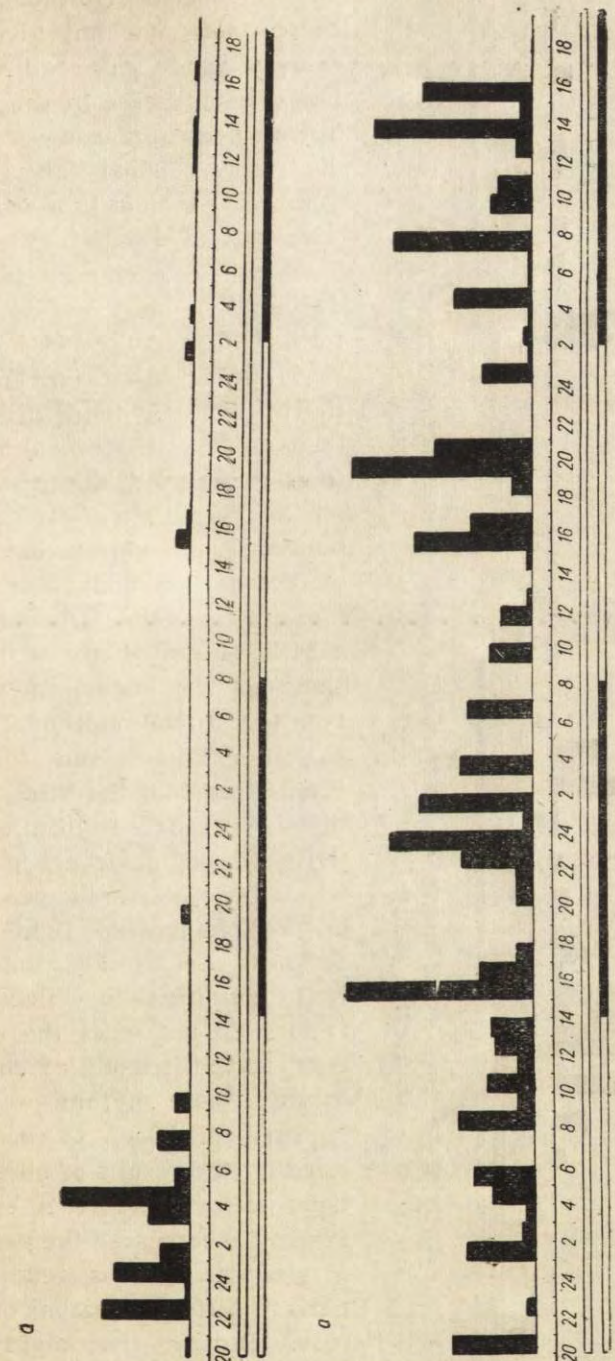

Fig. 22. Activity of the common buzzard (*Buteo buteo* L.) No 2 under the influence of constant lighting applied to test whether the 36-hour rhythm would be preserved in stable conditions. The upper stripe under the diagram denotes constant lighting, the lower one the changes in light and darkness in the 36-hour rhythm; a— the first day of experiments, b — the fourth day of experiments

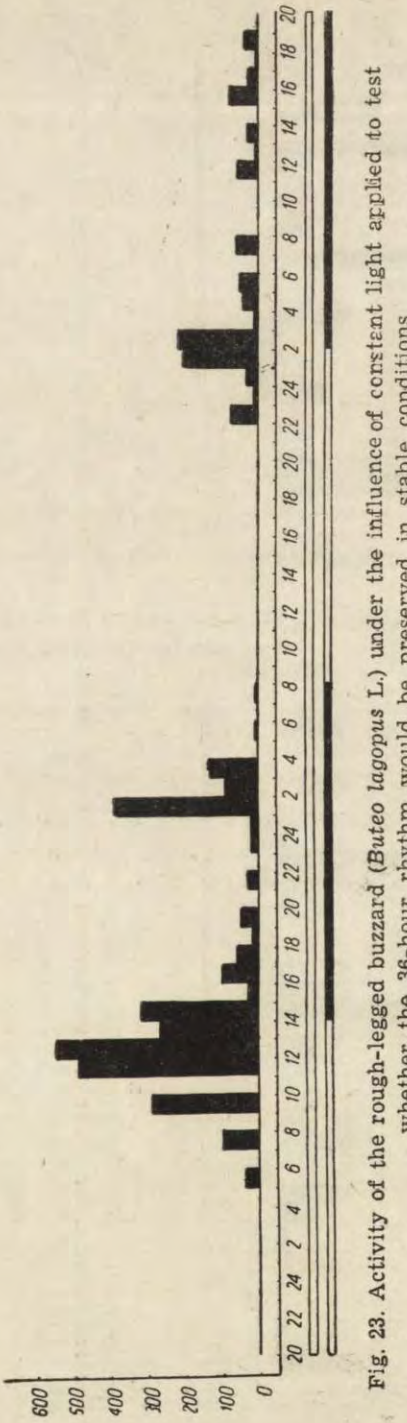

Fig. 23. Activity of the rough-legged buzzard (*Buteo lagopus* L.) under the influence of constant light applied to test whether the 36-hour rhythm would be preserved in stable conditions

for long weeks. Unfortunately the author does not give the value for the light required in luxes. In any case in the room in which the buzzards were studied there reigned light conditions weaker than in a room lit with normal day light.

F r a n z (1949) established during his observations performed beyond the polar circle that during the bright nights the birds living there preserve their 24-hour rhythm although their rest period is much shortened. W a g n e r (1930) also noticed in the whitethroat *(Sylvia communis)* kept in perpetual darkness the occurrence of a 24-hour periodicity with the phase activity beginning earlier than in natural conditions. These observations lead to the assumption that the birds have their own innate rhythm of activity which, however, is controled by external factors such as the changes in light and darkness occurring rhythmically. In conditions in which such factors do not exist the quiescent period is modified chiefly by the innate rhythm.

This conclusion is corroborated by the results of observations performed further on by Franz. He compared the periods of sleep in various genera of birds at different seasons of the year. During the night on

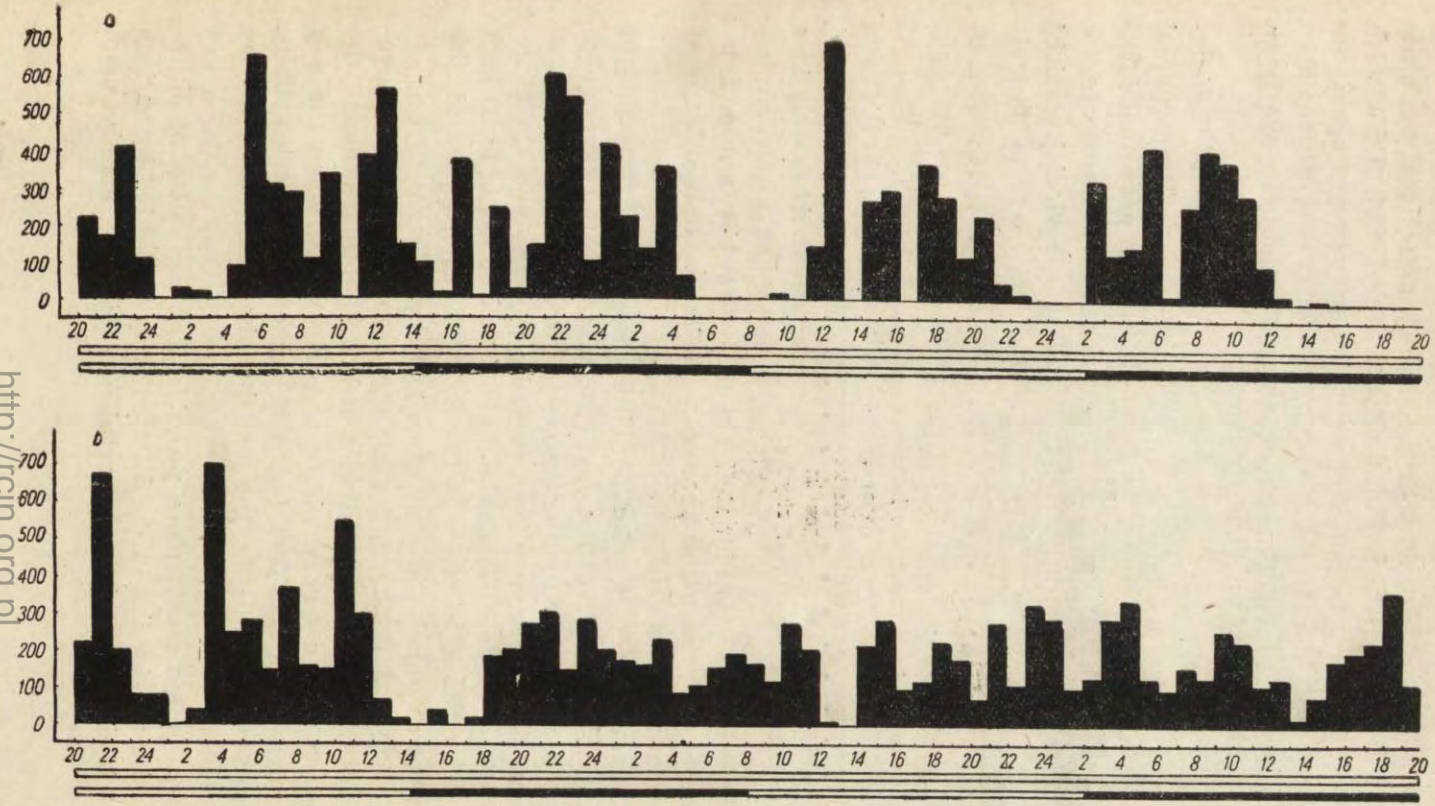

Fig. 24. Changes in the activity of the common buzzard (*Buteo buteo* L.) No 1 under the influence of constant lighting applied to test whether the 36-hour rhythm would be preserved in stable conditions; a — the first day of experiments. b — the fourth day of experiments

7/8th July, that is at the time of bright nights, generic differences in the time of sleep are distinctly marked, which means that the birds are guided mostly by the rhythm proper to every genus. On 12/13th August instead, when the sun sets and it is dark at night again, the differences vanish and the same or very similar period of sleep may be established in all genera, because external conditions exerts an influence which evens up their rhythm.

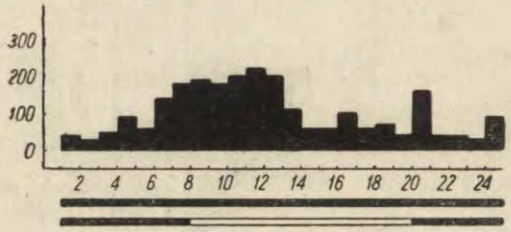

Fig. 25. Activity of the common buzzard (*Buteo buteo* L.) No 1 under the influence of constant darkness. The upper stripe under the diagram denotes constant darkness, the lower one the changes in light and darkness in a normal rhythm

The fact that the buzzard kept in perpetual lighting or perpetual darkness does not preserve the 24-hour periodicity might indicate that the bird either does not possess an innate rhythm or only such a feeble one that without the control of external factors it is not marked at all or only in a very slight degree. As, however, the existence of an innate 24-hour rhythm has been established in all birds investigated heretofore the latter supposition is of course by far more probable.

The buzzard differs from other birds also by the spreading of its activity in the course of the day. It results from the experiences of various research workers discussed at the beginning that there exist two peaks of movement-activity, the morning and the evening maximum. The evening maximum is not exhibited in birds kept in captivity if the light phase is too short in comparison to the length of the day at the time of experimenting.

In the buzzard neither morning nor evening intensive movement-activity has been noticed at a 24-hour lighting although the research was carried out in winter or early spring when the day lasts less than 12 hours. Therefore the lack of a two-peak activity cannot be explained only by the fact that the light phase was too short for the bird.

The assumption seems more probable that the cause of differences in the activity lies in the different way of life led by various genera of birds. Research was carried out hitherto mainly upon

small song birds. The biology of the buzzard, a bird of prey, varies so much from their biology that it may form the cause of the differences in the course of the diurnal activity.

For the sake of comparison it should be mentioned what the 24-hour rhythm is like in animals different in every respect from birds, that is in the invertebrates.

The problem of time memory of bees has been studied by several authors such as B e l i n g (1929), W a h l (1932) and F r i s c h (1948). Research on their rythm was conducted by way of training. Food was given them every day at the same time. After several days the food was removed, and in spite of that the bees used to come at the same time as they had been fed previously. In order to eliminate such obvious factor of orientation as the rhythmic changes of light and darkness the experiments were repeated at a perpetual lighting. The results were the same.

The question arose whether the bees remembered the period of time which elapsed between the feedings, or the time of the day. Therefore the syrup was given them every 19 instead of every 24 hours so that the feeding fell each time at a different hour of the day. In this case they failed to be accustomed to visit the spot of feeding at the hours of training. Thus, the bees obviously remembered the part of the day and not the given interval of time. In another test food was given at the same time but at intervals of two days. After several days the food was removed and control observations began. The bees arrived at the given hour every day, and not every other day. This proves that the bees possess an innate rhythm of activity and cannot be adjusted to another rhythm.

When compared with them the buzzard exhibits a more pronounced plasticity of the 24-hour rhythm and its behaviour depends distinctly on external factors.

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THE CELLS OF THE CHICK YOLK SACK AND THE PRODUCTION OF DIGESTIVE ENZYMES

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THE PROBLEM AND METHOD

The digestive enzymes in the embryonic cells and in the yolk sack have been known and confirmed many times (Albert and Hecht 1933, Kapel 1927, 1929, Amoroso, 1932, Poluszyński 1929, Remotti 1927, 1930). The cells of the chick yolk sack when cultivated in the solid medium liquefy it after some time (Grodziński 1930, 1947). The aim of the present work was to establish whether this phenomenon depends upon the influence of digestive enzymes, and to determine the course of the process which in Grodziński's publication was only touched.

The cultures of pieces of the chick yolk sack were used for that purpose. They were taken from the eggs incubated for 48 hrs. Explants were taken from the very edge of the sack in order not to use cells differentiated into ecto and endodermic. Explants from the area close to the vascular field consisted of the ecto and endoderme. The tissue was washed a few times in the Tyrode fluid of pH 7, then it was cut into small pieces and put by means of the

pipette on the cover glass. Then the Tyrode fluid was removed from the cover glass with pipette. The droplet of heparinized plasma, diluted with Tyrode fluid in relation 1 : 1; was put on the tissue. The same quantity of the extract of 10 days old chicken was added, — and it was also diluted with Tyrode fluid in relation 1 : 1. The cover glass with prepared in this manner culture was fixed to the metal ring, the diameter of which was 15 mm and the height 3—4 mm, and which was fastened with vaseline to the object glass. The plasma being mixed with the embryonic extract coagulates and gives a solid medium. This enables the growth of tissue in the hanging drop. The tissue cultures were kept in thermostat at +37°C all the time, in some cases even more than 400 days. The growth of tissues and the changes in the state of medium were controlled every 12—24 hours. More interesting fragments were photographed by means of the Zeiss microscope of a Miflex camera, and the Polish ortochromatic plates „Omega". The photographic filtres, blue, green and yellow were used.

Experiments were conducted since May 1952 to 30 of December 1953, observations lasted to May 1954. During this period 22 series of experiments, and 300 cultures were made. Among them 200 cultures were normal, it means that the substrate consisting of plasma and extract was inoculated with tissues and kept in thermostat. 100 cultures were modified as follows:

1. A drop of plasma was mixed with a drop of extract and kept in thermostat.

2. A drop of plasma was covered with a drop of liquefied substrate (taken from digested culture), and a drop of extract was added. The whole was kept in thermostat.

3. A drop of plasma was mixed with a drop of extract and after their coagulation a drop of liquefied substrate was added (composition the same as before, but successiveness changed). The whole was kept in thermostat.

4. The liquefied substrate was heated in thermostat at +53C° during 1 hour (in order to destroy enzymes), then it was mixed with plasma and extract (see point 2 and 3).

70 preparations without tissue (point 1, 2, 3, 4) were done. Other 20 preparations without tissues and 10 with inoculated tissue were kept at room temperature without light. We do not mention here

those cultures that were set on but they did not grow, or were infected.

THE MORPHOLOGY OF THE CELLS OF THE YOLK SACK „IN VITRO"

The growth of cultures may be noticed not earlier than after 24 hours (Pl. I, fig. 2a). On the edge of the explant there appear not numerous cells, which stretch out their protoplasmatic pseudo-

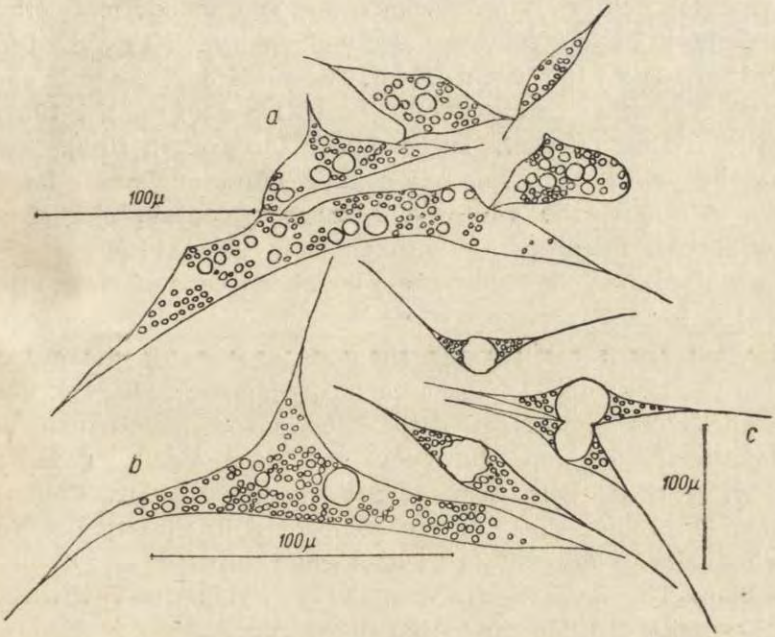

Fig. 1. The changes in the cells of the yolk sack cultivated in vitro. a — ectodermal cells with numerous fat granules, b — a single ectodermal cell with long protoplasmic threade, c — shrunken cells. Fat granules coalesced into big drops, the protoplasm drawn into long threads

podia, and they are followed by cells. After 48 hours the growth of culture is extensive (Pl. I, fig. 2b). The surface of growth region or „invasion" is many times greater than that of the explant. The cells of the yolk sack represent membranelike or epithelial kind of growth. They are flattened and stretched in direction of motion. They are connected together in a close membrane. If material was taken from the edge of the yolk sack, it consisted of indifferentiated cells. The outlines of the cells are not distinct, the nuclei placed at

the edge of the cell are hardly visible, as negatives on the ground of the granular content of plasma. Sudan III (Fig. 1a) stains inside the cell neutral fats present, appearing either as numerous, little round droplets or as 1—3 large drops. They are yellow, shiny and optically empty, showing no birefringence under the polarizing microscope. The fat drops exist both in ectodermal and endodermal cells.

Material taken from the part of the sack which lies nearer to the vascular field, consists of ectodermal and endodermal cells. The latter contain besides fat drops also yolk spheres (G r o d z i ń s k i 1930, 1947, D a t k ó w n a 1949). The surface of the yolk spheres consists of semipermeable membrane (G r o d z i ń s k i 1938) and inside there are fat drops suspended in the protein fluid. Some of them, the yellow yolk spheres, contain numerous small fat drops inside, — others, the white yolk spheres, contain one great or a few smaller fat drops. Cells taken from *Area vitellina externa* contain a yellow yolk, while the yolk of the cells of *Area vitellina interna* is for the most part white.

The migrating cells change their shape from the irregular and oval to the elongated, lobate, with long filaments (Fig. 1b). For the most part cells migrate round the explant and move equally in all directions. It happens however that some cells grow more quickly, — then their field of growth is irregular. Cells form smaller or greater sheat of the compact epithelial tissue. Young, growing cells do not contain vacuols while the older and dying ones have them. The vacuols stain with neutral red, and that is why they may be easily distinguished from the fat drop.

THE ENZYMES INFLUENCE UPON THE YOLK AND THE CELLS

The yolk sack cells are able to digest proteins and fat. For the proteins to be digested, it may be the culture medium or cell plasma, the presence of proteolytic enzymes is necessary. It is known that they occur not only in the yolk sack wall (R e m o t t i 1929, 1930), but also in the embryonic extract which is added to the culture medium (A l b e r t and H e c h t 1933). Even if the digestive influence of the proteolytic enzymes of the embryonic extract occurs — it is very week. It was seen on the fact, that when the culture medium composed of plasma and embryonic extract 1 : 1 (about 50 preparations) was kept in thermostat at +37°C for

several days — it did not liquefy. After adding there a slice of living tissue — the culture medium became liquefied, then possibly after a few days.

Yolk spheres contained in cells have at the beginning a round or oval shape, the bordering line is equal, smooth and clear. The cell enzymes digest the membrane of the yolk sphere. It begins to wrinkle, the outlines of the sphere become unequal, finally the pellicle breaks and the fat drops liberate and they disperse in the protoplasm. Then the membrane becomes completely liquefied and the yolk spheres — as independent morphotic components of the cell — disappear (Fig. 1).

The chick plasma when mixed with embryonic extract gives a stiff, jellied culture medium. The liquefaction of this substrate begins within a few days after establishing the culture. The first little drop of liquid substrate appears beneath the growing cells, and it augments till a complete liquefaction of the whole culture medium. The digested substrate is a watery, white liquid, which pours easily on a cover glass.

Table I

Rate of activity of enzymes

The changes observed \ Number of observations	1	2	3	4	5	6	7	8	9	10	Time in
Yolk spheres digested	96	120	192	150	/	/	/	/	/	/	
Culture medium liquefied	72	72	96	96	70	60	72	60	65	65	hours
Cells digested	9	13	20	17	7	8	12	5	6	6	
Fat crystallizes	275	285	291	300	290	285	270	265	268	302	days

The rate of liquefaction depends upon a few moments:

1. ectodermal cells liquefy a culture medium more quickly than endodermal ones. It is because the latter digest first the intracellular yolk spheres with which they are filled, and then having them partially digested they influence the substrate. I was no succesful in establishing pure cultures, composed of endo or ectodermal cells

only, — therefore it is impossible to determine the digestive rate of both kind of cells.

2. The culture that grows better, liquefies the substrate earlier than that which grows poorly, while the culture not growing at all does not liquefy the substrate.

3. Older plasma when mixed with embryonic extract coagulates more slowly, but this substrate liquefies later more difficult — on the other hand a young plasma coagulates quickly with an embryonic extract and the substrate is digested more easily, (it is liquefied more quickly). When speaking about the age of plasma, we understand the period from taking the blood till the moment of establishing the culture (a period of keeping plasma in the icebox) — and not the age of the hen which plasma has been taken from.

After digesting the culture medium — proteolytic enzymes influence cells. In this period the cells are dead already, what can be infered from cell nucleus turned turbid and distincly visible within the protoplasm. The cells — were poisoned with metabolic byproducts because the culture medium was not changed, nor washed. Cells migrating in the medium for few days are flattened, long with thin threadlike outgrowth. Digestion manifests itself by the fact that cell becomes smaller and smaller and more flat. Thin threadlike outgrowth keep their former position and remain joined with the cell. The fat drops in the cell begin to flow into greater ones (Fig. 1c). Few days later the cells begin to disappear, their fat drops enter the liquefied substrate and swim disorderly when the preparation is shaken. Cell bodies being digested completely, the plasmatic threads still exist for a long time but finally they disappear, to (Pl. I, fig. 2c, 2d). The time necessary for the cells to be completely digested is very different. Sometimes cells were digested after 8—12 days, but there were cultures, in which after a month and even later the remains of protoplasm were seen. Surely, it depends upon the quantity of enzymes secreted by a culture.

The fat exist in rather great quantities in the cell, in the shape of glittering, yellow drops. Young culture contains neutral fats only, which do not display birefringence under the polarising microscope. Their first change caused by enzymes is the loss of shiny appearence. Then their surface begins to wrinkle, their shape changes from the spherical to the irregular and elongated one, their outline become serrate, — at last the individual drops fuse together

into one great, fat mass. The physical pressure of the constantly diminishing cell body has much to do in this process.

Lipases dissolve emulgator which covers the fat drops surface, they liquefy fat and they enable fusing of individual drops. Not all drops fuse into one great drop, — there always remain a few isolated ones. All the changes mentioned here occur within the cell but not all the drops are involved in this process.

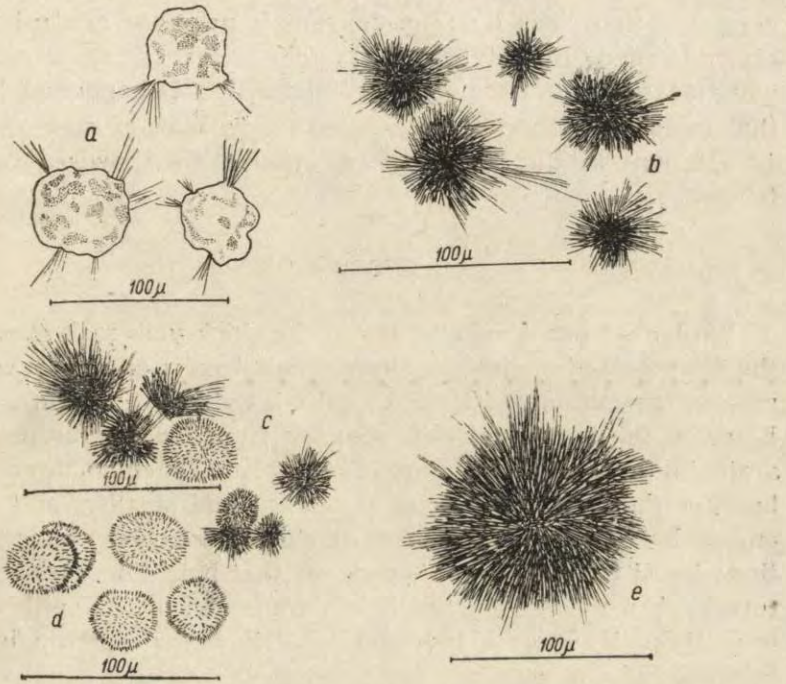

Fig. 2. Fat crystallisation. a — the first crystals protruding from the deformed fat droplets, b — crystals arranged radially in a shape of rosette (1st type of crystallization), c — the two types of crystals in the same field of view, d — fat drops covered on the surface by small needles (2nd type of crystallization, e — a single big crystalline rosette

The liquid culture medium together with fat drops, derived from digested cells, — when being kept henceforth in thermostat at +37°C — the fat changes again. Morphological changes will not be seen for a long time. After a year and even more — crystal formation begins. They appear in different places (Fig. 2a). In the initial period the wrinkled and folded fat drops can be found with single

needle-like crystale protruding from them (Fig. 2a). Later on the whole drop turns into a mass of needle-like crystals. They radiate in all directions from one point of the fat drop (Fig. 2b, 2c). Another kind of crystallisation may be met in the same preparations, namely: the fat drops become covered on the whole surface by minute crystals which do not grow any more, that means the crystallisation has stopped definitively. A fat drop remains round and looks as if bristled (Fig. 2d). Sometimes we meet both kind of crystallisation close by (Fig. 2c). Fat is no more neutral fat after crystallisation. Both types of crystals show strong birefringence when examined under polarising microscope. It happened, however, that in some cultures there existed fat without crystals even after 12—18 months. But even in these cultures fat showed strong birefringence.

DISCUSSION

Explants from *Area vitellina* of the chick yolk sack grow during the first 2—3 days just as the epithelial cells and they constitute compact membranes (G r o d z i ń s k i 1930, T h o m a s 1938). Later on the individual cells separate from the membrane and migrate in the culture medium. The rest of the membrane changes because the cells are becoming smaller and smaller, and the slits appear between them. The cells stretch at their very ends into long threads. G r o d z i ń s k i suggests that it is a degeneracy of culture, T h o m a s — that it is a transition of the epithelial cells into fibrocyts. Present investigations show that this might be the influence of enzymes.

G r o d z i ń s k i observed (1933) that the edge cells of *Area vitellina* grown in tissue culture liquefy the solid substrate rapidly. It appears, however, that cells from any region of *Area vitellina* do the same, but the ectodermal and indifferentiated cells from the margin of yolk sack do it more rapidly than endodermal ones. The latter digest the intracellular yolk spheres first, while the former ones produce proteolytic enzymes and liquefy at once the culture medium. The liquefied medium contains enzymes in the active state lasting within even 30 days. When added to a coagulated culture medium which does not contains yolk sack explants — liquefies it within 3—6 days. When heated to $+53^{0}$C during one hour it looses its activity and it does not postpone the coagulation

when mixed with a normal culture medium, while when it is added to a coagulated culture medium—does not liquefy it. Proteolytic enzymes are destroyed within one hour when kept at +53°C.

Proteolytic enzymes shed into the culture medium liquefy not only the substrate but they attack also cells. As yet, we do not know whether enzymes digest the cells degenerating or dead. Healthy cells are not attacked, as their influence on cells may be seen not earlier than after a few days, then in the period when cells are dying. Nevertheless it is sure, that the proteolytic enzymes dissolve cells completely within shorter or longer period of time. The fat drops get out of such cells, pass into the substrate and may move freely when seized by currents arising in liquefied culture medium. If the substrate is not washed with Tyrode solution for a long time, — surely the wast products of metabolism have a bad influence upon cells. Namely, there appear numerous vacuoles which stain with neutral red. At the same time the epithelial cells aquire the shape od stars stretched in one axle. T h o m a s suggests them to be fibrocyts, though it is more probably — they are degenerating cells which have been digested by proteolytic enzymes present in the culture medium.

The chick yolk sack cells produce also enzymes digesting fat. Their influence upon fat drops we observe much later than of proteolytic enzymes. First changes of the fat drops occur inside the cells. The droplets become turbid, stop to glitter, they wrinkle and fold. Then emulgator surrounding the fat drops is dissolved and fat is liquefied. As a result the fat droplets fuse together. When cell bodies are destroyed by digestion, the fat drops pass into culture medium, and undergo farther action of lipase. They show now a strong birefringence under polarising microscope. In the last stage of digestion, i. e. after several months, fat crystals appear.

The fat drops may crystallise so as to form a needle-like mass, or little needles stand out their surface like bristle. Those crystals show strong birefringence under the polarising microscope.

G r o d z i ń s k i (1947, 1949) digested fats of the hen, lizzard and sea trout yolk „in vitro" using the same steapsine all the time (lipase made by T. Schuchardt Görlitz). In all cases fat was digested and showed birefringence in the polarised light and then changed partially into needle-like crystals. The same lipase digests then fat, whatever its origin may be. The fat of a hen is digested, however, at higher temperature (+37°C). The fat of a lizzard or sea trout

are digested at room temperature. Another difference is that the fat kept in natural lipase transforms long (within weeks and months), in the manufactured one — quickly (within days). Efficacy of the latter depends surely upon its concentration (G r o d z i ń s k i 1949).

Yolk contained in the yolk sack is digested outside the cells thank to enzymes which are secreted by the wall of the sack. In the yolk sack carbohydrates are digested easily, proteins — less easily, while fats are digested with difficulty. This successiveness is characteristic for the metabolism of the chick in the process of embryonic growth (R e m o t t i 1929).

Fat is digested at the end of development. The influence of lipase in the first week of incubation is rather slow. At the end of second week their activity increases and reaches its maximum (N e e d h a m 1931, R e m o t t i 1930). Because of the folds starting to grow on the yolk sack in the sixth day of incubation, which augment its surface, the digestion of yolk within the sack increases. The same holds true for absorbing of dissolved elements (R e m o t t i 1930). In this time we observe considerable growth of vessels in the folds of the yolk sack which absorbe digested yolk. At the end of the embryonic growth the digesting activity diminishes, though it may be prolonged by adding a new yolk to the yolk sack, this additional yolk will be digested too (R e m o t t i 1930).

RESULTS

In tissue culture of the chick yolk sack was proved that the outer part of *Area vitellina externa* consists in the 48 hrs chick of indifferentiated cells, while *Area vitellina interna* — consists of ecto and endodermal cells. Both kinds of cells are able to secrete digestive enzymes proteolytic and lipases.

The influence of the proteolytic enzymes is seen thanks to the fact that they liquefy a solid culture medium on which the tissue was grown, and that they digest also the growing cells. Proteolytic enzymes in the substrate maintain their activity within several days. When added to the culture medium composed of coagulated embryonic extract and plasma but not containing any chick tissues — they liquefy the substrate. When heated to $+53^{0}$C within 1 hour they are destroyed and loose their activity.

The enzymes liquefy earlier in cultures of indifferentiated or ectodermal cells than in cultures of endodermal ones. The latter digest yolk spheres contained by their bodies first, and then they liquefy the substrate. Lipase starts digest fat in the cell body. It dissolves emulgator covering the fat drop, and causes fusing of the droplets together. The fat, which passed out of digested cells into the liquefied medium is brought by lipase to birefringence under the polarising microscope, and crystallisation as well. A complete digestion of the fat and crystallisation lasts very long, 12—18 months. The crystals show strong birefringence under the polarising microscope, too.

The work was carried out under the guidance of Professor Z. Grodziński, to whom my best thanks are due for the suggestion of the problem, and for his continuous help and advice.

SUMMARY

The method of tissue culture was used in this investigation, which was carried out in order to determine the ability of the blastoderm cells to digest the egg yolk, i. e. the ability to produce digestive enzymes. The explants were taken from the eggs incubated for 48 hrs, either from *area vitellina externa* (undifferentiated cells), or from *a. v. interna* (ectodermal and endodermal cells). The tissue cultures were kept in thermostat at +37°C, for 400 days, in coagulated medium which consisted of equal parts of plasma and embryonic juice. The plasma and juice were diluted with Tyrode fluid (1 : 1).

The yolk sack cells are producing lipases and proteolytic enzymes. The activity of proteolytic enzymes manifests itself in the liquefaction of the substrate. After 6—20 days the protoplasm of the cells is also completely digested.

The proteolytic enzymes liberated into the culture medium remain active for about 30 days (at +37°C). The enzymes can be inactivated by heating at +53°C for one hour.

The lipases dissolve the emulgator covering the fat droplets within the cells body. They turn also the fat more liquid. This results in fusion of fat into bigger bodies. After one year in the fat droplets needle-like crystals appear and the whole droplet can be transformed into a mase of needle-like crystals which exhibit the birefringence.

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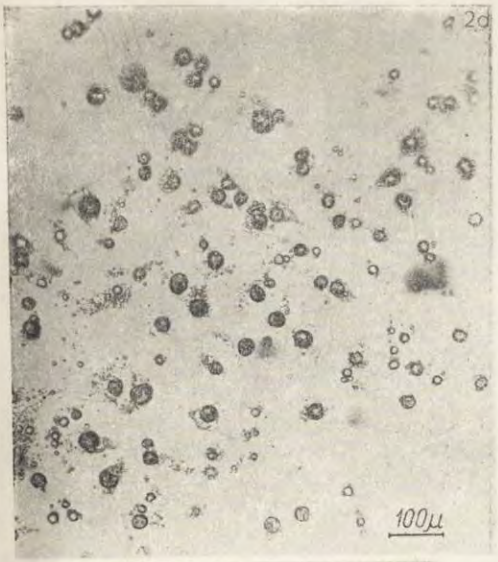
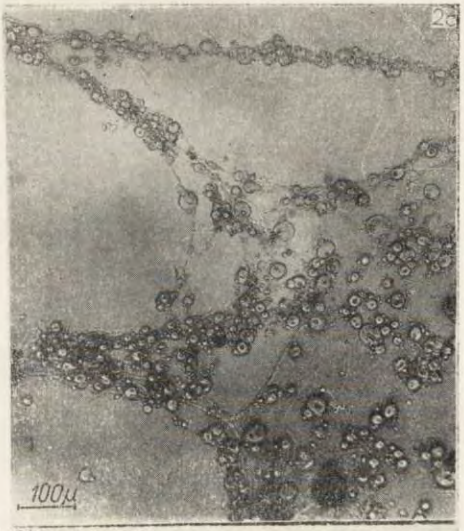
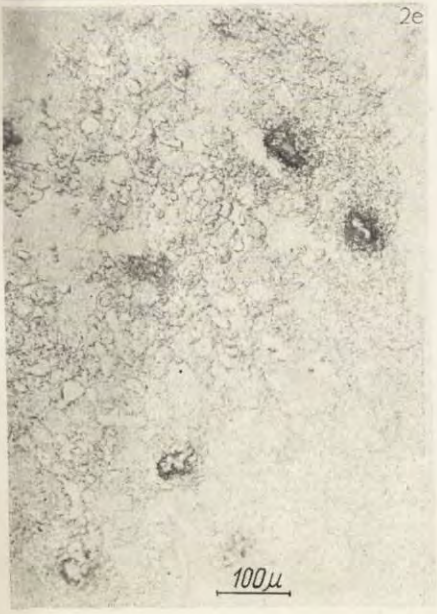
PLATE I

Fig. 2. The subsequent changes of tissue culture. a — the tissue culture 24 hrs old, b — the same, 48 hrs old, c — the culture, after the cells are partially digested (the remnants of the protoplasm), d — the culture after the cells are completelly digested (Fat drops liberated), e — the centres of crystal formation in the fatty mass







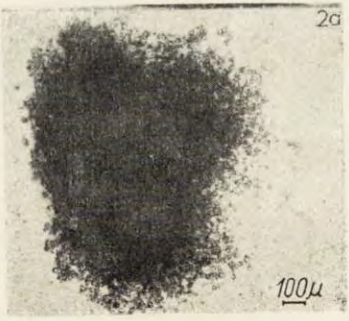


PLATE I

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SOME PHYSICO-CHEMICAL PROPERTIES OF THE YOLK OF THERMOPHILIC FISH

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PROBLEM AND METHOD

The yolk of Teleost fishes is a highly concentrated food. It contains a sufficient amount of substances to build the embryo's body and to maintain its basic metabolism. It lacks however more than half of the water needed for these processes. The quantity and quality of the yolk is different in various representatives of this fish order. Fauré-Fremiet and Garrault (1922 a, b) contrasted from the chemical point of view, trout eggs (*Salmo trutta* m. *fario* L.) with carp eggs (*Cyprinus carpio* L.). The first possess more fat (9,16%, carp 6,20%) and a little more protein, but less water. In the fat of the trout glycerids prevail (10%), in carp phosphatids (11,35%).

The morphological and physical properties of the yolk in the two fish groups differ considerably. The yolk of the Sea-trout *Salmo trutta* L. is a transparent, homogeneous, dense fluid. In the water it becomes rigid and milky, dissolving easily in watery solutions of NaCl. The liquid yolk, called ichthulin, contains many fat drops. They fuse during the development of the fish into bigger bodies, keeping constantly a spherical shape. When transferred with the ichthulin into a suitable NaCl solution (1—1/16 M) they emerge to the surface and flatten immediately. Under the action of steapsin, and exceptionally without it, birefringent crystals appear within the fat drops (Grodziński 1949).

The yolk of Bitterling (*Rhodeus amarus* Bl.), belonging to the carp family, has a quite different appearance, being above all opaque. It consists of a small

amount of ichthulin, many yolk spheres, 6—19 μ in diameter, and of tiny (1—1,5 μ) fat drops. The yolk when poured into hypotonic solutions (1/256 M NaCl) does not become turbid or rigid. Fat droplets suspended in the yolk are spherical, flattening when they emerge to the surface of the dilutor. The yolk spheres are vesicles with walls built of a semipermeable membrane. The vesicle contains optically homogeneous, yellowish fluid. Gentle currents of osmotic origin, which enter the yolk sphere, precipitate fat from the fluid in shape of tiny droplets (G r o d z i ń s k i 1954).

The above described observations were extended to three species of thermophilic fish namely: *Macropodus opercularis* L., *Betta splendens* Regan and *Pterophyllum scalare* Cuv. & Val.. The eggs of *Macropodus* develop successfuly at a temperature of +20° to +25°C, of *Betta* at +26° to +30°C and of *Pterophyllum* at +24° to +28°C (F r e y 1954).

The yolk of the mentioned fish was investigated like that in trout and bitterling. Into a drop of experimental fluid, placed upon the coverslip a soaked egg or young fish was put by means of forceps. The egg membrane or the yolk sac was torn off with fine needles in order to release the yolk. The morphotic components of the yolk, derived from different developmental stages proved to be of equal quality for experiments.

The preparations were mounted like explants for tissue culture; the coverslip was sealed to the objective glass with the aid of a high ring of vaseline. This arrangement protected the yolk from dessication and rendered the preparation suitable for microscopical inspection. The aim was to observe without interruption one and the same morphotic component of the yolk for several minutes, in order to follow the whole cycle of its transformations. The preparations were stored for several days, according to necessity, at room temperature (+ 18°), in a thermostat (+ 37°) or in the refrigerator (+ 2°). During this time they were looked over two or three time daily.

Many details were photographed with the aid of a Zeiss Miflex camera. As dilutor for yolk samples were used 1, 1/2, 1/4, 1/6, 1/8, 1/10, 1/12, 1/16, 1/32, 1/64, 1/128 M NaCl nad Tyrode fluid for cold-blooded animals. The preparations were treated with such colouring matters as neutral red, nile blue sulphate, cresyl blue, janus green, sudan III, and with chemical agencies such as sulphuric ether, iodine and dioxan (diethylen-dioxide).

OBSERVATIONS

Macropodus opercularis L.

The yolk in the egg of this fish is yellowish and opaque. From the very beginning one big fat drop and a small number of little ones is visible. The yolk pours out from the egg membrane or the yolk sac freely in all used dilutors, and even in plain water. It does not coagulate nor become milky. In the yolk observed with the aid

Thanks are due to Mgr Choczewski and Dr Czapik who offered me willingly the material for experiments.

of the microscope, when pouring upon dry glass, morphotic components are visible namely: a small amount of ichthulin, plenty of yolk spheres and fat drops. Fundamentally it recalls rather the yolk of bitterling than that of sea-trout.

Fat properties

Fat is one of the very characteristic components of the *Macropodus* yolk and performs an important hydrostatic function during development. Thanks to it the eggs move towards the surface of water and gather here beneath the nest built from air bubbles. Shaking the jar with the nest drives the eggs down, but they return quickly and immediately to the previous position. This proves that the eggs are lighter than water. Depending upon the place occupied by the large fat drop the embryo floats with the abdomen upwards, almost vertically or horizontally.

The fat drop occupies in the dividing egg its upper pole (Fig. 1a). The body of the embryo originates at the opposite, than at the lower pole. In the beginning the fish contained within the egg membrane floats with its back turned down (Fig. 1b). Shortly before hatching the embryos body encircles the yolk sac and the fish floats upon its flank (Fig. 1c). The fat drop again occupies the top of the yolk sac, but it changes its position in relation to the body, from the abdominal to the lateral one.

A few minutes after hatching the fat drop moves again from the flank towards the middle of the lower part of the body and at the same time closer to the head. As a result the fish floats almost vertically with the belly upwards. The yolk sac, oviform at this period, faces the head with the blunt end and the tail with the sharp (Fig. 1d). After some time the intestine becomes more pronounced and the liver makes its appearance. The intestine later forms a loop and wedges perpendicularly into the yolk sac. This shift results in the partition of fat into two drops of approximatively equal size. The rest of the yolk moves into the rear of the yolk sac and elongates it. The two fat drops now lie to the right and left of the intestine. Later they move upwards to the flanks of the trunk. Simultaneously with the replacing of fat the position of the floating fish changes. They pass from the vertical position into a horizontal one (Fig. 1e). The fat remains in this position for a long time after the

digestion of the yolk, even when the airbladder is filled with air and could equilibrate the weight of the fish's body with water.

The fat does not always divide under the pressure of the intestine into two equal drops. Sometimes there arise three, placed

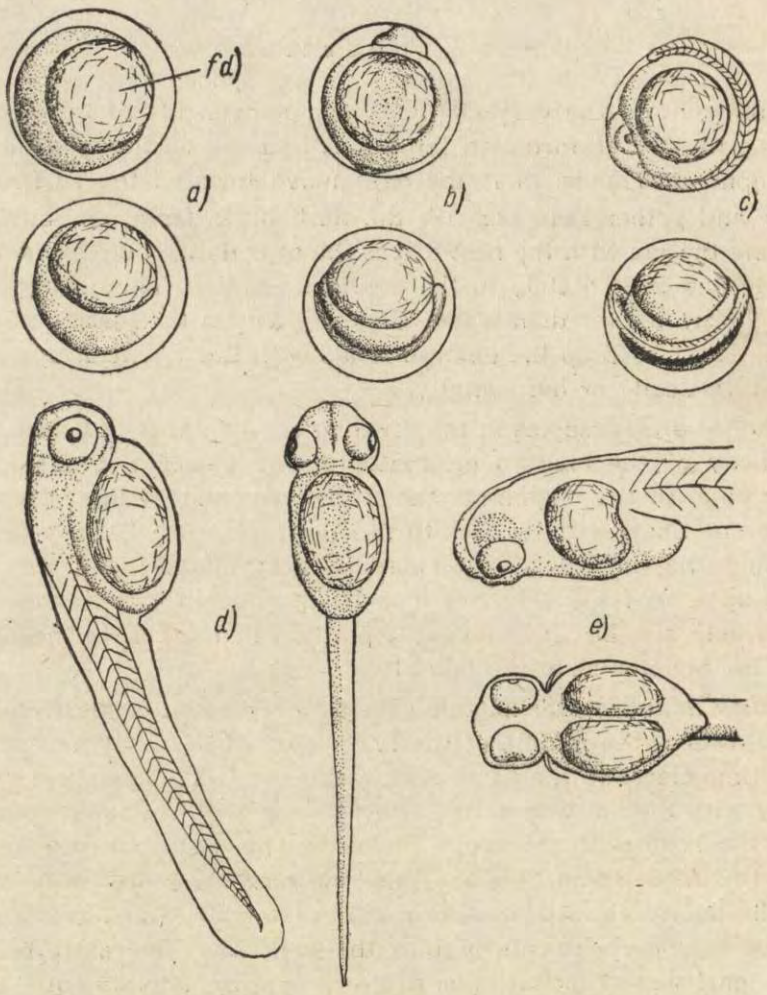

Fig. 1. Influence of fat drops (ft) upon the floating of developing *Macropodus*. a, b, c — development of the embryo within the egg membrane; upper row shows the top view, lower row the side view. a — freshly laid egg, b — body of the embryo forms at the lower pole of the egg, c — embryo floats upon its flank; d — embryo freshly hatched, floats almost perpendicularly, turned with abdomen upwards, e — fat drop divided into two parts, placed to both sides of the intestine and trunk. The fish swims in horizontal position

irregularly in the body. Such fishes float not quite horizontally or inclined to one flank.

The fat drop which passed from the yolk into some of the experimental fluids or even into water has a spherical shape, whitish colour and strong glitter. It soon rises to the surface of the fluid and flattens suddenly, becoming dull-greyish.

In the flattened drop small crystalline bodies appear within a few minutes. Some of them, not numerous and not present in every drop, have the shape of needles, single or gathered in bundles. In every fat drop, however, bodies are present, with the appearance of multilateral plates, single ones or clustered together (Fig. 2 a, b c, d). The needles are birefringent in polarized light at once, the plates

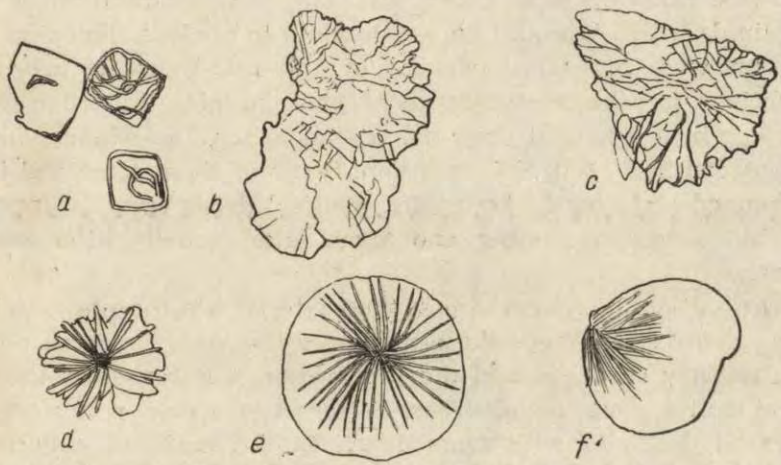

Fig. 2. The crystals precipitated in the fat drop. a, b, c, d — crystals from the yolk of *Macropodus*, a — in the undiluted yolk, b — in 1/12 M NaCl solution, c — in 1/4 M NaCl solution, d — in the spherical fat drop, 1/12 M NaCl solution; e — from the egg of *Pterophyllum* in Tyrode's fluid, f — from the egg of *Betta* in 1/128 M NaCl

after a few minutes. Their berefringence increases slowly. Neither the remainder of the fat drop nor the whole drop before the precipitation of crystals exhibits such qualities.

The crystalline plates have a rigid consistance. They may move within the liquid fat without change of shape. Sometimes the fat drop moves alongside the coverslip and partially or completely uncovers the plate. The plate sticks to the surface of the glass and maintains its original shape. The sharp ridges or folds on their surface do not round off and the plates do not fuse together. The

liquid fat does not react with neutral red, trypan red, janus green, cresyl blue. Sudan III gives it a yellow tint. The crystalline fat is resistant to all these dyes. The vapours of iodine quickly dissolve the crystals and give a now homogeneous fat drop a brown and later a cherry-red colour. The vapours of sulphuric ether also very soon dissolve the crystals. Neither the liquid nor the crystalline fat changes in osmotically active solutions. The surface does not wrinkle in hypertonic fluids, the hypotonic solutions do not induce the vacuoles. From these observations may be inferred that the fat lacks any protein component. The fat itself consists of two different substances, the liquid component and the crystallizing one. The first is a neutral fat (glycerid), the second probably a phospholipid.

There must be some causes inducing the precipitation of phospholipids which it would be worth while to analyse. The first supposition was that the flattening of the fat drops furnishes the conditions for the reappearance of phospholipids. In order to see whether in a spherical drop the crystal arises, the whole yolk sac was placed in 1/12 M NaCl solution. Some of them remained in an unchanged state for 24 hours. In spherical drops crystals appeared too, but fewer in number and much later, usually after several hours.

The salt solution was also supposed to enable the phospholipids to crystallize. Therefore the contents of the yolk sac were poured onto the dry coverslip and the preparation was sealed as usual to the objective glass. In all drops, flattened as a rule, many crystals appeared, but also with some delay. In the modified experiment the dead fish larve was placed in a drop of water upon the objective glass. As the water evaporated, the fish body and the yolk dried. Finally in the completely dry preparation the polarizing crystals were visible in the defaced fat drops.

The temperature exerts a decisive influence upon the appearance of the fat. When freshly made preparation was kept at a temperature of $+30^0$ or $+37^0$, no crystals were present even after 24 hours. The crystals visible in other preparations disappeared without any trace in higher temperature ($+37^0$) after several minutes. In such a homogeneous fat drop no birefringence was seen. The same preparation was several times transferred from room temperature ($+18^0$) into the thermostat ($+37^0$) and back again. It remained in each temperature for several hours. Constantly at $+18^0$ the crystals appeared and at the $+37^0$ dissolved.

In a freshly made preparation, kept in the refrigerator at about $+2^0$, the crystals very soon become precipitated. The whole fat drop congeals and becomes opaque acquiring a whitish spotted tint. Such a drop melts at room temperature during some minutes. The disappearance of whitish spots and the regaining transparency is visible. The plates of crystals show up distinctly against the transparent backgroud. After a while many plates scatter into rods and persist in this state. Similar changes can be observed in crystals formed at room temperature but after few days.

The body of live fish changes to a certain degree the influence of temperature. The fishes kept at room temperature ($+18^0$) possess optically homogeneous fat drops. But a fat drop released from its yolk sac into salt solution becomes crowded with crystals in a short time.

Yolk spheres

The yolk spheres, when present in Tyrode's solution, have a whitish colour and a distinct glitter. Their diameter oscillates between 5 and 13 μ, the mean for ten spheres present in one field of vision amounts to 9,3 μ. In osmotically active fluids it becomes apparent that the yolk spheres possess on the surface a semipermeable membrane and optically homogeneous fluid within. The latter consists mostly of proteins with dispersed submicroscopical fat particles.

The 1—1/6 M NaCl solutions appear to be hypertonic against the yolk spheres. The spheres contract and become deformed. In the majority of them pink dots appear, which later vanish partially or completely. The yolk spheres preserve mostly their regular shape and primitive appearance in 1/8—1/16 M solutions; these fluids are then more or less isotonic. The 1/32—1/126 M solutions act as hypotonic fluids. The spheres burst and the contents flow out through the hole in the semipermeable membrane. The empty membrane contracts and at the same time cleft closes, leaving no trace. Finally a small dull disc remains. In the fluid which pours out from the bursting sphere small grains are visible. In 1/64 M and especially in 1/128 M solutions the grains appear sometimes within the sphere before the membrane bursts. These grains may fuse into larger body. Judging from the glitter and transparency it is fat, precipitated from the protein fluid. In this fat pink spots appear, they enlarge, and later disappear. The contents of the sphere liquefy and become homogeneous again (Fig. 3).

The yolk spheres are distinguished by a small durability. The majority of them decay after 24 hours in all used solutions and disappear. Dull discs, derived from the semipermeable membranes and many fat droplets are the only remnants of the yolk spheres.

The same processes may be followed more distinctly, when the dyestuff (e. g. neutral red) is added to the preparation. The yolk spheres stain in hypertonic solutions comparatively weakly. The pink dots show distinctly against the stained background. They show a tendency to disappear. In isotonic solutions the whole spheres stain with more dark grains within. Similar pictures result in hypotonic solutions. The grains show a general tendency to fuse into bigger drops. When such a yolk sphere bursts, the coloured grains pour out and lose their tint. Similarly the semipermeably membrane, which remained in the shape of a disc, contains no dyestuff. It happens sometimes that the burst yolk sphere loses only a part of its contents and later the slit in the membrane closes. In such a case the sphere becomes paler, but the grains or drops contained within keep their dark hue unchanged. They now execute Brownian movements. In stained yolk spheres no formation was observed of huge bodies in strongly hypotonic solutions (Fig. 3)

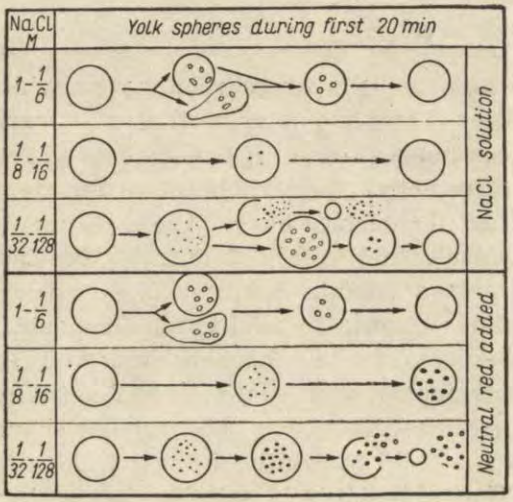

Fig. 3. The behaviour of yolk spheres of *Macropodus* in NaCl solutions of different concentration, with or without addition of neutral red

The superficial membranes of yolk spheres have some stickiness exhibited in NaCl solutions, but not in the yolk sac. When two spheres come into touch with each other, they remain mostly in this position. After a while two spheres may fuse into one. At first the double wall in the region of contact dissolves and later the newly formed dumbbell shaped body rounds off. The fusion occurs more

slowly in extremely hypertonic or hypotonic solutions. Neutral red seems to increase the stickiness of the membrane.

Two other dyestuffs, nile blue sulphate and cresyl blue, yield similar results to neutral red; their penetration however across the membrane seems to be slower. One see at first uniform coloration of the sphere and later the darker grains appear. Janus green and trypan red do not stain the spheres at all. In sudan III the spheres become lemon-yellow.

The superficial membrane of the yolk sphere exhibits weak elasticity. They lose their spherical shape and become irregular, oviform or ridged, when the hyper- or isotonic dilutor is stirred violently with the needle. After a time, lasting from a few to several minutes, they acquire the former regular shape. The readjustment occurs sooner in solutions of lower osmotic pressure. In hypotonic solutions the deformation of the yolk spheres occurs only exceptionally, because the water penetrating into the sphere strains the superficial membrane and prevents its deformation.

The yolk spheres, stained or not, burst in the vapours of sulphuric ether and their membrane decay, which prevents the formation of dull discs. The vapours of iodine give to the yolk spheres a lemon colour, which after a fairly long duration of this action turns cherry-red. The superficial membrane does not expand and does not become detached from the coagulated protein contents of the sphere. The spheres treated with iodine remain without further change for four days, at least this was the period during which they were observed.

Betta splendens Regan

The eggs of *Betta* develop like those of *Macropodus* in the nest built up of air bubbles. The male picks up with his mouth the eggs, which fall down after spawning, and spits them out in the direction of the nest. The eggs are at that time gluey and stick to the air bubbles and to each other. They float at the water surface only, when attached to an air bubble, even a small one. Otherwise, being heavier than water, they sink.

The turbid whitish yolk pours freely from the dissected egg into Tyrode's fluid. Under the microscope two morphotic components — fat drops and yolk spheres — are visible. The diameter of fat drops measures 1,5—9 μ (mean 5,7 μ), that of yolk spheres 3—9 μ (mean

6,8 μ). The fat drops are slightly smaller than the yolk spheres and the span of their dimensions oscillates between larger limits. Yolk spheres are considerably more numerous than fat drops. They gather in the lower layer of Tyrode's solution. The specific weight of the spheres equals the weight of the erythrocytes; the two bodies occupy the same level in the dilutor. Erythrocytes appear in preparations derived from the yolk of larvae provided with blood circulation. Fat drops always gather in the upper layer of the dilutor; they are apparently lighter than it.

Fat drops possess a faint yellow tint and are very shiny. They maintain the spherical shape for several days in all used solutions, regardless of whether they float from the side of coverslip or of the air. Their surface must be covered with some emulgator which preserves their spherical shape and prevents their fusion. The fat drops do not react to osmotically active solutions within the range of 1—1/128 M Na Cl, which proves that they do not contain any protein admixture. From all applied dyestuffs only sudan III gives them a lemon colour. No birefringent crystals appear in fat drops even after several days or in preparations kept in the refrigerator at a temperature of +3°C. When the fat drop consits of glycerids with the addition of phospholipids, the latter cannot be easily precipitated.

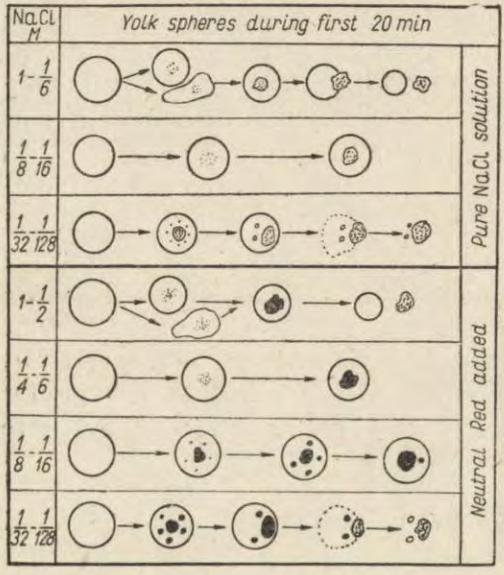

Fig. 4. The behaviour of yolk spheres of *Betta* in NaCl solutions of different concentration, with or without addition of neutral red

Among the yolk spheres of some preparations thin, dull discs are present, wich recall in their appearance flattened fat drops. They stain with sudan III. After some time birefringent crystals may be seen within them in the shape of needles radially arranged.

It is difficult to decide whether these fat bodies derive from the above described fat drops or are of other origin (Fig. 2f).

The yolk spheres, observed in Tyrode's solution, have a regular spherical shape, a predominantly whitish uniform tint and a shining surface. When the drop of dilutor is stirred with a needle, the yolk spheres seized by the whirlpool become irregular, oviform or ridged. After several minutes they acquire a spherical shape. The superficial membrane is not characterized by great elasticity or rigidity. In some yolk spheres darker dots, irregular in shape, are at once present in the freshly made preparation. Similar dots appear in almost all spheres of the yolk poured upon a dry objective glass.

NaCl solutions, of different concentrations, clearly influence the yolk spheres. Hypertonic solutions (1—1/6 M) induce the lasting or transitory deformation of spheres connected with changes in their liquid content. In the first moment tiny fat drops are precipitated from this fluid. Later they coalesce and enclose in small areas between them the protein component of the original content of the sphere. In this way platelets arise, consisting of clustered fat drops with tiny pink dots within (protein fraction). The fat platelet slips out from the sphere, tearing off its wall. The membrane of the sphere persists as a white disc for a long time, as also the platelets, which are still abundant in the preparation after 24 hours. The precipitation of fat and the liberation of platelets occurs quicker and more generally in solutions of higher hypertonicity than 1/6 M.

Isotonic solutions (1/8—1/16 M) precipitate fat in the shape of small droplets. They coalesce as in hypertonic solutions into irregular platelets with pink dots. But they escape only occasionally from interior of the yolk sphere.

1/32—1/128 M solutions are decidedly hypotonic; they change the yolk spheres more distinctly than isotonic fluids. The fat precipitates at once in the shape of numerous droplets, which unite with each other by means of slender bridges. Fusion into one uniform drop never occurs. Later fat plats are formed, with bulging drops and with irregular outlines. Finally, when the superficial membrane bursts, the plates pass into the dilutor. Here they are still visible after 24 hours.

All these transformations of the yolk spheres may be observed more distinctly when neutral red is added to the dilutors. This dyestuff enters the spheres in all solutions from 1—1/128 M, and gives them a uniform red tint. The fat drops precipitated within

the yolk spheres acquire always a darker colour. When the membrane of the sphere bursts and drops or platelets enter the dilutor, they decolorize very soon. From this peculiarity it may be concluded that the colourless platelets present in preparations are not covered by the membrane. This holds true also for platelets in hypertonic solutions. The membranes of the spheres deprived of fat may contain some protein fluid, which binds a certain amount of dyestuff. But when the membrane beginns to distend and deteriorate the tint fades and disappears.

The membrane of the *Betta* yolk spheres is in salt solutions not very elastic and somewhat sticky. The spheres often adhere to each other but only sporadically fuse together. The durability of the membrane is limited, in isotonic solutions a certain number of spheres persist even after 24 hours. In other solutions they are hardly ever seen after this time.

Pterophyllum scalare Cuv. & Val.

Pterophyllum glues the eggs singly to the water plants. When detached from the plant they fall to the bottom; they are then heavier than water. From the egg opened in Tyrode's solution mainly yolk spheres and quite abundant fat drops pour out. Yolk spheres are whitish with a slight shine, without grain within.

Fat drops, characterized by their strong shine, emerge at once to the surface of the Tyrode's solution. Here some of them flatten and become gray and dull, the others remain without any changes. When the coverslip is turned with the preparation upside down, the shining drops pass quickly through the Tyrode's fluid and gather beneath the glass. The flattened drops remain in their original position in one plane with the yolk spheres. This happens either because their specific weight equals the weight of the spheres or because the wide and flat discs are not able to force their way across the drop of dilutor. At present it must remain unsettled whether the two morphotically different fats are chemically identical.

After several minutes needlelike crystals appear in the flattened drops. They gather in bundles arranged radially. In one fat drop, depending upon its size, one or more bundles of crystals are present. The needles themselves are rigid, they may protrude beyond the drop and their tips do not bend or dissolve. Such crystals seldom appear in spherical fat drops. In such cases the needles are shorter

and placed very close to each other. Both crystals exhibit birefringence in polarized light. The rest of the fat, even the drops flattened but without crystals, does not polarize. The needles disappear in vapours of ether or iodine, after the addition of dioxan or when kept in a temperature of $+30^0$ and dissolve without trace in the fat drop. Sudan III gives the fat drops an intensive lemon-yellow colour, vapours of iodine a faint yellow.

The yolk spheres react towards osmotically active solutions. Hypertonic solutions (1—1/6 M) contract and deform them. In the whitish contents of the sphere small pink dots appear. After several minutes or some hours the spheres become round again and lose their dots. These changes occur sooner in fluids of lower osmotic pressure, but always some percentage of spheres remains deformed. In more or less isotonic solution (1/8—1/16 M) the deformation of spheres occurs to a much lesser extent and bears a transitory character. In hypotonic solutions (1/32 and especially 1/64, 1/128 M) small grey dots appear in almost always rounded yolk spheres. The contents of the sphere congeal and stiffen. The membrane slips down from it and persists for a while as a small white disc. The uncovered contents of the sphere form on the other hand a dull yellow-grey plate. In the plate small pink spots are visible, which have a tendency to fuse into bigger vacuoles. They correspond to the protein fraction enclosed within the fatty substance.

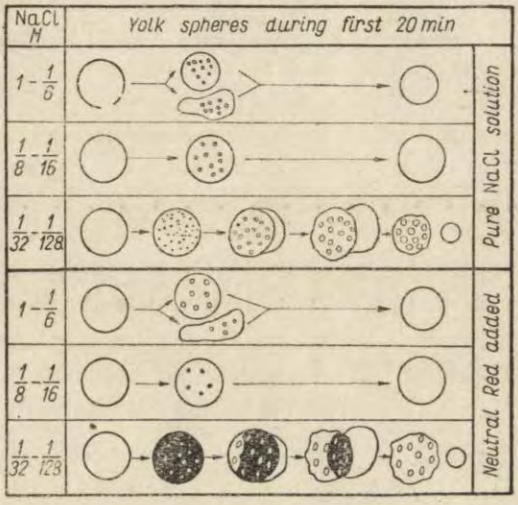

Fig. 5. The behaviour of yolk spheres of *Pterophyllum* in NaCl solutions of different concentration, with or without addition of neutral red

Neutral red, when added to all salt solutions, stains the yolk spheres pink. In hypertonic solutions the yolk spheres stain uniformly, in isotonic ones some spheres possess small dark grains

within. In hypotonic solutions the dark grains are universally present and very distinct. Later they fuse into stiff discs. At the moment when the membrane slips down the congealed contents loses their hue and conforms to similar bodies from unstained preparations.

The yolk spheres are not very stable bodies, disappearing from preparations after 24 hours. In a fresh preparation some spheres stick to each other and sometimes fuse even into one bigger sphere.

In vapours of iodine the yolk spheres become lemon-yellow, and after prolonged exposure cherry-red. The superficial membrane moves away only in exceptional cases and then only a short distance from coagulated contents. This is not the distention of the membrane, rather it recalls the slipping down of the membrane, which however very soon stopped. This phenomenon occures only exceptionally in Tyrode's fluid, more often in 1/32 M NaCl.

The yolk spheres suspended in 1/12 and 1/32 M NaCl solutions do not react to trypan red. But after treatment with vapours of iodine they become red. The vapours probably damage the superficial membrane and make it permeable to the particles of trypan red.

The vapours of sulphuric ether dissolve the superficial membrane and the contents of the yolk sphere disperse as tiny grains. Dioxan stiffens the yolk spheres, when added to the suspension in Tyrode's fluid. Within the sphere no precipitated grains appear nor fat droplets on the surface of the yolk sphere.

DISCUSSION

After having investigated the yolk of the sea trout, bitterling, three species of thermophilic fish, and from other vertebrates the yolk of the sand-lizard, turtle, and hen (Grodziński 1938, 1946 a, b, 1947, 1949 a, b, 1951 a, b) with the aid of similar methods one can try to analyse more closely its qualities. The yolk of the investigated vertebrates is an emulsion of the O/W type. It consists always of watery solution of protein bodies as a continuous phase and of fat drops or yolk spheres as dispersed phase. The yolk spheres are in their turn also emulsions. Apparently the yolk of sea-trout and lizard, characterized by fat drops only, is a simple emulsion, that of other species a multiple emulsion. The yolk of the latter posesses fat drops and yolk spheres. Since the investigated yolks persist in a state

of emulsion for a fairly long period of time, they must be provided with emulgator, which prevents the fusion of the dispersed phase.

The continuous phase, called in fishes ichthulin, is known in two forms. Ichthulin in sea-trout, very abundant, coagulates and becomes turbid in water, in other fish it is scanty and water soluble. The coagulation of ichthulin is a reversible process; the addition of NaCl induces the liquefaction of the coagulated mass and renders it transparent. These qualities probably depend upon the presence of globulins (G r a y 1920, R u n n s t r ö m 1920, G r o d z i ń s k i 1949).

The fat present in the yolk of Teleost fishes does not participate directly in the development of the embryo's body. It plays, at least in some fishes, the role of a hydrostatic organ. In the fertilized egg of the sea-trout the fat drops gather beneath the embryonic disc and thus keep the egg with the disc on trop. The hatched fish with a big yolk sac, lies upon the flank on the bottom of the dish. At this period of life the fat may help to lift the body, when the fish starts for a short swim. The pelagical eggs of fish possess a huge fat drop and float therefore in suitable layer of water. An extreme example of this type are the eggs of *Macropodus*. The presence of a very big drop determines their floating at the water surface and the position of the developing embryo or the young fish.

The fat present in eggs of all investigated fish is in the shape of more or less spherical drops, differing in seize. Whenever they find themselves within the yolk sac, they maintain their shape, shiny surface, and colour. When poured out into salt solution, they emerge to its surface and flatten violently. They turn into flat, grey discs, without any shine. The process occurs in almost all investigated fish.

The fat drops from the yolk of the sea-trout flatten in 1—1/16 M NaCl solutions. In fluids of lower concentrations the coagulating globulins surround the drops and prevent any change in their shape. In the 1/16 M solution the globulins precipitate upon the surface of the flattened drop as irregular white spots, which disappear after several minutes (G r o d z i ń s k i 1947).

In the yolk of other fishes the globulins are absent or scanty, therefore the flattening of fat occurs in all salt solutions and even in water. But the small drops of bitterling, 1—1,5 μ in diameter do not flatten at all. Similarly the fat drops of *Betta*, 1,5—9 μ in diameter, maintain predominantly their spherical shape, but some also

flatten. Many more fat drops flatten in *Pterophyllum*. The big fat drops of *Macropodus*, even when divided into pieces with the needle, flatten always. One may infer that the size of the fat drops plays some role in these processes, — the bigger the drops — the surer the flattening.

The change in shape of fat drops, as a physical phenomenon, is based upon the change of superficial tension. Low tension keeps the fat in spherical shape, a sudden rise in this tension results in the flattening of the drop. Danielli and Harvey (1935) measured the tension at the surface of the fat from the mackerel's egg, placed in sea water or in egg extract. In the first case they obtained the value of 7 dyn/cm, in the second 0,8 dyn/cm. In the fish's egg then some substances are present which lower the tension at the interface between oil and dilutor. The authors are of the opinion that these substances are proteins, probably globulins. In the sea-trout the globulins cover the surface of the fat drops and before they slip down from them into suitable salt solution, the drops are of a spherical shape. In the uncovered fat drop, at direct contact with the salt solution, the tension at the surface rises violantly and flattening occurs also violently. In the yolk of other fish the presence of globulins was not confirmed. When these substances are in fact absent, the role of emulgator may be played by some other substances, for instance lecithin, as in the hen's yolk (Grodziński 1938, 1946). The fat drops of thermophilic fish consist of glycerids and probably of phospholipids, which are dissolved in them. It is not certain whether all fat drops contain phospholipids. In any case, when present, they precipitate in certain conditions in the shape of birefringent crystals. They vary in appearance, being needlelike or platelike. In the yolk of sea-trout the crystals generally appear after treatment with steapsin (lipase) seldom without it. The crystallisation occurs however after some days, in thermophilic fish after several minutes.

No plausible reasons can be found to explain the role of phospholipids in the fat of yolk. Their precipitation depends upon several factors. The main ones are: temperature and the place occupied by the fatty drop, and of secondary importance — the state of the fat drop. The crystals make their appearance below a certain temperature, on condition that the fat is beyond the fish's body. The flattening of the fat drop only accelerates this process. If the crystals could be discovered also in other fish and if their shape and

temperature of precipitation differed, one would be inclined to accept the systematic specificity of the phospholipids described here.

The yolk spheres have a complicated and diverse structure in various representatives of vertebrates. They are as a rule vesicles with the wall built of semipermeable membrane and contain a liquid mixture of protein with fat. This liquid is optically homogeneous in fish's eggs and ovarian eggs of the hen. In the laid eggs of reptiles and birds the yolk spheres contain fat drops varying in size and number. These differences are only apparently fundamental, because the currents of osmotic origin cause precipitation in homogeneous spheres similar fat drops.

The superficial membrane, being semipermeable, lets water through in both directions, that is into the sphere and outside (hyper- and hypotonic solutions). Some basic vital dyestuffs (e. g. neutral red) penetrate across the membrane into the sphere. These substances leave the spheres only when the membrane is damaged. The acid dyestuffs (trypan red) do not enter the undamaged yolk sphere.

The semipermeable membranes e. g. the cell membranes, consist of two chemical components — proteins and phospholipids. The same holds true for the membranes of yolk spheres. In ether vapours, attacking the lipids, the membrane disperses completely. The presence of the protein component is indicated by the stiffening of the membrane in iodine vapours which coagulate these substances. The quantitative relation of both components is different in different animals.

The yolk spheres of the hen or the turtle treated with iodine show at first the coagulation of their liquid content. Later the membrane distends and moves away from the coagulate. The membranes of the fish's yolk spheres do not distend in similar conditions. We attribute the distension of the membrane to the liquefaction of their lipids under the influence of iodine vapours. The membranes of yolk of fishes contain less lipids than those of sauropsids. Similar consequences may be expected from the reactions of yolk spheres treated with strongly hypertonic solutions. Upon the surface of yolk spheres only in sauropsids do the myelin figures, characterictic for phospholipids, appear.

Stickiness characterizes the surface of yolk spheres of fishes, kept in watery NaCl solutions. Owing to it the yolk spheres coalesce

when coming in touch with each other. Later they may fuse into one body. This process occurs in all concentrations of used solutions. The yolk spheres of the hen coalesce only in strongly hypertonic solutions, never in isotonic. The stickiness is attributed to the presence of great quantities of proteins in membrane; they are more abundant in the fish than in the hen.

The yolk spheres show some elasticity, depending upon the tension at their surface. This tension was measured in the yolk spheres of the hen and amounts to 0,009 dyn/cm for yellow yolk and 0,004 dyn/cm for white yolk (P i g o ń 1951). No similar measurements were taken for fishes. Some indications concerning tension at their surface can be concluded from the behaviour of the spheres. When the yolk spheres of the hen, suspended in isotonic solution, are stirred with a needle, they maintain their spherical shape. In similar conditions fish's spheres undergo smaller or greater deformations, which vanish after some time. The tension at the surface of fish's yolk spheres is then lower than in the hens yolk. For similar reasons the membrane of the hen's sphere is more lasting. In isotonic solutions the yolk spheres persist for several days, those of fish decay in the same medium for the most part in 24 hours.

The semipermeable membrane of the yolk spheres recalls in some physicochemical peculiarities the cell membrane. The fundamental difference is that the cell membrane passes gradually into protoplasm. It may be torn off only with the aid of a micromanipulator needle. The membrane of the yolk sphere is much thicker and forms a kind of balloon, whose content pours out easily.

The content of yolk spheres is characterized by a greater diversity than the membranes, but it may be brought to a single common scheme. The ovarian yolk spheres of the hen and the spheres of the ripe eggs of the fish are filled with a homogeneous, shiny, and whitish fluid. The currents of osmotic origin break it down into protein and fatty components. The first form the continuous, the second the dispersed phase of the emulsion. Primarily the liquid of the sphere is a mixture of protein substances and submicroscopical particles of glycerids. This mixture is in a state of unsteady equilibrium or in a state close to oversaturation, because the fat precipitates very easily. This process occurs in ovarian yolk of the hen as a physiological phenomenon, in the yolk of the fish it must be induced experimentally.

The fat drops precipitated with the aid of vital dyes, in isotonic solutions, have a spherical shape and various size. Their number varies also; among the fish they are most numerous in *Betta*. These drops may fuse into larger bodies (drops or plates). In the yolk of the hen they maintain their primordial appearance, being small and numerous in yellow and few but larger in white yolk. Emulgator, probably lecithin, prevents their fusion (Grodziński 1947). The fish's yolk possesses a small amount of emulgator, because the fat drops have a tendency to fuse together.

The fat drops from the yolk spheres are homogeneous in appearance in all investigated animals except turtles. The latter possess in the fat drop irregular birefringent bodies. They liquefy in vapours of sulphuric ether or iodine, and dissolve in the rest of the fat drop. These peculiarities bring them close to the fat drop of *Macropodus*.

The reactions to dyestuffs (sudan III) indicate that the fat precipitated in yolk spheres belongs to glycerids. It contains however an admixture of protein content, which hydrates in hypotonic solutions and is disclosed in the form of pink vacuoles. Fat drops within the yolk spheres in all studied animals are the complexes of protein and fats.

SUMMARY

The yolk of *Macropodus opercularis* L., *Betta splendens* Regan, and *Pterophyllum scalare* Cuv. & Val. was investigated. The yolk was studied when diluted with osmotically different NaCl solutions, or treated with some dyestuffs and chemical reagents.

The yolk of these fish consists of a small amount of liquid ichthulin, a great number of yolk spheres and a variable amount of fat.

The yolk spheres consist of membrane, which forms its wall and of fluid contained within.

The fluid is a mixture of protein substances with submicroscopical particles of fat. The currents of osmotic origin precipitate this fat in the shape of drops or plates. The intensity and quality of this process depends upon the kind of currents (osmotic pressure, presence of dyestuffs) and upon the fish's species.

The membrane of the yolk spheres, as a semipermeable membrane, consists of proteins and phospholipids, the former being in

preponderance. The membrane exhibits in experimental conditions some stickiness, slight elasticity and short durability.

The fat has the shape of small droplets, in *Macropodus* one big drop is present (pelagic egg). Fat drops consist of glycerids with admixture probably of phospholipids. The latter precipitate in the form of birefringent crystals, varying according to investigated species. This phenomenon occurs in drops kept beyond the organism of the fish and in a temperature below $+20^0$C.

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MANCHE REGELMAESSIGKEITEN IN DEM RAUMVERHALTEN VON *PARAMECIUM CAUDATUM* EHRBG. („PERIPHERISCHE REAKTION")

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Die nicht selten auftretende Art des peripherischen Verhaltens eines Tieres, oft als peripherische Reaktion bezeichnet, hat keine reiche Literatur und stellt ein schwach bearbeitetes Problem dar. Diese Reaktion besteht darin, dass manche in einem beschraenkten Raum abgesperrte Tiere sich an den Raumperipherien sammeln, als ob sie den Zentralteil vermeiden moechten. Ein klassisches Beispiel dafuer sind die, sich an den Kulturgefaessperipherien ansetzenden Paramecium-Ketten.

Als erster hat dieses Problem exakt wissenschaftlich D e m b o w s k i (1926) im Laufe seinen Forschungen ueber das Verhalten von *Uca pugilator* behandelt. Wenn sich diese Krabben in einem beschraenkten Raum befinden, graben sie ihre Loecher in der Peripherienzone, trotz der im Gefaess als Hindernis aufgestellten Glasplaettchen.

Es wurde versucht die Zusammenhaeufung von *Paramecium caudatum* an den Kulturgefaessperipherien, auf Grund des Tigmotropismus zu klaeren; die Versuchsergebnisse aber, haben diesem widersprochen.

Wenn wir in ein walzenformiges Gefaess mit Pantoffeltierchen einen kleineren Zylinder hineinstellen (was der Wasserflaeche eine

Ringform gibt — Fig. 1), bzw. ein Plaettchen (Fig. 2) — werden sich die Tiere weiterhin an der Peripherie zusammenhaefen. Dagegen an dem Plaettchen, sowie an der inneren Peripherie des Ringes wird man sie nicht finden. Von diesem Standpunkt aus ist die Kritik der tigmotropischen Konzeption begruendet. Wenn wir also das Bestehen einer eigenartigen Reaktion (peripherische Reaktion genannt) anerkennen — bleibt das Problem ihres Mechanismus zu klaeren.

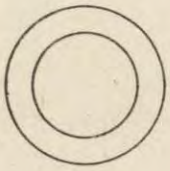

Fig. 1. Querschnitt durch zwei ineinander gestellte Gefässe

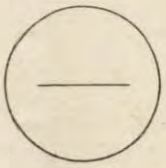

Fig. 2. Querschnitt durch das zylindrische Gefaess mit hineingestellter Glasplatte

Die Arbeit von D e m b o w s k i (1921—22) ueber die Bewegung der *Paramecium caudatum* in ungleichfoermigen Gefaessen kann das Problem der peripherischen Reaktion ins neue Licht setzen. Den dort enthaltenen Angaben gemaess, die auch durch die Beobachtungen der anderen Forscher bestaetigt wurden, prallt das Paramecium von dem begegneten Hindernis unter dem Winkel von 70^0 im Verhaeltnis zur senkrechten Linie des Hindernises ab (im Falle eines Bogens — im Verhaeltnis zu seinem Radius).

Die Vorversuche der Autoren erwiesen, dass im *Paramecium*-kultur enthaltenden Kolbenhals, dicht unter der Fluessigkeitsflaeche (in Bedingungen der guten Saettigung mit Sauerstoff) die Bewegung der Pantoffeltierchen regelmaessig ist und den, von D e m b o w s k i fuer seichte Gefaesse angewiesenen Verhaeltnissen, entspricht. Wenn die freie Kulturflaeche gering und kreisfoermig ist, schreiben die Infusorien ihren Weg in Form eines Achtecks ein. Auf Grund diesen Angaben kann man vermuten, dass die sogen. peripherische Reaktion durch einfache Bewegungsregelmaessigkeiten des Pantoffeltierchens bedingt ist. Die Paramecium haeufen sich an den walzfoermigen Gefaessperipherien, und nicht in seiner Mitte, zusammen, denn bei Beruecksichtigung der konstanten Groesse des Reflexionswinkels, die Zone ihrer massenhaften Unterbringung, nur ein Ring, von Gefaessperipherie und von der in Achteck

des Parameciumsweges eingeschriebene Kreisperipherie gebildet, sein kann (Fig. 3).

Im Zusammenhang damit koennte sich die konstante Kette der sitzenden Infusorien nur an der Peripherie bilden, denn nur dort kann das Tier, waehrend seines Weges auf die Wand stossen. Im Prinzip sollten die Infusorien sich an der Flaeche des ins Gefaess hineingestellten Zylinders bzw. Plaettchens nicht ansetzen, da eine minimale Aussicht ihres Treffens dorthin besteht.

Fig. 3. Grafisches Schema des Weges welchen das *Paramecium* beschreibt wenn der Umfang des inneren Gefaesses zustaendig ist dem in dem aeusseren Zylinder eingeschriebene Achteck

Wenn aber die äussere Flaeche des eingestellten Zylinders sich der Gefaessperipherie naehert, so wie es fuer den Zylinder die Fig. 4 darstellt, sollten solche Verhaeltnisse nicht vorkommen. Dann soll die Kette der angesetzten Infusorien an beiden Flaecheperipherien sich bilden da die Aussicht des Treffens eines einzelnen Individuums gleich ist.

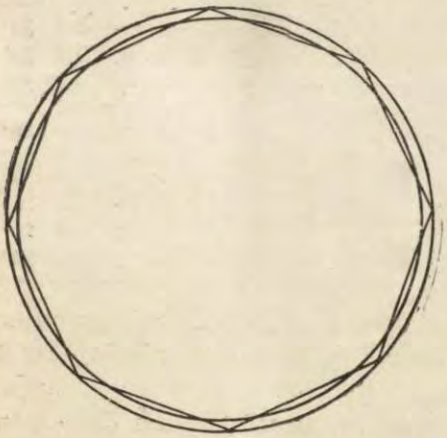

Fig. 4. Grafisches Schema des Weges welchen das *Paramecium* beschreibt wenn der Umfang des inneren Zylinders das in den aeusseren Zylinder eingeschriebene Achteck durchschneidet

Infolgedessen ist das Auftreten einer Kette an der inneren Peripherie des Gefaesses mit einem ringfoermigen Durchschnitt, von einem einfachen Zahlverhaeltnis der Radien der beiden Kreise $r_1 : r_2$ bedingt.

Die Abbildung 5 (Fig. 5) stellt die Grenzverhaeltnisse dar, d. h. solche, dass die Vergroesserung diesen Verhaeltnisse eine Infusorienansetzung an der inneren Peripherie veranlassen soll, und Verkleinerung diesen Verhaeltnisse dieser Ansetzung stoeren. Dieser Verhaeltnisgrenzwert $r_1 : r_2$ wird von dem konstanten Reflexions-

winkelswert $\alpha = 70^0$ bestimmt. Infolgedessen betraegt der Winkel β, zwischen beiden Radien, in diesem Falle 20^0 und das Radiusverhaeltnis cos 20^0. Es ist also zu vermuten, dass die Kette der angesetzten Infusorien wird, wenn das Verhaeltnis $r_1 : r_2$ groesser als cos 20^0 ist, an den beiden Peripherien auftreten, und wenn das Verhaeltnis $r_1 : r_2$ kleiner als cos 20^0 ist, wird sie nicht vorkommen.

Tabelle I

Das Auftreten der peripherischen Reaktion an den inneren Peripherien der Ringe verschiedener Groessen

\+ — Kettevorkommen, ± — Vorkommen einzelner Infusorien, — Mangel an Infusorien

Serie	$2r_1$	$2r_2$	1	2	3	4	5	6	7	8	9	10	$r_1 : r_2$
I	16,7	18,7	+	+	+	+	+	+	+	+	+	+	> cos 20^0
	15,4	18,7	±	±	±	+	±	±	±	+	±	±	< cos 20^0
	11,7	18,7	—	±	—	—	—	—	—	—	—	—	< cos 20^0
	6,6	18,7	—	—	—	—	—	—	—	—	—	—	< cos 20^0
	3,7	18,7	—	—	—	—	—	—	—	—	—	—	< cos 20^0
II	13,8	14.8	+	+	+	+	+	+	+	+	+	+	> cos 20^0
	10,5	14,8	±	—	±	±	—	—	—	±	—	—	< cos 20^0
	6,6	14,8	—	—	—	—	—	—	—	—	—	—	< cos 20^0
	3,7	14,8	—	—	—	—	—	—	—	—	—	—	< cos 20^0
III	9,6	10,5	+	+	+	+	+	+	+	+	+	+	> cos 20^0
	6,6	10,5	—	—	—	—	—	±	—	—	—	—	< cos 20^0
	3,7	10,5	—	—	—	—	—	—	—	—	—	—	< cos 20^0
IV	6,0	6,6	+	+	+	+	+	+	+	+	+	+	> cos 20^0
	3,7	6,6	—	—	—	—	—	—	—	—	—	—	< cos 20^0
V	41,2	44,9	+	±	+	+	±	+	+	—	+	+	> cos 20^0
	38,8	44,9	±	—	±	+	—	—	±	—	+	+	< cos 20^0
	32,0	44,9	—	—	—	±	—	±	—	—	—	—	< cos 20^0

Dasselbe soll stattfinden im Falle des zentral hineingestellten Plaettchens, wenn unter r_1 die Haelfte seiner Breite hineingestellt wird. So stellt sich die Annahme, die den Mechanismus der sogen. peripherischen Reaktion zu erklaeren versucht, dar, die dann von den Autoren experimental geprueft wird.

Die Parameciumkultur wurde in Kolben von Druchmessern ($2r_2$) 18,7 mm, 14,8 mm, 10,5 mm, 6,6 mm und 44,9 mm mit Zugabe von etwas Leitungswasser eingegossen und dann wurden diese Kolben, um ein intensives Bild durch gleichzeitige Ausnutzung der

geotropischen Erscheinung zu erhalten, einige Zeit geschuettelt. Nachdem wurden in die Kolbenhaelser die Glasroehrchen von entsprechend kleineren aeusseren Durchmessern ($2r_1$) hineingestellt und es wurde die Bildung der Ketten der angesetzten Infusorien an den inneren Peripherien festgestellt. Den schematischen Durchschnitt zeigt die Fig. 1 und die Versuchsergebnisse die Tabelle I.

Die Ergebnisse der Experimente ausser dem letzten weisen deutlich darauf hin, dass die Ketten der angesetzten Infusorien immer an der aeusseren Peripherie auftreten; erscheinen aber auch an der inneren wenn das Radiusverhaeltnis der beiden Kreise den Grenzwert ueberschreitet ($r_1 : r_2$ groesser als cos 20^0). Infolgedessen werden nur dann zwei Ketten entstehen, wann der Ring so schmal ist, dass das *Paramecium* nicht im Stande ist in derem einen Achteck einzuschreiben, denn waehrend des Weges es nicht nur die aeussere, aber auch die innere Gefaesswand trifft. Diese Verhaeltnisse ziemlich deutlich in den zwei ersten Versuchsserien, verschaerfen sich noch in der dritten und vierten Serie, dagegen ver-

Tabelle II

Ansetzung der Pantoffeltierchen an dem ins walzenfoermiges Gefaess zentral hineingestellten Plaettchen

$2r_1$	$2r_2$	1	2	3	4	5	6	7	8	9	10	$r_1 : r_2$
26,0	44,9	—	—	—	—	—	—	—	—	—	—	$< \cos 20^0$
26,0	36,5	—	±	—	±	—	—	—	—	±	—	$< \cos 20^0$
26,0	28,6	+	+	+	+	+	+	+	+	+	+	$> \cos 20^0$

(Bezeichnung wie in der Tabelle I)

wischen sich sichtbar in der 5. Serie. Dies ist vollstaendig uebereinstimmend mit der Annahme, die die peripherische Reaktion auf Grund der Reflexionswinkelsbestaendigkeit klaert, unter Beruecksichtigung dass, laut D e m b o w s k i (1921—22), die hier bindende Bewegungsregelmaessigkeiten deutlich in kleinen Gefaessen auftreten und in groesseren gestoert werden.

Die am Anfang geaeusserte Annahme ueber den Mechanismus der sogen. peripherischen Reaktion bei *Paramecium,* wurden durch die Versuchsergebnisse ueber seine Kultivierung in Gefaessen mit ringfoermigen Durchschnitten, bestaetigt. Um diese zu ergaenzen

wurden folgende Versuche durchgefuehrt: In ein walzfoermiges Gefaess mit Pantoffeltierchen stellte man zentral ein Glasplaettchen hinein (Fig. 2). Dieses Plaettchen mit Durchmesser von 26,0 mm ($r_1 = 13$ mm) wurde abwechselnd in drei Gefaessen mit inneren Durchmessern von 44,9 mm, 36,5 mm und 28,6 mm hineingestellt.

Diese Versuchstechnik entspricht vollstaendig der vorigen. Die Ergebnisse zeigt die Tabelle II.

Wie daraus folgt, gibt es keine Anhaeufung der Infusorien an dem Plaettchen, wenn das Verhaeltnis $r_1 : r_2$ kleiner als cos 20° ist. Im Falle wenn das Verhaeltnis groesser als cos 20° ist, erscheint die Anhaeufung, aber nur an den Plaettchenraendern. Dies ist in voller Uebereinstimmung mit den Beobachtungen waehrend der vorigen Versuchen.

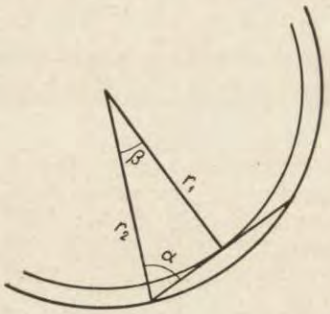

Fig. 5. Die Grenzgroesse des Radiusverhaeltnises $r^1 : r^2 = \cos 20^0$

Die angefuehrten Angaben betreffen die Periode, waehrend welcher die Ketten der angesetzten Infusorien in der Kultur deutlich merkbar sind. Dies kommt in einigen bzw. einige zehn Minuten vom Anfang des Versuches vor. Es wurde aber vielmals in der Experiment-Anfangsphase beobachtet eine schwache Erscheinung der angesetzen Infusorien an den Roehrchen oder Plaettchenflaechen in den Orten die naeher der Gefaessmitte sind und das Radiusverhaeltnis viel kleiner als cos 20° haben. Dem Anschein zuwider entheben diese Tatsachen nicht die am Anfang vorgebrachte Annahme, sondern bekraeftigen sie. Solche „unregelmaessige" Anhaeufungen sind immer schwach, koennen am Anfang des Versuches auftreten und zerstreuen sich sehr schnell. Den Beobachtungen von D e m b o w s k i (1921—22) gemaess faengt die regelmaessige Bewegung der *Paramecium* in einem Gefaess erst nach einer gewisser Zeit an. Die beobachteten Anhaeufungen entsprechen wahrscheinlichst der kurzdauernden Anfagsphase der ungeordneten Bewegung, die auftretet bis das Milieu genuegend und gleichmaessig mit Sauerstoff gesaettigt ist. In dieser Periode haben die unregelmaessig schwimmenden Infusorien gleiche Aussicht an alle Gefaesswaende zu treffen. Dieses Stadium geht schnell vorueber und est befestigen sich die oben beschrieben Verhaeltnisse.

Paramecium, das sich unter dem konstanten Winkel von 70° in den vieleckigen Gefaessen, mit Winkeln groessern als 70° abprallt, beschreibt einen der Gefaessform entsprechenden Weg (z, B, Quadrat). In diesem Falle ist die Wahrscheinlichkeit des Zusammentreffens der Infusorien mit der Wand fuer jeden Peripherienpunkt dieselbe. Daher soll in einem Gefaess von Quadratform eine gleichmaessige Kette der, die ganze Gefaessperipherie entlang, angesetzten Infusorien, auftreten. Wenn die Ecken der gegebenen Figur (z. B. eines gleichseitigen Dreiecks) kleiner als 70° sind, werden sie eine Falle fuer die Tierchen bilden und die Zusammenstoesse der Tierchen mit den Gefaesswaenden, von aehnlichen Durchschnitt, werden vor allem in den Ecken stattfinden. In solchem Gefaess soll die Kette der angesetzten Infusorien am schwaechsten in den Zentralteilen jeder Seite und am staerksten in den Ecken sein.

Fig. 6. Die Ansammlung der Infusorien in einem Gefaess mit dem Querschnitt eines gleichseitigen Dreiecks

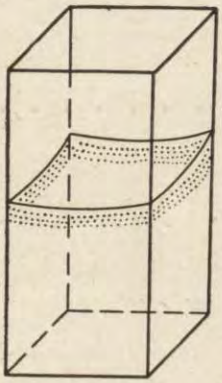

Fig. 7. Die Ansammlung der Infusorien in einem Gefaess mit dem Querschnitt eines Quadrates

Fig. 8. Schema des grossen Gefaesses mit hineingestellter Platte, welche die Form eines Halbzylinders hat

Dieses bestaetigen vollstaendig die diesen Angaben gemaess durchgefuehrten Versuche. Im einen Gefaess vom Quadratdurchschnitt (Fig. 7) setzen sich die Infusorien in einer gleichmaessigen Kette die ganze Peripherie entlang, an. In einem anderartigen Gefaess (Fig. 6), ist die Kette sehr schwach abgezeichnet, dagegen in den Ecken bilden sich sehr dichte und weite Anhaeufungen, die ohne weiteres aus Mangel am Platz die Gestallt „herabhaengender

Bindfaeden" annehmen. Diese Versuche weisen ueberzeugend darauf hin, dass die Infusorien sich dort ansetzen, wo sie die geometrischen Regelmaessigkeiten ihrer Bewegung lenken.

Zusaetzlich werden von den Autoren die Versuche in groesseren Gefaessen, die die Bewegungsmoeglichkeiten des Parameciums nicht begrenzen, gefuehrt. In ein ziemlich grosses Aquarium, Parameciumkultur enthaltend, wurde ein Glasplaettchen in eine Halbwalzenform gebogen (Fig. 8) hineingestellt. Wenn die Annahme, die die peripherische Reaktion mit der Reflexionswinkelsbestaendigkeit verbindet, richtig waere, so sollte sich an der inneren Seite des Halbwalzens eine schwache Kette bilden (Kreis ist nicht geschlossen), und an der aeusseren Seite sollte ueberhaupt keine Kette auftreten. Dieses ergibt sich aus der Wahrscheinlichkeit des Zusammenstossens von einzelnen Individuen mit den beiden Plaettchenflaechen. Es wurde die Parameciumansetzung an der gewoelbten Seite wirklich nicht bemerkt, dagegen an der hohlen Seite, zwar kleine, aber trat immer vor. (ca 30 Individuen/1 cm^2 der Flaeche fuer die Kultur die in 1 cm^3 ca 1000 Individuen enthaelt). Alle, ohne Ausnahme, mit *Paramecium caudatum* duchgefuehrten Versuche haben die Annahmen bestaetigt, dass die sogen. peripherische Reaktion, bei diesem Organismus, von der Winkelsbestaendigkeit der Reflexion von dem begegneten Hindernis bedingt, ein Efekt der Bewegungsregelmaessigkeit ist. Diese Versuche beweisen, dass die sogen. peripherische Reaktion nicht auf einer besonderen „Freiheitsstrebung" des Tieres beruht, sondern auf einfachen Verhaeltnissen. Diese Reaktion, deren Grund bei anderen Tieren im Tigmotropismus oft liegt, ist im Falle des *Paramecium caudatum* eine Konsequenz der geometrischen Bewegungsregelmaessigkeit des Tieres. Die, von dem begegneten Hindernis, unter dem Winkel von 70^0 abprallenden *Paramecien*, haeufen sich zusammen in der Peripherienzone, die genau durch die Reflexionswinkelsgroesse bezeichnet ist (Verhaeltnis $r_1 : r_2$ groesser als cos 20^0).

Das Auftreten der peripherischen Reaktion in einer reinen Form kommt selten vor. Sie laesst sich sehr leicht durch die chemische, thermische, Futter- u. a. Faktoren zu stoeren. Klare Ergebnisse werden nur bei ganz reinen Versuchen und weitgehender Elimination der Zusatzfaktoren erreicht.

Infolgedessen scheint die peripherische Reaktion ein Sympthom der ganz einfachen und ursprunglichen Relationen zu sein. Sie ist auch nicht leicht zu absondern, besonders bei den evolutionsfort-

geschrittenen Organismen, wo, im Zusammenhang mit der progressiven Komplikation des Verhaltens, die Anzahl und Vielfaelltigkeit der sie verwischenden Faktoren vermehrt sich.

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AUTOPROTECTION IN *PARAMECIUM CAUDATUM* BY INFLUENCING THE CHEMICAL PROPERTIES OF ITS MEDIUM

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We consider the three ways of autoprotection of infusory *Paramecium caudatum* against changes of chemical properties of the medium: influencing the chemical medium, isolation from influence of medium and adaptation to medium agents.

We begin the investigations on autoprotection of paramecia by influencing the medium with an analysis of regularities of the toxic action of some substances and the resistance phenomena of infusoria. In the second part we investigate the changes occuring in the media of cultures and their causes. In the third part we compare the reactions of dispersed and condensed cultures. The reactions of dense aggregations are connected with processes of the organism influence exerted on the medium and is of great importance in evolutionary synthesis.

RESISTANCE OF INFUSORIA TO CHEMICAL AGENTS

Regularities of the toxic actions of salts of inorganic and organic acids, of alcaloids, organic stains were examined as well as the changes of pH and rH and the contents of waste products of metabolism in the medium. Infusories were bred in cultures fed with

dry milk powder. For experimental purposes they were taken from the bulk of the culture liquid and from the thigmotropic agglomeration in such a proportion as to obtain the sample density of about 500 Protozoa in 1 ml. Liquid with infusories was mixed (1 : 1) with the experimental solution of a concentration exceeding twice the concentration required. The highest concentration of the substance which did not cause the death of the Protozoa after 24 hours was regarded as the toxic limit. When the differences between survival times of individual infusoria were very great, we chose as the toxic limit the concentration killing 50% of the individuals within 24 hours.

The concentrations of alcaloids and stains are calculated in weight proportions, those of other substances in mM per litre.

Ionic regularities of toxicity

In order to distinguish between the roles played by cations and anions in the salt toxicity, the resistance of infusoria to 15 chlorides and to 20 sodium salts was examined. One ion Cl^- as a constant in the cation series was chosen and one ion Na^+ in the anion series, in consideration of the small toxicity of these ions and the high solubility of almost all of their salts.

Table I gives the toxic limits of chlorides investigated.

Table I

Toxic limits of chlorides of different metals in mM per 1 litre

LiCL 50	MnCl 2	$SrCl_2$ 30
NaCl 50	$CoCl_2$ 0,25	$CdCl_2$ 0,03
$MgCl_2$ 50	$NiCl_2$ 0,008	$BaCl_2$ 3
KCl 45	$CuCl_2$ 0,003	$HgCl_2$ 0,0005
$CaCl_2$ 45	$ZnCl_2$ 0,045	$PbCl_2$ 0,03

Results of analogical investigations of sodium salts are given in the table II.

All sodium salts, no matter of what kind of anion, prove to be only slightly toxic in comparison with many chlorides. Salt toxicity depends chiefly on the kind of cation. This conclusion corresponds to the results obtained by Dryl (1952) for chemotropism and is explained by the slight dispersion of the toxic limits for anions (from

60 to 0,5 mM/l), and huge dispersion for cations (from 50 to 0,0005 mM/l). This thesis is confirmed by the effect of cyanides. NaCN and KCN are harmless to paramecia even in high concentrations (about 10 mM/l). Physiologically neutral $Hg(CN)_2$ is deadly to paramecia in concentrations somewhat surpassing 0,01 mM/l. This proves the decisive role of cation and the secondary role of anion in the toxicology of *Paramecium caudatum*.

Table II

Toxic limits of different sodium salts in mM per 1 litre

NaF 20	Na_2SO_4 45	Na_3AsO_4 15
NaCl 50	Na_2CrO_4 25	Na_2CO_3 5
NaBr 55	Na_2WO_4 30	$NaBO_2$ 20
NaJ 60	$NaNO_2$ 50	NaCN 10
Na_2S 0,5	$NaNO_3$ 55	NaCNS 15
$Na_2S_2O_3$ 30	Na_2HPO_4 20	$Na_2Fe(CN)_5NO$ . . . 35
Na_2SO_3 15	$NaAsO_2$ 5	

This rule is valid for many organic substances. The comparison of toxicity of acetic salts of sodium, potassium, copper and lead with toxicity of sodium formate, acetate, oxalate and citrate showed that the toxicity series of acetates agrees with that established for chlorides, and the differences in the degrees of toxicity in these series are very great while for anion series they are much smaller.

Alcaloid salts in which a molecule of alcaloid functions as a cation have also shown great differences in toxicity (concentrations 1 : 100 for morphine and 1 : 120 000 for quinine in weight proportions).

The determination of the toxicity of organic stains gave consistent results. Three acid stains and seven basic stains were examined. The thesis regarding the slight toxicity of acid stains, well known in the literature of the subject, was also confirmed (1 : 250 for Congo red and 1 : 1 000 for Orange G) and also that of the high toxicity of basic stains (1 : 60 000 for methyl-green and 1 : 4 000 000 for Nile-blue).

But the division of stains into acid and basic ones chiefly known in cytology is not strictly chemical and seems to be physiologically incorrect. This is also suggested by M a k a r o w (1948). We would formulate the said principe differently: The toxicity of the stain depends on the character of the charge of the coloured ion.

Salts with coloured cation are characterised by a strong toxic action, while salts with coloured anion are only slightly toxic. This thesis is confirmed by the reaction of infusoria to solutions of fuchsine, not investigated by us, which in cationic form is deadly in action in a concentration lower than 1 : 25 000 (B a l l, 1927), in anionic form, however, it is harmless in a concentration of about 1 : 100 (C h e j f e c, 1937).

Table III

The role of cation and anion in the toxicity of compounds of salt type

Toxicity of cations			Toxicity of anions		
Group ot compounds	min.	max.	Group of compounds	min.	max.
Chlorides	50 mM/1	0,0005 mM/1	Sodium inorganic salts	60 mM/1	0,5 mM/1
Acetates	40 mM/1	0,002 mM/1	Sodium organic salts	65 mM/1	20 mM/1
Cationic azo-dyes	1:60 000	1 : 400 0000	Anionic azo-dyes	1 : 250	1 : 1 000
Alcaloids	1 : 100	1 : 120 000	—	—	—

In table III we compare the toxic action of different substances depending on the character of the charge of the acting ion taking into consideration the minimal (min.) and maximal (max.) toxicity in each group of compounds.

Taking inorganic sodium salts as an example we will discuss the small differences noticed in the action of different anions. Differences in the toxicity of sodium salts enable us to drawn up the following toxicity serie viz:

$$S^{--} > CO_3^{--} \geqslant AsO_2^{-} > CN^{-} > SO_3^{--} \geqslant CNS^{-} \geqslant AsO_4^{---} > BO_2^{-} \geqslant HPO_4^{--} \geqslant F^{-} > CrO_4^{--} > WO_4^{--} \geqslant S_2O_3^{--} > Fe(CN)_5NO^{--} > SO_4^{--} > Cl^{-} \geqslant NO_2^{-} > Br^{-} \geqslant NO_3^{-} > J^{-}$$

In this series sulphide and carbonate are the most toxic. The strong toxic action of sulphide is explained by its strong hydrolysis causing high pH (= 13 for concentration 0,1 M/l). The 0,1 M solution of Na_2CO_3 is also characterised by its strong alkaline reaction (pH = 11,6). A slight change of pH explains the low toxicity of substances usually so strongly toxic as nitroprusside, chromate and tungstate. These facts prove that the differences in anion toxicity depend largely on the reaction of the medium.

Table IV
Correlation betwen the pH of the 0,1 M sodium salt solutions and their toxicity

	pH < 8	8 < pH < 10	pH > 10
>30 mM/l	NaCl, NaBr, NaJ, $Na_2S_2O_3$, Na_2SO_4, $NaNO_2$, $NaNO_3$, $Na_2Fe(CN)_5NO$		
10—30 mM/l	NaF	Na_2SO_3, Na_2CrO_4, Na_2WO_4, Na_2HPO_4, Na_3AsO_4, $NaBO_2$, NaCNS	
<10 mM/l			NaCN, $NaAsO_2$, Na_2CO_3, Na_2S

pH of 0,1 M solutions of the examined salts was determined by use of a potentiometer and the correlation table (table IV) was drawn up. A neutral (below 8), slightly alcaline (between 8 and 10) and strongly alcaline (above 10) pH was distinguished; it never fell below 6. The intensity of toxic action was also determined in three ranges: lethal concentration above 30 mM/l, between 30 and 10 mM/l and below 10 mM/l. Correlation is quite distinct — from among 20 salts only one compound NaF does not show it. Toxicity series of the organic sodium salts also shows correlation with the dissociation constants of particular acids.

In certain cases a slight influence of anion itself on toxicity of the salt was noticed. It may be found in neutral salts. So, for example, a toxicity series of sodium compounds with halogens may be

established: NaF> NaCl> NaBr> NaJ. In this case small differences in toxic action are arranged according to atomic weights.

The ionic regularities of toxicity result from the facts quoted: toxicity depends primarily on the kind of cation and secondly on the hydrogen-ion concentration. The specific action of the anion itself is very small and is only sometimes noticeable in solutions of neutral salts.

Toxic action of cations

Toxicity series of cations combined with ion Cl may be established as follows:

$$Hg^{++} > Cu^{++} > Ni^{++} > Cd^{++} \geqslant Pb^{++} > Zn^{++} > Co^{++} > Mn^{++} > Ba^{++} > Sr^{++} > K^{+} \geqslant Ca^{++} > Mg^{++} \geqslant Na^{+} \geqslant Li^{+}$$

This series is not subject to any change when acetate ion is substituted for the chloride. The alkaline earth metals may be considered as the least and the heavy metals, particularly Hg, Cu and Ni — the most toxic. Woodruff and Bunzel (1909) consider that the toxicity depends on the ionic potential of metals. We give below lethal concentrations arranged not according to atomic weights but in accordance with the ionic potential series:

50	45	3	45	50	30	50	2
Li^{+}	K^{+}	Ba^{++}	Ca^{++}	Na^{+}	Sr^{++}	Mg^{++}	Mn^{++}
0.045	0.03	0.25	0.008	0.03	0.003	0.0005	
Zn^{++}	Cd^{++}	Co^{++}	Ni^{++}	Pb^{++}	Cu^{++}	Hg^{++}	

It appears that the said regularity exists within broad borders and may be considered as a general rule.

The examined heterocyclic poisons: alcaloids and histamine gave the following toxity series: quinine> strychnine> nicotine> scopolamine≥ atropine> morphine≥ histamine. This series forms an almost entire reversal of the toxicity series of alkaloids established for mammals. Beside the strong action of plasmatic poisons the absolute harmlessness of the potent poisons of the nervous system is striking. A comparison of weight proportions shows that histamine and morphine do not act so strongly on paramecium as for example NaCl.

These facts are important in the study of the physiology of conductive fibrilla system in Protozoa.

We give below a table of lethal concentrations of the examined organic stains (table V). These stains are arranged according to their

degree of toxicity and the first seven substances are characterised by their coloured cation.

The lethal concentrations obtained by us for stains previously examined by other authors generally agree with those given by more modern literature particularly by Ball (1927) and Chejfec (1937). Only the methylene-blue toxic concentration found by Chejfec (1937) is, we consider, incorrect. We base our opinion on the fact that our own result conforms to that of Ball (1927), and which is more significant, it is nearer the toxic limits of the similar stain — toluidine blue.

The dependence of toxicity on the kind of coloured cation is approximately illustrated by the following series: oxyazines> thiazines≥ azines> amidazines> phenylmethanes.

This series is probably connected with the degree of affinity of stains to lipids, which degree is measured by the intensity of the lipid colouration and solubility in lipid solvents. This explains the

Table V

Lethal concentrations of some organic stains (in weight proportions)

Nile-blue . . .	1 : 4 000 000	Pyronine . . .	1 : 75 000
Toluidine-blue .	1 : 200 000	Methyl-green .	1 : 60 000
Neutral-red. .	1 : 180 000	Orange G . .	1 : 1 000
Methylene-blue	1 : 90 000	Haematoxyline .	1 : 500
Bismarck-brown	1 : 80 000	Congo-red. . .	1 : 250

fact that a typical lipid stain such as Nile-blue is the strongest poison among the known drugs for paramecium and that the toxicity of pyronine and methyl-green compounds insoluble in lipid solvents is considerably decreased. Such an explanation would confirm and widen Nirenstein's opinions (1920) and seems probable on account of the various penetration abilities of these stains through the pellicle, it is not, however, absolutely proved as the number of compounds investigated by us was small. It is certain, however, that the organic stains confirm excellently the ionic regularities of toxicity.

Resistance to the pH-changes of the medium

pH ranges given in literature as resistance limits of *Paramecium caudatum* are striking in their discordance. Morea (1927)

obtained a range 6,0—9,5, Darby (1930) — 5,3—8,2, Gaw (1936) — 5,8—8,9 and Wichterman (1948) reduces this range to 6,2—7,2. Górski (1938) obtained for tartaric acid the value 4,6 and for acetic acid — 5,8. The cause of their divergence is probably the complexity of the pH actions on infusoria. Our first experiment proved that resistance depends on the state of the culture, its age, kind of medium etc.

In order to eliminate secondary influences we used in each experimental series infusoria taken simultaneously from the same culture, and pH changes were defined in buffer media: $C_6H_4(COO_2)$ HK + NaOH, $Na_2B_4O_7$ + HCl, $Na_2B_4O_7$ + Na OH. pH was measured at the beginning and at the end of the experiment by use of a potentiometer. In acid media the survival times diminish slowly together with the fall of pH, e. g. when pH = 4,28 death occurs after 30 minutes, when pH = 4,40 after 70 minutes, when pH = 6,64 — 10% of the Infusories survive for 12 hours and with pH = 4,68 after 24 hours 50% survive. In alkaline media the difference is marked: when pH = 9,12 all infusoria survive after 24 hours and when pH = 9,22 all of them perish after 2 hours.

The use of buffer solutions removes differences of resistance between infusoria of various origin (they do not exceed 0,1 of pH value). This shows that the divergences depend on the culture media. The range of survival so obtained comes within the limits of pH = = 4,68 to 9,16.

For further analysis of the influence of pH changes the resistance to 10 acids and 5 bases has been examined. The inorganic acids (HCl, HNO_3 and H_2SO_4) were used as well as organic acids, (such as formic, acetic, lactic, oxaslic, succinic, malic and tartaric). Before experiment the infusoria were washed with distilled water. The lethal concentration was established first only for the sake of orientation by adding to the liquid with Infusoria some dissolved acid from the microburette. Then the occurate dilution series were prepared. 5 ml of the liquid with Infusoria were mixed with 5 ml of each solution and the lethal concentration was looked for. pH was measured by use of a potentiometer immediately after mixing and also at the end of the experiment.

All the acids were determined at the same time using Infusoria from the same culture. The measurements were repeated ten times. Different infusoria have shown considerable differences of resistance to the same acid (up to 1 pH unit). As these oscillations of re-

sistance appear in nonbuffered media and disappear when buffers are used, it may be considered that they depend on various peculariities in the medium of the original culture. Probably the direct influence causing the neutralisation and increase in the buffer capacity of the medium are of importance as are also the indirect ones which influence Infusoria before the experiment. Consequently the experiments are in principle unrepeatable and that is probably the chief cause of the divergence of results obtained by the authors previously quoted. Also in case of bases (NaOH, KOH, $Ca(OH)_2$, $Ba(OH)_2$ and AgOH) nonbuffered medium makes the experiments unrepeatable.

The experiments however are repeatable and comparable for infusoria from the same culture. It is shown that although different Infusories respond variously to the same acid yet the same infusoria respond in the same manner to different acids. Quite independently of the chemical nature of the anions present the infusoria perish in the same concentrations of hydrogen ions. The average range of resistance differences is only 0,16 pH. It rises however to as much as 1,76 pH for bases. So consequently the same Infusoria respond variously to different bases and in this case the role of the chemical nature of ions interferes seriously with the role of pH. These data are the natural supplement of ionic regularities of the toxic action. G ó r s k i (1938) reached different conclusions but he applied only two acids and did not compare their action on the same infusoria.

We have calculated the general average of lethal concentration for all acids and for all bases. pH range found in this way is 4,62—9,03, so it corresponds strictly enough to the range 4,68—9,16 obtained by applying buffer solutions. In all acidified media a slackening of the movements of infusoria took place, and in all alkalised media — an acceleration of short duration. In basic solutions a ciliary reversal always took place. This phenomenon is not characteristic for any acid. However, it was observed in varying intensity in solutions of all salt compounds.

A c t i o n o f o t h e r a g e n t s

The relation of *Paramecium caudatum* to redox potential changes of the medium is a difficult and completely uninvestigated problem. Only E f i m o f f, N i e k r a s o f f and E f i m o f f (1928) took this agent into consideration, but only from the point

of view of potential changes connected with pH changes. In our opinion the resistance of infusoria to reducing substances is considerably higher than to oxidizing substances. This results from our data in the toxicity of particular salts, acids, bases and azo-stains and especially from a certain relation of the ion toxicity to its E_0 value. This is also confirmed by ecological works affirming the presence of *Paramecium caudatum* in waters of a markedly low rH (see Jahn 1934 and Wichtermann 1953) as well as by experimental investigations on anaerobic conditions (see Brand 1946 and Wichterman 1953) which support the theory of anaerobiosis (Lindeman 1942).

Cationic stains are strongly condensed by Infusoria in food vacuoles and then deposited in cytoplasm in a harmless form (Grębecki and Kuźnicki 1955b). This permits the determination of intracellular rH by a strictly vital method. Neutral-red, Nile-blue and Capri-blue are never reduced. The degree of reduction of methylene-blue, toluidine-blue and thionine is variable and depends on the physiological condition of the Infusories; consequently intracellular E_H lies within the E_0 limits of these stains. Accepting Makarow's (1948) pH of the cytoplasm and Hewitt's (1950) methods of calculations we get the rH of the protoplasm in the range of 13—18. The internal medium of the infusory lies on the border between neutral and reducing media.

Resistance of paramecium as regards changes of rH in the surrounding medium can only be investigated in systems with stable pH and free from cations acting oligodynamically. Quinone-hydroquinone system in buffered media with pH = 7—7,1 was applied. The lethal concentration of hydroquinone was 3 mM/l, and that of quinone — 0,002 mM/l. The toxicity of the standard quinhydrone solution and other quinone and hydroquinone mixtures of various percentage always depended on the real concentration of quinone. Consequently the real resistance seems to be relatively low to oxidizing substance and the toxicity of the redox system is decided by the action of the oxidized form.

In considering the action of the products of metabolism we avoid the discussion met with in the literature on this subject (see Weatherby 1941 and Wichterman 1953) as to what paramecium really excretes and we take into consideration all the substances which in fact can be found in cultures. Urea acts very weakly and only by osmosis so that it cannot be noxious in natural

culture conditions. Its lethal concentration exceeds 350 mM/l. Uric acid is so slightly toxic and little soluble that it is possible to keep normal cultures for a long time in saturated solutions of this compound. Urea, uric acid and other nonvolatile substances which occurred in cultures can be condensed by evaporation of the culture filtrate. The infusoria can live for more than 2 hours even in their own medium condensed 100 times. A direct or osmotic action of metabolites is impossible. In order to explain the checking of the development of the cultures by autointoxication (W o o d r u f f 1911 and 1913 as well as D i m i t r o w a 1932) one must turn towards other factors strongly influencing the physicochemical properties of the medium. The pH and rH changes which can be accomplished by NH_4^+ and HCO_3^- ions are probably such factors.

CHANGES OF THE CULTURE MEDIA

Further investigations aimed at stating whether the *Paramecium caudatum* can defend itself against noxious changes of the chemical properties of culture medium by influencing this medium to neutralise the action of the toxic factor.

We establish in each case whether the observed regulation processes are passive or active. By passive regulation we mean such influencing of the chemical medium which results from normal vital activity i. e. from the metabolism of the infusory. The term „active regulation" is given to the direct responses of the animal which result directly from the action of a noxious agent.

The same substances and agents the action of which had already been determined were taken into account, namely chlorides and acetates of metals, inorganic and organic sodium salts, organic stains, pH and rH changes of the medium and waste products of metabolism.

P r o c e s s e s o f a p p a r e n t r e g u l a t i o n

In the literature on the subject the data regarding the influencing of the chemical medium by *Paramecium* are scanty and there is almost no information regarding substances introduced into the cultures. Only B o r e n s t e i n's investigations (1938 and 1939) and those of G ó r s k i (1940) concerning the protective aggregation influence suggest the existence of regulation processes. B o-

renstein (1938), demonstrating that the toxic action of mercuric chloride is better tolerated by aggregated than by separated infusories assumes that the difference causing the divergence in reaction lies in the culture medium.

These assumptions are confirmed in certain conditions. The culture samples proceeding from cultures fed on dried milk powder were condensed by centrifugation to obtain 4 000 infusoria in 1 ml. The solutions of the substances examined were prepared corresponding to their lethal concentration and mixed with infusoria culture in the proportion of 1 : 1.

The culture of 2 000 infusoria in 1 ml of the liquid corresponding to 0,5 of the lethal concentration was kept 5 days. Some liquid was taken out every day and the actual concentration of the substance investigated was defined in the filtrate by titration of the anions or by use of a Pulfrich colorimeter. The control consisted of analogical solutions prepared on tap water alone and on the filtrated culture medium. In this way action of chlorides of the following metals introduced into the culture media was examined: lithium, sodium, magnesium, potassium, calcium, manganese, cobalt, nickel, copper, zinc, strontium, cadmium, barium, mercury and lead as well as the following sodium salts: fluoride, chloride, bromide, iodide, sulphide, thiosulphite, sulphite, sulphate, chromate, tungstate, nitrite, nitrate, bibasic phosphate, arsenite, arsenate, carbonate, metaborate, cyanide, rodanide and nitroprusside.

The results indicate that the decreased concentration of each chloride introduced into paramecium culture is an undeniable fact in the conditions described. This process takes place almost completely in the first phase of the experiment. The relatively smallest changes occur in the concentration of chlorides of monovalent cations, the greatest changes, however, concern the salts of heavy metals, so that, in fact, it is impossible to discover these salts by the methods applied by us. The increased concentration of inorganic sodium salts introduced into cultures occured in all cases investigated. It is impossible to prove the dependence of the process on the kind of anion. As already described in the case of metallic chlorides, the process discussed takes place almost completely in the first phases of the experiment.

Borenstein (1938) assumed the possibility of the concentration change of the dye introduced but was not able to decide whether it depends on absorpion of the noxious substance or on its

neutralization by the infusoria. But he was rather inclined to the supposition that the infusoria excrete substances which neutralize poisons. Or experiments contradict this, as the anion examined would be discovered by titration even after transition into another type of compound.

The only explanation is afforded by adsorption and a problem arises as to the kind of adsorbent. B r e s l a u (1924) supposes adsorption on trichocysts, such a small adsorbing surface does not however explain the disappearance of ions. The supposition that the whole infusory absorbs the diluted substance cannot be explained on the basis of the osmotic theory which in its present form seems to be questionable and is definitely contradicted by the following calculation.

From the paramecium volume and sample density it results that the infusoria hardly occupy 0,2% of the sample volume. In the case of NaCl the loss after 5 days amounted to 4 mM/l whence the salt concentration in the cell should amount to almost 12%. Consequently the infusory cannot be the chief absorbent and probably neither can any living cell in the culture — protozoan or bacterial. The cause of adsorption should be looked for in the abiotic compounds of the culture.

*

* *

Colloid detrite proved to be the adsorbent sought for. It appears at the bottom of cultures and in smaller degree on their surface. The chief role here is played by the same adsorption process mentioned by C u l l e n (1922) in the case of the introduction of the pH indicator into liquid containing protein and called by him „the protein error", which term we use here.

This interpretation explains the characteristic features of the processes described, among others the relatively greater disappearance of salts of heavy metals. The strongly toxic substances are applied in the lowest concentrations, consequently the adsorption on proteins becomes the regulative mechanism improving gradually and parallelly to the increase of toxicity, as with the fall of concentration of the solution only the absolute adsorption value decreases, while the relative value increases conspicously.

For the analysis of the role of detrite in the resistance phenomena it is necessary to elaborate a method of preparing dense

aggregations of infusoria, combined with their separation from foreign suspensions. The requirements of the experiment are best satisfied by the galvanotropic method decribed by us in another work (G r ę b e c k i and K u ź n i c k i 1955a).

In order to control the process of the disappearance of salts diluted in the cultures, the detrite was gathered, crushed in a homogenizator and centrifugated. In this way a condensed detrite deprived of living infusoria was obtained. The infusoria were purified by the galvanotropic method. The experiments were carried on in 4 series: a. infusoria with detrite, b. infusoria purified, c. water with detrite, d. pure water. The series „infusoria purified" corresponded to the previous ones, but was free from impurities. After the addition of the detrite (some 50 ml to 1 litre of liquid) a series of „infusoria with detrite" was obtained. „Water with detrite" was a detrite suspension 20 times diluted with water. „Pure water" was the control series. Each sample was mixed with the solution of the substance investigated, and after 3 days the actual concentration established. 5 metallic chlorides and 4 metallic acetates were applied as well as 5 inorganic and 4 organic sodium salts. The results of some of those measurements are shown on the table VI.

Table VI

Salt adsorption on the detrite of Paramecium cultures

Substance	Initial concentration in m/Ml	Concentration after 3 days in m/Ml			
		series a	series b	series c	series d
NaCl	25	21.2	24.8	21.3	25.0
$CaCl_2$	25	21.3	24.9	21.3	25.0
NaBr	25	21.5	24.9	21.5	25.0
$NaNO_3$	25	22.2	25.0	22.3	25.0
$C_2H_3O_2Na$	20	17.4	19.9	17.5	20.0
$C_2H_3O_2K$	20	17.7	20.0	17 8	20.0
CHO_2Na	30	25.2	30.0	25.1	29.8
$C_2O_4Na_2$	10	8.1	9.9	8.1	10.0

If the concentration fall depended on living Protozoa, it should occur only in series a (infusoria with detrite) and b (infusoria purified). However the titration shows it in series a (infusoria with

detrite) and c (water with detrite). Consequently, the adsorption of the poison takes place on the organic detrite.

It certainly plays a big protective role in laboratory cultures and unquestionably also in natural conditions. But it should be understood, however, that this is not the active autoprotection of the organism against the noxious agents by neutralization in the medium. The regulation is apparent and is accomplished by the medium itself.

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* *

In the purified aggregation the protein suspensions may appear only where bacterial infection exists. Paramecia do not produce the protective protein. Their chief sources are the food given (dry milk powder containing about 30% of protein) and microflora. The protein error is not, however, the only factor of the apparent regulation.

The substance introduced may disappear even from the galvanotropically purified culture, if its concentration is greater than the lethal concentration. The cause of the phenomenon lies in the fact that in the samples freed from nonliving proteins they appear repeatedly during the experiment. The dead infusoria contain them and they adsorb the toxic substance since intoxication consists in adsorption of the poison and in the increased permeability of the pellicle. So the first dead infusoria save the next living ones and the defense is the more effective the more advanced is the experiment, i. e. the more dead infusoria exist in the medium: A large number of infusoria were killed by electric current and four kinds of samples consisting of 100 living infusoria each were prepared. To each sample containing 0,002 mM/l of mercuric chloride were added successively: 400, 2400 and 12400 of corpses. According to the increase of the number of corpses the survival times increased to 85, 100, 325 and ∞ minutes.

Processes of passive regulation

In further investigations of *Paramecium caudatum* regulation capacities three ecologically important changes of chemical medium were taken into account, i. e. pH and rH changes and changes of concentration of waste products.

pH changes in culture media were investigated by Bodine (1921), Jones (1930), Szulzinger and Kałuska (1936) and also by Wichterman (1948). According to these authors pH value initially lies in the range of 5—6 and subsequently rises to 8 and above.

However the mechanism of hydrogen-ion concentration changes is not thoroughly known neither is the participation of infusoria in this process. The changes observed are ascribed hypothetically by the majority of authors to the action of microflora or to the effect of food supply.

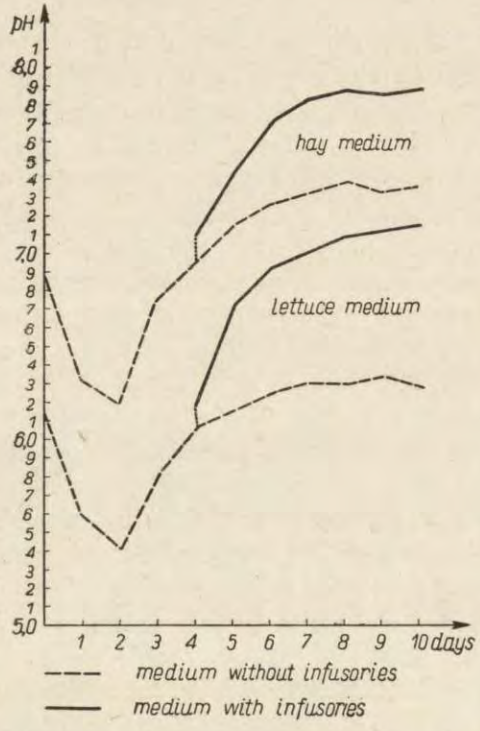

Fig. 1. Diagram showing pH changes in hay and lettuce media and in hay and lettuce Paramecium cultures

For observation of pH changes in milk cultures a medium on distilled and tap water was prepared (400 mg of dry milk powder to 1 litre of water). Each sample was divided introducing the infusoria to one part (the final density 2000 individuals in 1 ml) and the second part was left free from Protozoa. For 7 days every 24 hours pH was measured potentiometrically. Uniform pH increase is not observed but in the initial period (about 2 days) a decrease of its value takes place and only later on a gradual increase. pH oscillations are larger in media containing the infusoria. The differences are not, however, considerable and the hypotesis as to the participation of infusoria in pH changes requires further test on cultures reared in different way, as the milk itself has a certain definite influence on pH of the media, considerably raising its buffer capacity and at the time being a source of food for protozoa.

Consequnetly its concentration is not equal in both samples and these differences become greater with the time flow.

Further experiments were carried on in hay and lettuce media (5 g of hay or 2 g of dried lettuce to 1 litre of distilled water). The observations were continued for 10 days. The fourth day each sample was divided into two equal parts of which one was observed under the original condition, the second inoculated with 2000 infusoria per 1 ml of liquid. The results of daily measurements are given in the diagram (Fig. 1).

The distinct pH decrease usually observed during the first 2 days is probably connected with the exuberant growth of microflora; the participation of the infusoria themselves in this phase of the process does not seem to be decisive, as the infusoria placed in distilled water alkalize it at once. The direct influence of infusoria acts, as the diagram shows, in one direction and brings about alkalization. It occurs most quickly in the first period, later on the pH increase is limited. The slackening of the rate of medium alkalization may be connected with the slackening of culture growth, but probably the more real cause is the increase in the buffer capacity of the culture liquid. The alkalization can however proceed considerably further than in our experiments. In old cultures observed by us the pH value reached 8,4 but it never passed beyond this limit.

From the above results it may be infered that in the case of changes of hydrogen-ion concentration in *Paramecium caudatum* cultures regulation processes take place. It is a passive regulation as it always occurs and depend on the normal metabolism of the infusory. In asserting passive regulation we do not presume the existence of a specific agent catalysing the culture growth, but attribute the phenomenon of autocatalysis and allelocatalysis to the physico-chemical properties of the medium, resulting from the metabolism of the organisms living in it, as does D a r b y (1930) and J a h n (1934). The aggregated infusoria produce the optimal pH easily, and, which is more important — they buffer the medium quickly giving it a considerable stability and intensifying the resistance of the culture. Further alkalization would in turn lead to autointoxication. However we never observed a pH approaching lethal concentration in the cultures.

Jahn (1933, 1934 and 1935) as well as Efimoff, Niekrasoff and Efimoff (1928) endeavouring without result to determine the influence of redox potential changes on culture growth also showed that the gradual decline of the redox potential is parallel to the development of the culture. In order to acquaint ourselves better with this phenomenon we measured the E_H changes in milk and hay cultures and in distilled water containing infusoria. In order to eliminate the influence of the pH changes, the media were buffered within the limits of 7,02—7,12. The preparation method of media and experimental samples was the same as in the experiments on pH changes. The initial density of milk and hay cultures was about 2 000 infusoria in 1 ml and in the distilled water 5 000 infusoria in 1 ml. Measurements were made on a Cambridge potentiometer applying platinum and calomel electrodes in a saturated KCl solution.

The results of daily measurements made during one week are shown in volts in table VII.

Table VII

Eh changes in hay medium (A), hay culture (B), milk culture (C) and in distilled water inoculated with infusoria (D)

Days	0	1	2	3	4	5	6	7
A	+0,378	+0,356	+0,224	+0,192	+0,204	+0,218	+0,232	+0,240
B	—	—	—	+0,184	+0,112	+0,086	+0,082	+0,075
C	+0,372	+0,226	+0,120	+0,096	+0.072	+0,066	+0,060	+0,068
D	+0,398	+0,202	+0,148	+0.112	+0,104	+0.110	+0.116	+0.134

The comparison of E_H values in hay media without infusoria and in hay cultures with infusoria and also the data concerning the potential changes in distilled water containing a dense aggregation of infusoria show that paramecia themselves decrease the E_H of the medium. The data quoted indicate a more acute course of this process, than is usually observed in ordinary cultures, where E_H value hardly ever falls below + 0,100 V. The reason for this is the great density of our experimental samples. When the E_H value approaches + 0,100 V a checking of the culture growth is observed.

This inclines us to the assumption that the decline of the culture growth may be caused not only by pH changes but also by redox potential changes.

As the last element of the influence of infusoria on their own chemical medium, osmotic pressure changes (Δ) in the liquid of highly condensed cultures were observed. The observations were limited to the general measurement of Δ without detailed analysis of the role of particular waste substances, as it is not known which of the compounds discovered in the culture belong to the paramecium metabolism waste products, and the percentage composition of the end products of metabolism is various in different cultures and also in the same culture at various periods. Milk cultures of an initial density of 2 000 specimens in 1 ml were used as well as samples containing 5 000 specimens in 1 ml of distilled water. Because of the slow rate of the changes ocurring, the experiments were carried on for 20 days and measurements were made by the cryoscopic method at the beginning of the experiment and after 5, 10 and 20 days. The data are given in table VIII.

Table VIII

The influence of infusoria on the Δ changes in their medium

Time in days	0	5	10	20
Milk culture	0,014°	0,032°	0,042°	0,050°
Distilled water	—	0,008°	0,012°	0,018°

It is obvious that the influence of the infusoria on Δ of their media is relatively small. Even in very dense and old cultures Δ as a rule does not exceed 0,065°, consequently it remains harmless. Urea solution of such a osmotic pressure does not even cause any traces of plasmolysis.

Summarizing the above it should be to state that the infusoria, by their metabolism, influence the pH, E_H and Δ of their surrounding medium in an experimentally determinable degree. These changes easily create the optimal medium conditions. This is evidence of passive regulation. Changes proceed in one direction and finally they may exceed the optimum but usually they are then too slight to become the cause of the autointoxication of the culture; probably they may then check the growth of the culture.

Processes of active regulation

The responses of galvanotropically purified infusoria to organic stains introduced into the medium were investigated (Bismarck brown, Orange G, Congo-red, haematoxyline, neutral-red, Nile-blue, toluidine-blue, pyronine and methyl-green). Microscopic observations show that the solution in which condensed infusoria are present may in a very short time lose its coloration. This fact results supposedly from regulation accomplished by Protozoa. The colour of the medium containing coloured anions does not change, if however we introduce a cationic stain, the colour turns pale very quickly. Two reasons of this process are possible, either reduction of the stain in the medium or its condensation in the paramecium body.

It is known from previous works that paramecium can reduce many azo-stains in its cytoplasm, nobody however has observed the reduction of the stain or of other compounds of similar E_0 directly in the medium.

We found that the oxidation of the culture medium, from which the stain has disappeared, never produces intensification of the colour, so we reject the possibility of regulation by reduction outside the cell body.

The hypothesis of regulation by stain condensation inside the infusory body leads to the problem of the presence and concentration of the stain in food-vacuoles. Stain condensation in vacuoles was first demonstrated by Dembowski (1922). The investigations were continued by Strelnikow (1929) and Chejfec (1937). Strelnikow (1929) having examined cationic stains claims their condensation in vacuoles up to 25 000 times which he verified by comparing directly the colour of vacuoles. with the colour of standard stain solutions in glass capillaries. Chejfec (1937) rightly considers this method as primitive but himself draws different conclusions without applying any quantitative measurements. He denies the results of his predecessors and attributes the intensification of colour to chemical changes of the vacuole contents. Strelnikow's observations (1929) would explain the mechanisms of the regulation and Chejfec's (1937) views would make our results incomprehensible.

The inaccuracy of Chejfec's (1937) observations resulted from the incomparability of the examined condensations, which

always oscillated round the lethal concentration, that is for anionic stains in dilutions of the range 10^{-2} and for cationic stains at least 10^{-4}. In order to avoid this inaccuracy we always placed the infusoria in the solutions of concentration 1 : 100 000. Vacuoles were observed after one hour. In the case of all cationic stains the coloration of the vacuoles is stronger than that of their environment, for the anion stains both colours seem to be uniform. C h e j f e c's observation (1937) that the vacuole detaching from the bottom of the oesophagus is pale and becomes coloured only during cyclosis, is confirmed but only in the case of anionic stains. Consequently cationic stains are condensed in vacuoles, while the anionic stains are not.

To obtain quantitative data colorimetric measurements were carried out. A series of cultures in solutions of Congo-red, orange G, neutral-red and toluidine-blue were prepared. The average quantity of food vacuoles for one paramecium during one hour and the average volume of these vacuoles was calculated. After one hour the samples were centrifugated and the actual stain concentration determined by use of a Pulfrich colorimeter. Knowing the number of infusoria, number and volume of their vacuoles as well as the loss of the stain the coefficient of its condensation in the vacuoles was found. Results of measurements are shown in table IX. Concentration values have been rounded off to tens of thousands, the number of vacuoles to the whole numbers, their volumes to hundreds μ^3 and condensation coefficients to hundreds.

Table IX

Condensation of some stains in food-vacuoles

Substance	Initial concentration	Concentration after 1 hour	Number of vacuoles	Volume of the vacuole	Condensation coefficient
Congo-red	1 : 30 000	1 : 30 000	24	4 700μ^3	1
Orange G	1 : 30 000	1 : 30 000	21	4 400μ^3	1
Neutral-red	1 : 100 000	1 : 1 000 000	16	3 200μ^3	3 600
Toluidine-blue	1 : 100 000	1 : 210 000	11	3 500μ^3	2 700

So we see that the regulation consists in condensation of the stain in food vacuoles and takes place only in the presence of compounds with coloured cations. S t r e l n i k o w (1929) was right though he suggested the possibility of condensation to 25 000 times. We find the condensation within the range of several thousand times, but these data are not entirely comparable. S t r e l n i k o w (1929) calculated the condensation coefficient for the single vacuole, and our data give the average of all vacuoles formed during one hour.

The stain concentration decline occurs very quickly. The first vacuoles contain much more and the last ones much less stain than is shown by the average value. For the more accurate analysis of the process the experiments with Congo-red, toluidine-blue and neutral-red were repeated and the concentration fall in the medium determined after 15 and 30 minutes and also after 1 and 24 hours. The process of disappearance of the stains is presented in diagram (Fig. 2).

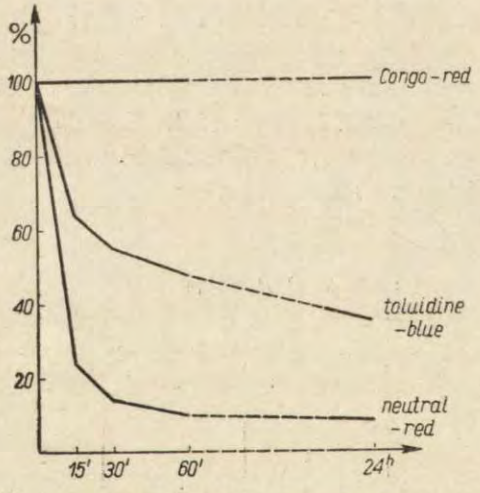

Fig. 2. Diagram showing gradual fall of concentrations of the three vitals stains introduced into the medium of a dense culture *of Paramecium caudatum*

The infusory deposits the condensed stain from its vacuoles into it cytoplasm and does not excrete it even after one month. The regulation rate and intensity for various stains depend probably on the ionic potential value of the latter. This dependence will be studied in a separate work. The mechanism of the stain condensation in vacuoles is perhaps an electrophysiological process and it cannot consist in any kind of stain „filtration" in view of the fact that the paramecium is unable to filtrate during one hour a quantity of water corresponding to the quantity of stain ingested.

The ingestion by vacuoles of the stain and its storage in the cytoplasm is harmless, while the penetration of the stain through the pellicle causes diffusive staining and death. Autoprotection consists consequently in the conspicuous decrease of the surrounding concentration so that the intoxication through the entire cell surface becomes impossible. Protective reaction appears only when the toxic substance is introduced into the medium, so it can be considered as a process of active regulation.

INFLUENCE OF DENSITY OF CULTURES

Our chief tasks are to establish whether culture resistance changes are dependent on culture density and to determine the role of regulation as a cause of this phenomenon. It is necessary to state how the apparent, passive or active character of the regulation is reflected in the character of the protective influence of aggregation. From the evolutionary point of view we try to determine if the process observed is an example of group reaction qualitatively different from individual reactions, or only the sum of the latter.

Protective influence of aggregation in the apparent regulation

The protective aggregation influence according to Borenstein's (1938 and 1939) and Górski's (1938 and 1940) methods in solutions of all previously mentioned chlorides was determined. The infusoria used were taken from cultures fed with milk powder suspension. The mixed culture was washed and condensed by centrifugation. After 48 hours a part was diluted with its own medium. Paramecia from the initial sample (10 000 infusoria/ml) were called „aggregated", and those from the second one (200 specimens/ml) „separated". To both samples the experimental salt solution was added simultaneously in the proportion of 1 : 1. The survival time of the separate infusoria and the percentage of the aggregated infusoria alive up to that time was noted. The last result noted concerned the survival time of the aggregated infusoria which was marked with ∞ if it exceeded 24 hours. The result of the experiment is given in table X. Concentration were prepared so that separate infusoria might live up to 100 minutes.

The percentage of the aggregated surviving infusories is usually small and their general survival time differs very little from the

survival time of the separate infusories. Yet there are exceptions. We found protective aggregation influence in the case of $HgCl_2$, $CuCl_2$, and $NiCl_2$. This process occurs in a less marked degree in the case of $PbCl_2$.

Table X

Protective influence of aggregation of Paramecia in the presence of metallic chlorides

Substance	Concentration mM/l	After min	Survive % of infusoria		Survival time of aggregation
			Separate	Aggregated	
LiCl	100	20	0	1	22
NaCl	75	70	0	4	85
$MgCl_2$	75	45	0	2	50
KCl	75	50	0	1	55
$CaCl_2$	75	100	0	1	105
$MnCl_2$	15	110	0	4	120
$CoCl_2$	10	60	0	5	85
$NiCl_2$	0,05	30	0	75	300
$CuCl_2$	0,05	70	0	85	∞
$ZnCl_2$	0,2	90	0	10	110
$SrCl_2$	80	120	0	3	125
$CdCl_2$	0,2	80	0	15	125
$BaCl_2$	40	65	0	4	75
$HgCl_2$	0,002	120	0	96	∞
$PbCl_2$	0,3	120	0	31	190

Table XI shows the correlation between the intensity of the protective aggregation influence and the lethal concentration of the salt. Substances little toxic ($>$ 1 mM/l), moderately toxic (1 mM/l — 0,01 mM/l) and strongly toxic ($<$ 0,01 mM/l) were distinguished. Three classes for the protective influence of aggregation were established: 1) absence of the process (less than 5% survives in the aggregation), 2) indistinct process — (5% up to 25% of infusoria survive) and 3) distinct process (over 25% survive). The correlation is distinct; from among 15 salts it is not shown only by $PbCl_2$. This allows of the apparently paradoxical inference that the

more poisonous the substance is the stronger is the protective influence of aggregation it causes.

Subsequently the resistance of separate and aggregated infusoria in solutions of inorganic sodium salts (fluoride, chloride, bromide, iodide, sulphide, tiosulphate, sulphite, sulphate, chromate, tungstate, nitrite, nitrate, monophosphate, arsenite, arsenate, carbonate, metaborate, cyanide, rodanide and nitroprusside) were investigated. The protective influence of aggregation is not observed in any of the solutions regardless of the kind of anion. The ratio of surviving specimens in aggregations never exceeds 5%. Consequently there is no correlation between the resistance of aggregation and the salt toxicity. It is due to the very slight toxic action of sodium salts and to the relatively small differences in their toxicity.

Table XI

Correlation between salt toxicity and intensity of the protective influence of aggregation

	>1 mM/1	1 mM/1-0,01 mM/1	<0,01 mM/1
<5%	$LiCl$, $NaCl$, $MgCl_2$, KCl, $CaCl_2$, $MnCl_2$, $SrCl_2$, $BaCl_2$.		
5% — 25%		$CoCl_2$, $ZnCl_2$, $CdCl_2$	
>25%		$PbCl_2$	$NiCl_2$, $CuCl_2$, $HgCl_2$.

The above mentioned principles of the appearance or absence of the protective influence of aggregation were confirmed when four organic sodium salts and four metallic acetates were examined. The density of the samples does not influence the resistance in the case of alcaloids of low toxicity but it is of considerable importance in the cases of quinine and partly also strychnine.

Borenstein (1938) showed that the infusoria from the bottom of the culture are most resistant than those taken from the thigmotropic surface agglomeration. This observation was confir-

med experimentally. It was found in addition that the freely swimming infusoria are still less resistant. Hence the conjecture that the observed resistance increase of aggregation is due to protein error and that the process depends on apparent regulation which is most intense for the most toxic substances. The methods applied heretofore (B o r e n s t e i n 1938, 1939 and G ó r s k i 1938, 1940) prove to be insufficient in view of the fact that the separate and aggregated infusoria differ in the quantity of nonliving proteins accompanying them. In condensing the protozoa by centrifugation all suspensions were also strongly condensed. The separate infusoria however were diluted with the medium centrifugated and consequently purified from detrite. Thus the samples differed not only as to the number of infusoria and that is why the experiments were not exact. Taking this into consideration we used only galvanotropically purified infusoria in our further investigations.

The resistance of the separate and aggregated infusoria, both purified and unpurified, to $HgCl_2$, $CuCl_2$ and $NiCl_2$ was compared. The resistance series is as follows: aggregated unpurified > separate unpurified > aggregated purified > separate purified. A sample containing non-living proteins independently of population density is always more resistant than a purified aggregation.

So e. g. in the solution of mercuric chloride of concentration 0,002 mM/l the separate purified infusoria die after 90 minutes. At the same time 60% of the aggregated purified infusoria, 85% of separate unpurified and 95% of aggregated unpurified survive. These samples do not die entirely even after 24 hours. In 0,05 mM/l $NiCl_2$ the separate purified Infusoria die after 35 minutes, while 25% of the aggregated purified survive and die after 190 minutes; 65% of separate unpurified survive and die after 200 minutes and 84% of the aggregated unpurified survive and die after 390 minutes.

In the next experiment to the media with separate infusoria 10% per volume of detrite of *Bacillus subtilis* culture, 10% of hen's eggwhite, 20% of human blood plasma or 1% of gelatine were added. The percentage of the living infusoria remaining in 0,002 mM/l $HgCl_2$ and 0,05 mM/l $CuCl_2$ was determined after 2 hours. 100% of infusoria survived in the solutions of mercuric chloride mixed with detrite mixtures. The purified infusoria all persihed. The sample in the solution of copper chloride mixed with blood plasma survived in 100%, in the solutions of copper chloride with

eggwhite, copper chloride with gelatine and copper chloride with bacterial detrite 90%, 80% and 72% respectively. No infusoria survived in the purified sample. Consequently it is evident, that the protective influence is not a specific feature of suspensions occurring in protozoa cultures.

In spite of the removal of all impurities, however, the condensed samples live somewhat longer than the non-condensed. This fact is to be connected probably with the processes of selection. The range of oscillations of individual resistance is considerably wider in the dense culture. Therefore the survival times of the last infusoria are somewhat longer in it and also the first dead Protozoa appear earlier.

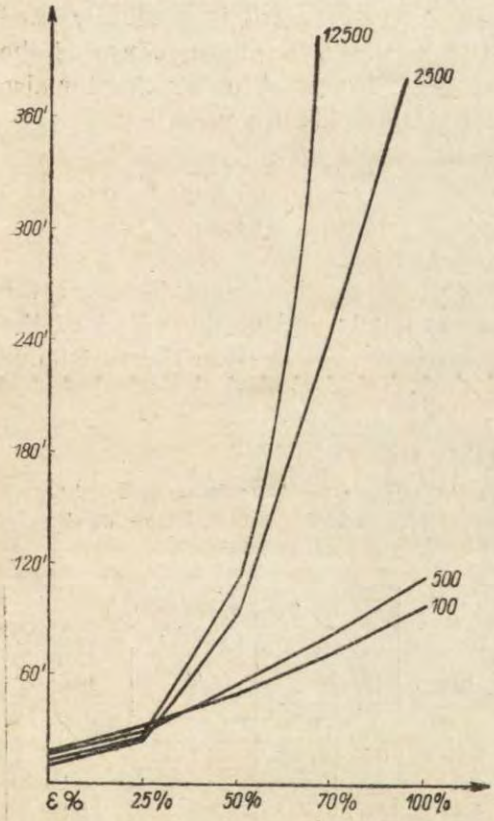

Fig. 3. Diagram showing the dependence of the survival times of a group of infusories on the quantity of previously dead specimens in the cases of varying density of samples

The samples containing 200, 1 000, 5 000 and 25 000 of infusoria in 1 ml were poisened by mercuric chloride usual solution. The moment of appearance of the first corpses (ε%) and the times of dying of 25%, 50%, 75% and 100% of the sample contents were noted. The development of the process is shown in diagram 3. At first the mortality is somewhat higher in the dense samples, but later on the relation is reversed. Selection alone does not adequately explain the great decrease in the final mortality. Selection is supplemented by the protective influence of the dead

infusoria which die during the experiment and absorb the poison thus creating the appearance of regulation. It is not the percentage that conditions the survival but the absolute number of dead infusoria and this is higher in the aggregation. So in the first place the greater resistance of the organism itself is decisive, and later the weakening of the noxious agent by the corpses of less resistant infusoria becomes the essential factor.

The selection consisting in survival of more resistant organisms becomes particularly marked as the toxic agent is gradually used up and the dying of the „unfit" becomes the direct cause of the survival of the „fittest". All processes described in this chapter also take place when organic salts and alkaloids are used.

Protective influence of aggregation in the passive regulation

As stated previously, processes of passive regulation are the result of accumulation of waste products and of the effect of the latter on pH, rH and Δ of the medium. We analyse the resistance of aggregations to changes of these three factors.

Table XII

The effect of age and density of the culture on its resistance to H_2SO_4 (N—normal concentration of the acid applied, T — survival time of the sample, pH — actual acidity of sample medium)

Age of the culture in hours	100 Infusoria/ml			5 000 Infusoria/ml		
	N	T	pH	N	T	pH
0	$2{,}1 \cdot 10^{-4}$	80′	4,2	$2{,}1 \cdot 10^{-4}$	12h	4,5
12	$3{,}5 \cdot 10^{-4}$	17′	4,2	$3{,}5 \cdot 10^{-4}$	∞	5,1
24	$5{,}2 \cdot 10^{-4}$	118′	4,3	$5{,}2 \cdot 10^{-4}$	∞	6,1
48	$8{,}9 \cdot 10^{-4}$	55′	4,3	$8{,}8 \cdot 10^{-4}$	∞	6,3
72	$10{,}5 \cdot 10^{-4}$	25′	4,3	$10{,}5 \cdot 10^{-4}$	∞	6,7

Differences of resistance to pH changes of separate and aggregated Infusoria were investigated by G ó r s k i (1938). He stated, that the protective influence of aggregation occurred, when tartaric and acetic acids were used. We applied hydrochloric, sulphuric, formic, acetic and tartaric acids. The technique of the experiment

corresponded to that applied previously, but instead of placing the separate and aggregated infusoria in the medium of 48 hours aggregation we washed them with distilled water enabling then the aggregation and the separate infusoria to change their media gradually. The resistance was determined immediately after washing and also after 12, 24, 48 and 72 hours. In this way we could simultaneously analyse the role of density according to Borenstein (1938 and 1939) and Górski (1938 and 1940) and also the role of the age of the culture according to Mitrophanowa (1925).

Each time the characteristic concentration of the acid killing the infusoria after some minutes was found. Both kinds of samples were placed in the acid solution of such a concentration. The pH was measured instantly and after the survival time of the infusoria. Results for H_2SO_4 are given in table XII.

The condensation of Protozoa aggregations always prolongs their survival time. This result is common for all acids investigated. Changes of the medium are the reason for the increased survival times. This is ascertained by the measurements of pH. The same quantity of acid added to the sample of aggregated Infusories diminishes pH of their medium in a smaller degree than when added to the sample of separate infusories. Hence the survival times of infusories in the condensed sample is longer. Differences between separate and aggregated infusories appear more distinctly with time flow. The older is the thin (less crowded) culture, the more acid it is necessary to add in order to obtain death after some minutes. The divergencies in the survival time and in the actual acidity of the two kinds of samples becomes however more conspicuous.

Therefore the protective aggregation influence in relation to acids exists as regards potential acidity but not in regard to actual acidity. The phenomenon increases with the age of the aggregation which, as time goes on, alkalizes and buffers its medium more intensely than do the separate infusoria. Thus it can stand higher concentrations of the acid, as it is more difficult to lower pH in its medium.

In view of the actual acidity the resistance of the aggregation is even somewhat lower and it decreases slightly with time.

Analogical investigations applying NaOH and KOH did not give clear results. In the case of the artificial increase of pH of the me-

dium we do not find any distinct influence of the density of the sample on resistance.

In order to demonstrate definitely that the protective aggregation influence on pH changes does not depend on the active reaction of the infusoria but on the medium, buffer solutions were applied. The medium of the culture may have a strong influence on the hydrogen ion concentration when diluted acid or base is added; if buffer solution is added it will not change the intensity of the acting agent. Therefore the separate infusoria and the aggregated ones are helpless and protective aggregation influence does not take place. In the solution of $C_6H_4(COO)_2HK + HCl$ of a pH = 4,42 the separate infusoria live for 67 minutes, while the aggregated live only for 70 minutes. In the solution of $Na_2B_4O_7 + NaOH$ with a pH = 9,24 the separate infusoria live for 105 minutes and the aggregated ones for 112 minutes. There is no protective aggregation influence.

The toxic action of buffer solutions does not decrease even with age of experimental samples.

The problem of the resistance of aggregated infusoria from the point of view of redox phenomena has up till now been dealt with only fragmentarily by B o r e n s t e i n (1939). We applied in our investigations the quinone-hydroquinone system. The quinone was used in the concentration of 0,005 mM/l. The separate infusoria die in such a solution after 46 minutes and 85% of the aggregated still survive after 24 hours. Distinct protective aggregation influence may be connected causally with the reduction capacities of the infusoria, especially in the case of the system of a relatively high E_0.

The dependence of the process on the number of Protozoa is confirmed by the use of H_2O_2. A rapid formation of little oxygen bubbles takes place — a phenomenon hardly noticeable in the sample of separate infusoria. Simultaneously about 50% of the infusoria of the aggregation die after some minutes. The separate infusoria behave normally. They die after 75 minutes, in the aggregation however almost one half of the infusoria survive and about 5% survive still after 24 hours.

Protective aggregation influence should therefore not take place in the presence of reducing substances. Nevertheless in the 6 mM/l solution of hydroquinone strong chemotropic aggregation of infusoria takes place in the area farthest from the free surface of the liquid. The separate infusoria die after 55 minutes and the chemo-

tropic aggregation in the condensed sample after 14 hours. Mechanical dispersion of the aggregation makes it die in a short time. Shaking every 5 minutes shortens the life from 14 hours to 70 minutes. The medium of oxidisated culture transforms the hydroquinone into quinone and in fact the action of this second compound is decisive. Oxidisation of 0,1% of the contents of hydroquinone is sufficient to make the solution toxic. From the surface to the bottom of the vessel the gradient of the quinone concentration is then formed. Consequently a limit concentration conditioning the existence of chemotropism appears. The concentration of quinone in the liquid surrounding the aggregation is naturally the smallest and it continues to remain on a low level thanks to the reduction capacities of the infusoria. In the closed glass tubes the lack of free surface does not permit the establishment of the gradient of hydroquinons oxidisation which fact causes chemotropism to disappear and results in a longer survival time. If boiled water is used the survival time becomes several times longer.

The appearance of the protective aggregation influence in the presence of oxidizing substances is an undeniable fact. When reducers are present this process is spurious and is only decided by the most toxic oxidisated form.

Resistance of the aggregations to Δ changes of the medium was investigated applying foreign substances and waste products. In the presence of glucose, saccharose, glycocoll and alanine the protective aggregation influence is not manifested, which is synonymous with the absence of regulation capacities. No differences of resistance in the thin and condensed samples to the action of urea was found. Uric acid was not investigated as it is not lethal even in a saturated solution. The own medium of infusoria concentrated a hundred times acts in the same manner regardless of the density of the sample. A contrary process however takes place, namely the harmful influence of condensation of the samples of infusoria after putting them into distilled water. At first both kinds of samples stand the dilution of their own medium well, but later on this experiment becomes gradually more harmful to the aggregation while the separate Infusoria are indifferent to it regardless of the sample age. The reason for this process consists in the transformations of the medium liquid of the condensed culture which (passive regulation) are fuller and deeper than those of the non-condensed culture.

Protective influence of aggregation in the active regulation

The influence of the density of the culture on its resistance in the case of active regulation was investigated by putting the infusoria into solutions of 10 organic stains. The results of the experiment are shown in table XIII.

Table XIII

The protective influence of aggregation in the presence of some stains

Substance	Concentration	After min.	% of living Infusoria		Duration of life of aggregation
			separate	aggregated	
Congo-red	1 : 80	35	0	1	40
Haematoxyline	1 : 200	90	0	5	125
Orange G	1 : 500	85	0	5	120
Methyl-green	1 : 20 000	55	0	100	∞
Pyronine	1 : 20 000	35	0	100	∞
Bismarck-brown	1 : 20 000	25	0	90	630
Methylene-blue	1 : 40 000	30	0	100	∞
Neutral-red	1 : 100 000	35	0	100	∞
Toluidine-blue	1 : 100 000	20	0	100	∞
Nile-blue	1 : 1 000 000	25	0	100	∞

There is no protective aggregation influence in the presence of slightly poisonous compounds with coloured anion. This influence in the presence of cationic stains is more marked than in the case of any other substances investigated. Protein error was excluded by galvanotropic purification and the protective role of corpses eliminated as a matter of course, as in some solutions 100% survival was observed during 24 hours. Consequently apparent regulation cannot be the cause of the processes described above which must result from active regulation as previously shown for stains. The course of the regulation depends on the density of the culture. When there are more infusoria in the sample the concentration of the poison falls more rapidly to a much larger degree, and in this

way the protective role of aggregation in the presence of cationic stains becomes manifest.

The regulation capacity of the infusory living in aggregation is not greater than that of the separate specimen. This was found by analysing the non-condensed and condensed cultures after 15 minutes of neutral-red action. The separate infusoria form about as many vacuoles with the stain as the aggregated, and there is no great difference in their average volume. Colorimetric comparison of the falls in stain concentrations in the media shows that the differences depend only on the number of infusoria. The calculation of the condensation coefficients of the stain in food vacuoles gives almost uniform results. The separate infusoria condense neutral-red rather more intensely presumably because of the high concentration of neutral-red continuously maintained in the medium of non-densified sample. Consequently the aggregation does not exert any specific activating influence as it does not augment the regulative ability of the individual.

In this case the protective role of aggregation is directly dependent on the number of infusoria. Changes in the concentration of the substance and in the density of the sample give similar results. A series of cultures containing 200, 1 000, 5 000 and 25 000 infusoria in 1 ml was prepared. The samples in each series contained neutral-red in concentrations of 1 : 25 000, 1 : 75 000, 1 : 150 000 and 1 : 250 000. The percentage of infusoria alive was counted after 24 hours (see table XIV).

Table XIV

The effect of sample density and stain concentration on the ratio of infusoria surviving after 24 hours

Density of the sample of infusoria /ml.	Stain concentration			
	1 : 25 000	1 : 75 000	1 : 150 000	1 : 250 000
200	0%	0%	0%	100%
1 000	0%	0%	100%	100%
5 000	0%	100%	100%	100%
25 000	100%	100%	100%	100%

It is evident that the regulation in aggregation is dependent in direct proportion on the number of infusoria taking part in regulation.

SUMMARY

The toxic action of compounds of the salt type on *Paramecium caudatum* is determined by the ionic regularities of toxicity.

It is primarly the kind of cation secondary the pH value which condition the toxicity. The kind of anion is not of great importance. A relation exists between the toxicity of the cations and their affinity for lipids and proteins. A correlation between their toxicity and their ionic potential value is specially evident. This rule also applies to organic stains which are highly poisonous only in cationic form.

The toxicity of acids depends on hydrogen ion concentration and the nature of the anion does not play any perceivable role. On the other hand the toxicity of bases depends simultaneously on pH and on the kind of cation. The infusoria can live in buffered media more than 24 hours in pH limits of 4,68—9,13.

Resistance to reducing substances is higher than resistance to oxidisating substances and the toxicity of redox systems is decided by the concentration of oxidisated form of the compound.

Resistance to the osmotic action of waste products is so high that autointoxication may only be caused by a change of pH and rH of the medium.

One of the way in which *Paramecium caudatum* cultures achieve autoprotection against noxious changes in their medium are regulation processes consisting in the weakening or neutralization of the noxious agent in the medium outside the organism. The regulation may be apparent, passive or active.

The process of apparent regulation comprises the adsorption of the poisonous substance by the colloid suspensions and sediments originating from the culture or by the dead infusoria. The live infusoria take no part in the process. This process takes place with all absorbable substances and was studied in detail using inorganic and organic salts.

The process of passive regulation consists in the transformation of certain physicochemical properties of the medium. It results from the animal's usual course of metabolism and is always accomplished independent of the presence of a noxious agent. The infusoria alkalize their medium, decrease redox potential value and augment the osmotic pressure.

We define the process of active regulation as the distinct response of the animal given ad hoc to the action of a noxious agent. This process has been found to exist in solutions of organic stains with coloured cations which are condensed in food-vacuoles and deposited in a harmless form into cell plasma. Thus the concentration of the stain in the surrounding medium is reduced and this prevents poisoning through the pellicle.

Regulation processes in *Paramecium caudatum* cultures cause the process known as protective aggregation influence. This process may result from any type of regulation: apparent, passive or active.

In the case of apparent regulation the protective aggregation influence depends only in a very small degree on the density of the cultures. This dependence is conditioned by selective dying of Infusoria and the adsorption of the poison by the dead Infusoria whose absolute number is higher in aggregation. More often however the cause of the increased resistance of aggregations is the protein error — i. e. adsorption of the poison on colloidal suspensions condensed during the condensation of the Infusory cultures.

In the case of passive regulation the protective aggregation influence depends directly on the density of the cultures. As, however, passive regulation does not result from the application of a noxious agent, but from the metabolism of the animal, the phenomenon consists in changes in the medium and not in the increased resistance of the infusory. Consequently this type of the protective aggregation influence depends directly on the age of the examined culture.

In the case of active regulation the protective aggregation influence also depends directly on the density of the culture, but the active regulation is achieved here ad hoc and as a result there is no direct dependence between the protective aggregation influence and the age of the sample.

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THE ELECTRICAL NEGATIVITY OF THE DAMAGED AREA OF FROG MUSCLES IN THE STATE OF POLARIZATION CONTRACTURE

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„If the surface of a resting cell is broken at any point, the lead-off from the damaged area, which is equivalent to a lead-off from the cell contents, will... show to be negative to the resting and uninjured outer cell surface" (L o v a t E v a n s, 1949). On this general statement is based the preexistence theory of electrical phenomena in living tissues. Although the electrical negativity of the cell contents has been also found by the mean of the intracellular electrodes (Cole and Curtis, 1941, Hodgkin and Huxley, 1945), there are at least some states of the muscle, in which the negativity of the damaged area can not be observed (S z a b u n i e w i c z 1938). Namely, in polarized and contractured frog muscles no electrical negativity was found. In this state, solely a longitudinal „polarization" of the fibres of the muscle is apparent (S z a b u n i e w i c z 1933).

In the present work, the depth of the electrical negativity in muscles, being in polarization contracture of different grades, has been investigated.

METHODS

The gastrocnemius muscle of frogs (mostly *R. temporaria*) has been prepared without touching it with the skin secretion (Szabuniewicz, 1930). Potential differences were measured with small silver electrodes (Szabuniewicz and Gibiński, 1948). One of the electrodes remained on the cartilage of the Achilles tendon, the other one being slided along muscle fibres. The potential differences were measured at points distant 2 mm from each other.

First, the potential of the normal muscle surface was investigated, as a rule only small differences being found. Then, the muscle surface has been damaged by means of an iron wire (about 2 mm in diameter) heated in the flame. Other means of damaging of the muscle surface were also tested. The burning has been chosen because it gives a sharp and distinctly visible limit on the muscle surface. The heated wire was applied perpendicularly to the direction of the fibres. A burn of about 2 mm along muscle fibres resulted.

After the burning has been made, the potential of the muscle was reinvestigated every 2 mm. This time the negativity of the damaged area was found. Also a small grade of longitudinal polarization of muscle fibres generally resulted, as found before (S z a b u n i e w i c z 1938). Subsequently the polarization of the muscle was produced. This was done either by solutions of $HgCl_2$ (S z a b u n i e w i c z 1933), or of polarine (S z a b u n i e w i c z and G i b i ń s k i 1948). After this has been done, the potential differences along muscle fibres were investigated once more every 2 mm. The external surface (the skin side) and the internal one (the bone side) of the gastrocnemius were investigated in different experiments.

The sequence of the proceeding was so short, that the usual diminishing of the negativity with the time was only very small. Corresponding results were also obtained if the muscle has been first treated with the polarizing agent, and afterwards damaged by incision or by burning. However, no investigation of the unpolarized muscle could be made in such cases.

RESULTS

The negativity, stated after injury, disapeared after treating with the polarizing agents. With the increasing polarization the negativity vanishes. This is shown in two experiments presented on fig. 1 and 2.

In order that the grade of polarization could be compared with the depth of the electrical negativity, following generalizations has been made. As the g r a d e o f p o l a r i z a t i o n was accepted the maximal potential differences between the proximal and the distal ends of the muscle fibres. This polarization grade was nearly zero in normal muscle before injury. After the incision or the burning, a small polarization grade (lesser than 10 mV) has been generally found. The grade of polarization after treatment with the

polarizing agent varied from about 20-to 75 mV, and was depending upon the concentration of polarizing agents ($HgCl_2$ or polarine).

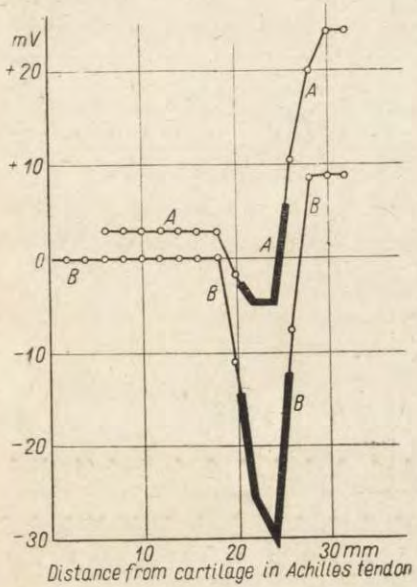

Fig. 1. The potential along muscle fibres of the internal surface of a Gastrocnemius (No 20-1). B — after damaging before the treatment with the polarizing agent. A — after the treatment with a diluted solution of polarine. The hollow circles — spots in which the potential has been measured on uninjured muscle surface. The thick lines present the damaged area of the muscle. After the treatment with the polarizing agent, only a small grade of polarization resulted (maximal potential differences amount to 21 mV). The negativity of the damaged area diminished visibly

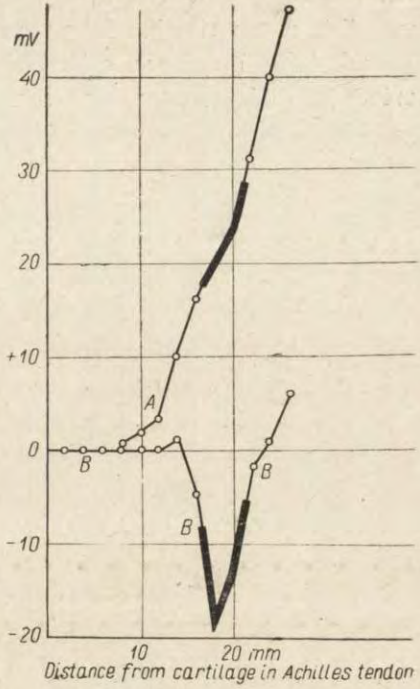

Fig. 2. The potential along muscle fibres of the internal surface of a Gastrocnemius (No 10-r). A and B — as in the fig. 1. In this experiment the polarization has been produced with a more concentrated solution of polarine. A greater degree of longitudinal polarization resulted. The negativity disappeared completely. The steepness of the longitudinal polarization is a little diminished in the damaged area

The depth of negativity was calculated in the following manner. Two measurements of muscle potential, made on normal

muscle surface closest to the injured area, one distally and the other one proximally, were taken into consideration. The arithmetical mean from these two measurements has been calculated, and from this mean the lowest potential of the damaged area was subtracted. This has been called the depth of negativity. This depth varied in different muscles (before treatment with the polarizing

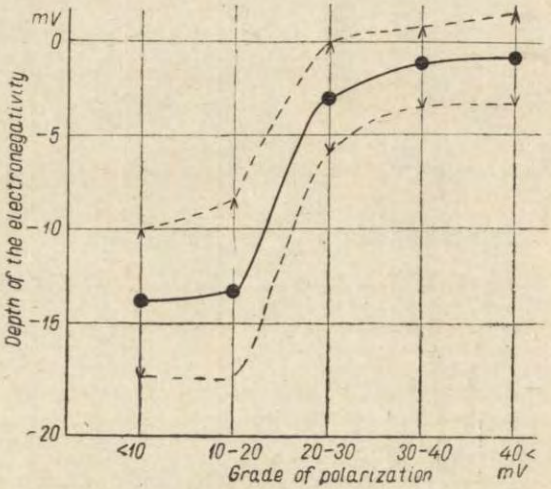

Fig. 3. The depth of the electrical negativity in muscles, polarized in different degres. The internal side of the muscle. Black discs and full line show the average mean for different polarization grades. The dashed lines present the limits of the standard deviation

Table I

The electrical negativity of the damaged area in dependence from the grade of polarization of the muscle

	Grade of polarization of the muscle				
	10 mV and less	10—20 mV	20—30 mV	30—40 mV	40 mV and more
External side	—13,8 mV ± 3,9	—13,1 mV ± 4,8	—3,0 mV ±3,0	—1,2 mV ±2,0	—0,9 mV ±2,3
Internal side	—14,5 mV ± 5,8	—13,0 mV ± 6,8	—2,0 mV ±2,7	—0,9 mV ±2,1	

agent) from about 10 to about 35 mV. Muscles of strong and healthy frogs gave the greatest negativity.

Results obtained in 55 experiments performed on the external muscle surface, and 46 experiments performed on the internal surface, are collected in tab. I, and partly, in fig. 3.

Tab. I and fig. 3 show, that the negativity disappears with the appearence of longitudinal polarization. Tab. I presents a small negativity in muscles, showing a polarization grade of more than 30 mV. This results only from the mode of estimation, being not the true expression of the low potential of the given place, but rather the consequence of a marked weakening of the longitudinal polarization in the injured area (fig. 2). The diminishing of the negativity is not parallel to the increase of the polarization, but rather preceds this phenomenon.

SUMMARY

The dependence of the depth of the electrical negativity of the damaged area from the grade of longitudinal polarization was investigated on frog gastrocnemius. The electrical negativity diminishes and vanishes completely with the beginning of the longitudinal polarization. Fully polarized muscles do not show any negativity of the damaged area.

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SHAPE OF MYELINATED NERVE FIBRES AND PROXIMO-DISTAL FLOW OF AXOPLASM

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A medullated peripheral nerve fibre increases in diameter near the nodes of Ranvier forming bulbous enlargements on both sides of the nodal constriction. It was shown in a previous paper (L u b i ń s k a 1954) that these enlargements differ both in shape and in volume according to their location. In motor as well as in sensory fibres the bulb on the proximal side of each node is more voluminous and extends over a longer stretch of fibre than the bulb on the distal side. This allows to recognize even on an isolated fragment of the fibre its original direction in the body.

As the content of the fibre is liquid and its neurilemmal tube in the internodes is distensible and elastic (C a u s e y 1948, L u b i ń s k a 1952), it may be presumed that the shape of the system would reflect the action of forces that maintain its equilibrium, and that the departures from cylindrical outline would show the loci where the relationship between the intraaxonal pressure and the tension of the neurilemma is altered.

The distension of the fibre above each node has been interpreted, similarly to the deformations obtained artificially by W e i s s

and H i s c o e (1948), as a morphological manifestation of the proximo-distal flow of axoplasm, dammed at the nodes by the relatively inextensible cementing disc of the neurilemma. If this interpretation is correct, the study of the shape and of the degree of asymmetry of the bulbs at various distances from the perikaryon may show whether the flow of axoplasm is progressively damped towards periphery or whether it is maintained throughout the fibre at a steady rate.

A quantitative analysis of the shape of living fibres at various distances from the cell bodies was therefore undertaken, special attention being paid to the juxtanodal region. The results obtained show that the shape and dimensions of the bulbs and of the nodes proper do not vary along the fibres. On this ground it can be inferred that the intensity of flow is kept constant along the fibre, whatever the distance from the perikaryon.

MATERIAL AND METHODS

Qualitative observations of the nodal region were made on living teased fibres from various peripheral nerves of cats, rabbits, dogs, and rats, as well as on amphibian fibres.

Quantitative results reported here were obtained on fibres from branches of sciatic nerve of cats. In order to detect the influence of the distance from the cell body on the shape of the fibres, they were taken from two levels: the upper — just below the trochanter, after separation of the branch supplying the hamstring muscles, and the lower — at the ankle. The approximate distance between the two levels has been some 160—200 mm.

About 700 fibres from 20 cats were examined.

Small fragments of nerve, 10—15 mm long, were removed under barbiturate anesthesia, and ligated at one end, either peripheral or central, in order to mark direction and to facilitate the handling of the nerve.

The epineurium was then either split lengthwise and removed, or bundles of fibres were drawn from the epineurial sheath. They were put into heparinized blood plasma, serum, or Ringer, and teased carefully with fine needles at one end. At the other end the fibres remained in bundles. Only those fibres which could be followed from their cut ends to the entry into undissociated bundle, i. e. those whose original direction in the body was known, were measured. The portions of the fibres near the cut ends and fibres showing signs of deterioration were discarded. All measurements were made within the first hour after removal from the body.

When the teased preparations were mounted on a flat slide, many fibres were compressed by the coverslip in places where they crossed each other. Sometimes even single large fibres appeared deformed. These deformations were avoided when the preparations were placed in chambers some 60—

100 μ deep. They were sealed to avoid concentration changes of the bathing fluid.

As the fibres floated in the chambers in various planes, it was necessary to refocus the microscope for examination of each fragment. It was not practicable therefore to make measurements from photographs, where only small fractions of the image appeared in focus.

After various trials with ocular micrometer, the measurements were made with a compass on a ground glass projection of the image under a total magnification of 1 000 x. This method proved to be more reliable. Successive readings made on the same spot did not differ by more than 0,1 mm on the medullated portions of the fibre and by 0,2—0,4 mm on the node proper. Some nerves were teased in the proximo-distal, other in the opposite direction although it was shown previously, by statistical analysis of the results obtained by both procedures, that the direction of teasing does not affect the asymmetry of the nodal region.

Various media in which the fibres were examined influenced their ultimate fate and the mode of deterioration but left the shape unaffected in the initial period after removal from the body.

All results of measurements, whatever the direction of teasing or the bathing fluid employed, are pooled together and divided in groups differing by 2 μ, according to mean diameter of internodes. The fibres from the upper and the lower levels are analysed separately.

RESULTS

Appearance and behaviour of the juxtanodal region in teased living fibres

Juxtanodal bulbs in apparently undamaged teased fibres differ from one another in the smoothness of their outline and in the number and pattern of myelin folds (Plate I, fig. 1—7). It is not known whether similar variability occurs in the body or is introduced by strains produced during the isolation of the fibres, or by inadequacy of the bathing fluid.

Certain features of the nodal region appear nevertheless in all fibres and in all bathing fluids used in the present experiments. They consist in a conspicuous asymmetry of the shape of the bulbs on both sides of the node and in much larger volume of the proximal bulb. Detailed measurements of various dimensions of the juxtanodal region show that the diversity of appearance is mainly due to variable configuration of myelin at the nodes, whereas the external outline of the fibre remains relatively constant. The diameters and the lengths of proximal and distal bulbs vary within narrow limits in fibres of similar size (Table II).

The participation of various components in the architecture of the nodal region cannot be resolved on living fibres by light microscope. In fresh preparations it is the outer border of myelin that traces the contour of the fibre. The neurilemma is invisible except at the node proper and in the middle of internodes where the myelin is pushed away from the neurilemmal wall by the Schwann cell. The outer endoneurium is frequently visible at the nodes bridging the distance between the largest portions of the bulbs. Sometimes it may also be discerned along the internodes as a faint loose wrapping of the fibre. The Schwann cell cytoplasm overlying the bulbs, seen in all electron micrographs (G a s s e r 1952; R o z s a, M o r g a n, S z e n t G e o r g y i and W y c k o f f, 1950; H e s s and L a n s i n g, 1953), is completely invisible in fresh fibres.

The nodal region is extremely vulnerable and unstable. The fibre deteriorates at the nodes when the internodes are still completely unchanged. The deterioration is manifested by swelling of the juxtanodal material and by a progressive retraction of myelin from the nodes. The Schwann cell cytoplasm becomes then visible. N a g e o t t e (1921) thinks that it is pushed there from the internodes at the beginning of degeneration. The alteration of the nodal region seems due to metabolic disturbances brought about by the change of environment. The onset of deterioration is delayed when glucose is added to the bathing fluid. It may be noticed that marked morphological alteration of the nodal region does not interfere for a time with the ability to generate unimpaired action potentials (S c h n e i d e r 1952).

Q u a n t i t a t i v e s t u d y o f t h e s h a p e o f t h e f i b r e s

N o d a l r e g i o n. The measurements were made on unaltered nodes with both adjacent internodes intact. As all bulbs located on one side of the node appear geometrically similar in fibres of various sizes (except for the smallest diameter group), the mean internodal diameter was employed as a yard-stick in determination of the shape of the juxtanodal bulbs. This diameter (A) was calculated from 6 measurements taken along adjacent internodes, and following dimensions (Fig. 1) were measured at each node:

The external diameter of the „cementing disc" — C

The largest diameter of the proximal bulb — P_1

The largest diameter of the distal bulb — D_1

The diameter of the bulb at the distance of one internodal diameter (A) from the largest diameter, P_2, for the proximal bulb and D_2, for the distal bulb. (All diameters of myelinated portions were determined by the outer border of myelin).

The distance between the node proper and the maximal enlargement, P_3, in the proximal, and D_3, in the distal bulb.

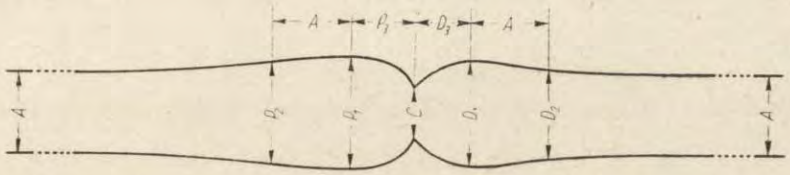

Fig. 1. Designations of various dimensions, measured in juxtanodal region. A — mean internodal diameter, C — external diameter of the node, P_1 — the largest diameter of the proximal bulb, P_2 — the diameter of the proximal bulb at distance A from the largest diameter, P_3 — the distance between the nodal constriction and the largest diameter of the proximal bulb, D_1, D_2, D_3 — corresponding dimensions in the distal bulb

As the bulbs merge imperceptibly into the cylindrical parts of the internodes it is very difficult to evaluate their total length. The dimensions P_2 and D_2 may serve as useful indications of the way the bulbous enlargements subside in the proximal and in the distal direction.

The measurements of some 650 nodes in fibres ranging from 5 to 21 μ in diameter have shown that all dimensions are consistently larger in the proximal bulbs than in the distal ones, indicating a surplus of material accumulated on the proximal side of each node.

The bulbs are more enlarged in thin fibres than in the thick ones. Thus the relation of the largest diameter of the proximal bulb to internodal diameter is 1,4 in the smallest size group and 1,2 in the largest. The nodal constriction, however, is more pronounced in large than in small fibres, the relation of nodal to internodal diameter being 0,5 for the largest and 0,7 for the smallest group.

The measurements of successive nodes in one fibre show small variations of bulbar diameters not exceeding several per cent of the mean. The nodal diameters fluctuate more and this can be attributed partly to larger error of measurements in unmedullated portions.

The largest scatter, reaching some 20 per cent of the mean is observed in longitudinal dimensions P_3 and D_3. A typical example is shown in table I.

Table I

Scatter of juxtanodal dimensions at 4 consecutive nodes of a fibre (in microns)
Symbols are explained in figure 1.

		A	C	Proximal bulbs			Distal bulbs		
				P_1	P_2	P_3	D_1	D_2	D_3
Mean values	Nodes	18.1	10.7	22.9	21.2	14.0	22.4	19.2	10.1
Deviations of individual measurements from the mean	I	0.1	0.9	0.5	0.1	2.0	1.0	0.4	1.1
	II	0.1	0.2	0.1	0.2	3.0	0.4	0.2	1.9
	III	0.1	0.6	0.5	0.3	1.0	0.3	1.2	1.1
	IV	0.1	0.7	0	0	0	0.3	0.6	0.4
Maximal deviation from the mean in per cent		0.5	8.4	2.2	1.4	21	4.4	6.2	19

The dimensions of the nodal regions (mean values and confidence limits) in fibres of equal sizes from the upper part of the thigh and from the ankle level are shown in table II, where the fibres from both levels are grouped according to the diameters of internodes. It follows from this table that the homologous dimensions at both levels fall well within confidence intervals of each other. The change of nodal dimensions with the increase of caliber of the fibres is shown in fig. 2; hollow symbols refer to magnitudes at the lower, and filled symbols to those at the upper level. The results obtained show that, over a length of some 200 mm of the nerve trunks, the distance from the perikaryon has no influence either on the shape or on the size of the nodal region.

Shape of the internodes. It has been observed during the measurements of the nodal region that the internodal diameter outside the bulbs is smaller on the distal side of the node than on its proximal side. The difference, though small, was visible at every node. It seemed worthwhile therefore to determine accurately the shape of the internodes by methodical measurements of fibre diameters taken every 20 or 30 μ along several successive internodes. However unexpected difficulties, due to the instability of fresh fibres were encountered. The teasing of long stretches of

fibres and the measurements of diameters every 20 or 30 μ took so much time that the outline of the fibre changed to unduloid before the measurements could be completed. Although at the beginning the undulations were not very conspicuous, the measurements taken

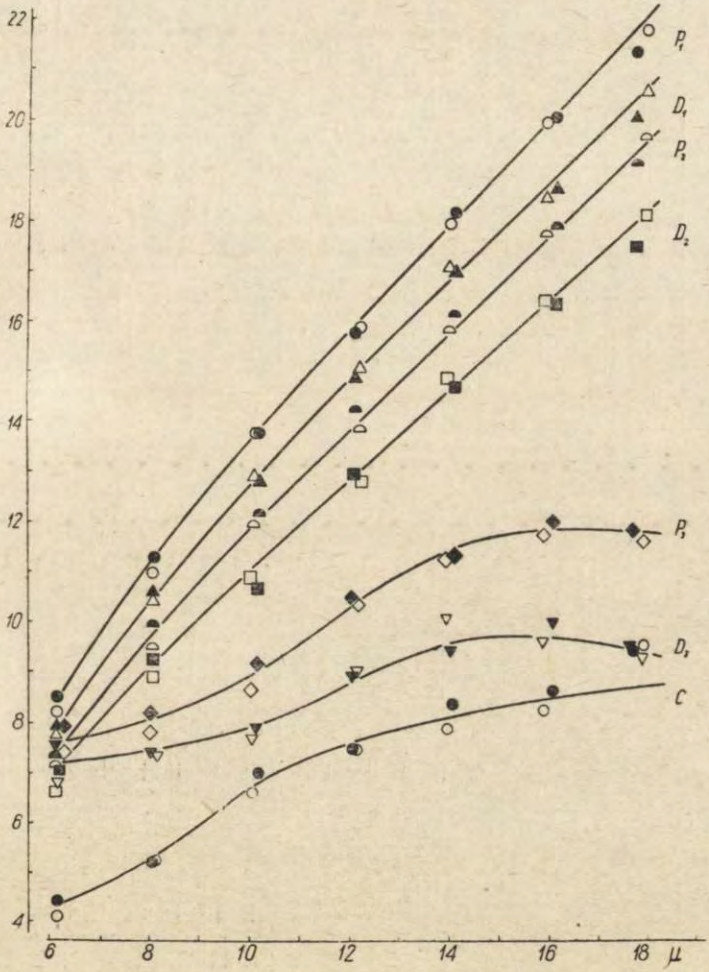

Fig. 2. Dimensions of juxtanodal regions in fibres from the upper and the lower level plotted against mean internodal diameter. Upper level — black symbols. Lower level — hollow symbols. Designations explained in fig. 1

at constant intervals showed a wide scatter of values that obscured to a large degree the faint systematic change of diameter along the internode. This could be detected, however, when mean diameters

Table II

Dimensions of juxtanodal regions in fibres from tbe upper and the lower level. Mean values and confidence limits (t — test) at 5 per cent level of probability (in microns)

Level	Number of nodes	Internodal diameter A	Nodal diameter C	Proximal bulbs: largest diameter P_1	Proximal bulbs: diameter at the distance A from the largest diameter P_2	Proximal bulbs: distance between node and the largest diameter P_3	Distal bulbs: largest diameter D_1	Distal bulbs: diameter at the distance A from the largest diameter D_2	Distal bulbs: distance between node and largest diameter D_3
Upper	32	6.2 ± 0.1	4.4 ± 0.3	8.5 ± 0.3	7.5 ± 0.3	7.9 ± 0.8	7.9 ± 0.3	7.0 ± 0.3	7.6 ± 0.9
Lower	45	6.2 ± 0.1	4.1 ± 0.2	8.2 ± 0.3	7.1 ± 0.3	7.5 ± 0.5	7.7 ± 0.3	6.6 ± 0,2	6.8 ± 0.4
Upper	36	8.2 ± 0.2	5.2 ± 0.4	11.3 ± 0.4	10.0 ± 0.4	8.2 ± 0.7	10.6 ± 0.5	9,3 ± 0.4	7.4 ± 0.7
Lower	51	8.0 ± 0.2	5.2 ± 0.3	11.0 ± 0.4	9.5 ± 0.3	7.8 ± 0.5	10.4 ± 0.3	8.9 ± 0.3	7.4 ± 0.3
Upper	35	10.1 ± 0.2	7.0 ± 0.4	13.8 ± 0.4	12.2 ± 0.4	9.2 ± 0.9	12.8 ± 0.4	10.7 ± 0.3	7.9 ± 0.8
Lower	33	10.0 ± 0.2	6.6 ± 0.5	13.8 ± 0.5	12.0 ± 0.3	8.7 ± 0.6	12.9 ± 0.3	10.9 ± 0.3	7.7 ± 0.6
Upper	57	12.0 ± 0.2	7.5 ± 0.3	15.8 ± 0.3	14.3 ± 0.2	10.5 ± 0.6	14.9 ± 0.3	13.0 ± 0.3	9.0 ± 0.7
Lower	61	12.1 ± 0.2	7.5 ± 0.4	15.9 ± 0.3	13.9 ± 0.3	10.4 ± 0.7	15.1 ± 0.3	12.9 ± 0.3	9.1 ± 0.6
Upper	58	14.0 ± 0.1	8.4 ± 0.3	18.2 ± 0.3	16.2 ± 0.3	11.4 ± 0.6	17.0 ± 0.3	14.8 ± 0.3	9.5 ± 0.6
Lower	59	13.9 ± 0.2	7.9 ± 0.3	18.0 ± 0.3	15.9 ± 0.3	11.3 ± 0.7	17.1 ± 0.3	14.9 ± 0.4	10.2 ± 0.7
Upper	51	16.0 ± 0.2	8.7 ± 0.3	20.1 ± 0.3	17.9 ± 0.3	12.1 ± 1.0	18.7 ± 0.3	16.4 ± 0.3	10.1 ± 0.6
Lower	21	15.8 ± 0.4	8.3 ± 0.5	20.0 ± 0.7	17.8 ± 0.6	11.8 ± 1.6	18.5 ± 0.5	16.4 ± 0 8	9.7 ± 0.9
Upper	48	17.6 ± 0.1	9.5 ± 0.3	21.4 ± 0.2	19.2 ± 0.4	11.9 ± 1.0	20.1 ± 0.2	17.6 ± 0.3	9.5 ± 0.6
Lower	16	17.8 ± 0.3	9.6 ± 0.9	21.8 ± 0.5	19.7 ± 0.5	11,7 ± 2.0	20.6 ± 0.6	18.2 ± 0.3	9.4 ± 0.9
Upper	39	19.5 ± 0.2	9.8 ± 0.5	24.0 ± 0.3	21.3 ± 0.3	13.4 ± 1.7	22.6 ± 0.4	20.0 ± 0.4	10.5 ± 0.9
Lower	—	—	—	—	—	—	—	—	—

of the initial and terminal halves of each internode were calculated. The proximal half proved consistently to be about 5 per cent thinner than the distal part. These results are shown in table III. For reasons explained below, the diameters of the medial portion of the internode, containing Schwann cell nucleus and extending over some 40—60 μ, were rejected and did not enter in calculations of the mean diameter.

Table III

Mean diameters of the proximal and distal halves of internodes in fibres of various sizes (In microns.)

Fresh fibres			Fixed fibres			
Proximal half. Mean diameter	Distal half. Mean diameter	Difference in per cent of the distal diameter	Proximal half. Mean diameter	Distal half. Mean diameter	Difference in per cent of the distal diameter	fixative
7.0	7.4	5.4	11.9	12.8	7.0	osmium tetroxyde
8.2	8.4	2.4	12.0	12.8	6.3	„ „
8.2	8.6	4.7	12.4	13.1	5.3	formaldehyde
11.0	11.9	7.6	12.4	13.3	5.3	„
11.3	12.6	10.0	12.5	12.4	6.7	„
11.5	12.9	11.0	12.5	13.3	6.0	„
11.7	12.4	5.6	12.7	13.2	3.8	osmium tetroxyde
11.7	12.3	4.9	16.5	17.0	2.9	formaldehyde
12.5	12.7	1.6	16.7	17.6	5.1	„
12.6	12.7	0.8	16.8	17.7	5.1	osmium tetroxyde
12.7	13.2	3.8	16.9	18.0	6.1	„ „
12.9	13.4	3.7	17.1	17.6	2.8	„ „
13.0	14.0	7.1	17.2	17.7	2.8	„ „
15.0	16.5	9.1	17.7	18.8	5.9	„ „
15.9	16.1	1.3	18.1	19.5	7.2	„ „
16.0	16.6	3.6	18.2	18.3	0.6	„ „
16.1	16.5	2.4	18.4	19.2	4.2	„ „
16.6	17.2	3.5	18.6	19.4	4.1	„ „
17.1	18.4	7.1	18.9	19.0	0.5	„ „
18.5	18.9	2.1	19.0	19.9	4.5	„ „
			19.3	19.8	2.5	„ „
Mean difference in per cent		4.89			4.51	

In order to obviate the difficulty created by rapid transition to unduloid shape in fresh fibres, a more detailed information concerning the shape of intact internodes was sought on fixed fibres. The nerves were fixed, in their natural length, in 0,5 per cent osmium tetroxyde dissolved in Ringer (Young 1935) and the fibres were teased after fixation. Owing to the very slow penetration of the fixative, the fibres in the middle of the nerve trunk often presented enlarged incisures and partly demyelinated nodes. Only undamaged fibres from the superficial layers were chosen for measurements. The results are shown in fig. 3, where the shape of several successive internodes from 2 fibres is presented. In order to make the sequence of changes of diameter along the internode more conspicuous, the scale of the long axis of the fibre has been reduced to 1/100 of that of the diameter. Owing to this change of scale the smooth variations of width of the fibre look jerky. Moreover, as only the variable fraction of the fibre thickness is shown on the diagram, the small accidental variations appear unduly magnified.

The mean diameter of the initial half of internode is, as in fresh fibres, several per cent smaller than the diameter of the terminal half (Table III).

Other constant features of internodes are a relatively deep depression, spreading over some 100—150 μ, that appears at the beginning of each internode immediately after the distal bulb and the enlargement of the fibre toward the middle of internode. Another depression, much less marked than the initial one, appears sometimes near the end of the internode just before the proximal bulb. In order to avoid confusion, it should be noted that the terms „proximal" and „distal" for the bulbs were introduced to mark their relation to the node. In considering the internode, it must be remembered that the „distal" bulb is located at the beginning and the „proximal" one at the end of the internode.

The medial widening of the fibre is due to Schwann cell nucleus and perinuclear cytoplasm. Interposed between the neurilemma and the myelin, the Schwann cell distends locally the neurilemmal tube and depresses the axoplasm and myelin. The distortion is not symmetrical in relation to the axis of the fibre and the degree of widening observed in the microscope depends on chance differences in spatial location of the Schwann cell with respect to the optical section of the fibre.

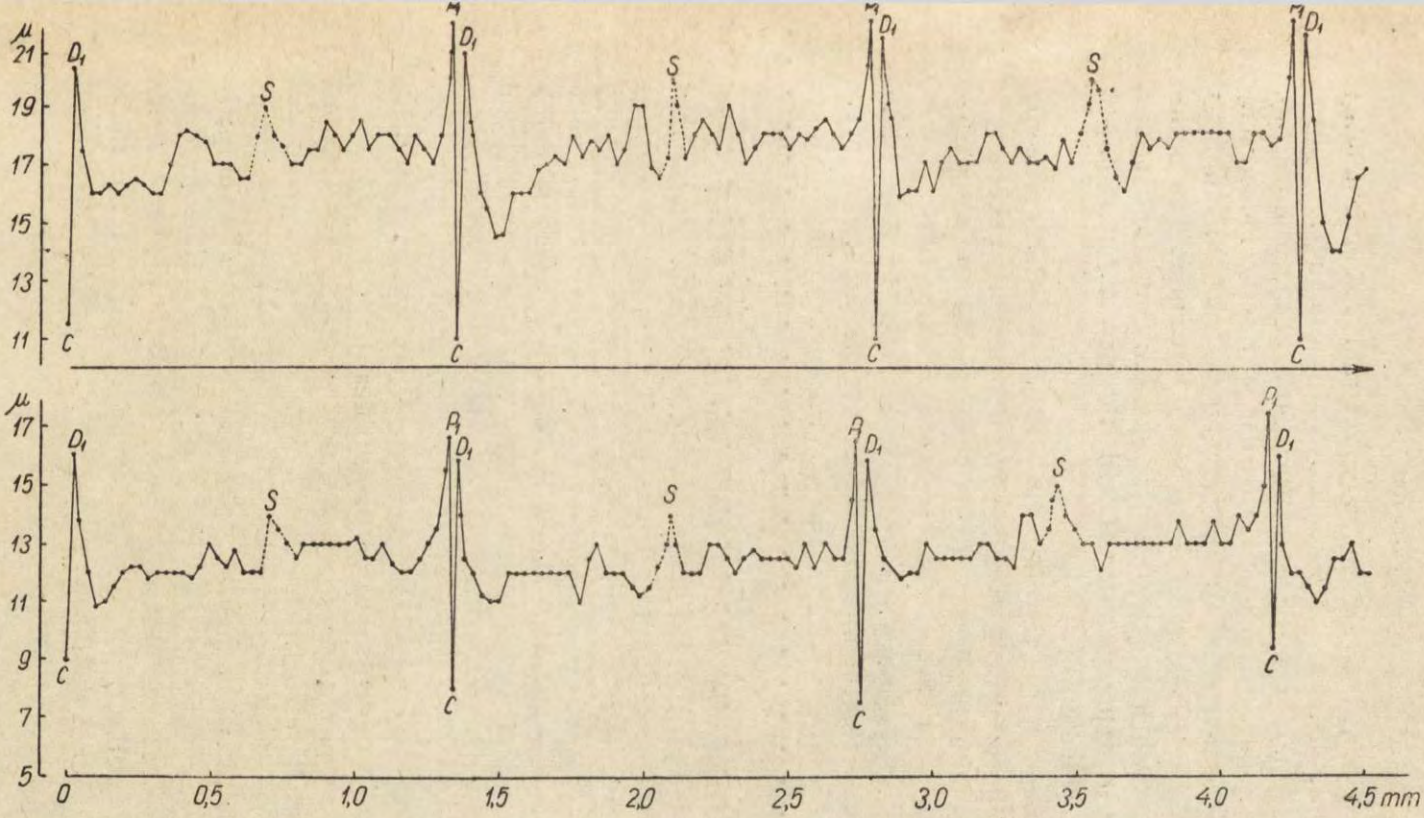

Fig. 3. Shape of several successive internodes in 2 fibres. Abscissae — length of the fibre (mm). Ordinates — diameter of the fibre (μ). The scale of the longitudinal axis of the fibre is reduced to 1/100 of that of the diameter. The arrow marks the proximo-distal direction. S — Schwann cell nucleus. C, P_1 and D_1 indicate, as in fig. 1, the nodal diameter, the largest diameter of the proximal, and the largest diameter of the distal bulb respectively. The distension of the fibre due to the Schwann cell is shown by dotted line.

DISCUSSION

A nerve fibre is, considering its proportions, a most unusual cytological formation. Owing to fluidity of its content, it is physically unstable (Young 1944, 1945) and is bound to break into droplets under influence of surface tension. It does so indeed some time after separation from the cell body but as long as the unity of the neuron is preserved the fibre remains threadlike. The nature of the factors which maintain axoplasmic continuity and normal organization in a fibre connected with the perikaryon is largely a matter of conjuctures.

As the shape of the fibre may be looked upon as a reflection of forces acting at its surface, detailed information concerning it could contribute to the understanding of the mechanism responsible for maintainance of the normal organization of the fibre.

Quantitative research on morphology of nerve fibres has been going on along several lines related to various aspects of nerve activity. The caliber of fibres and the thickness of myelin were studied primarily in order to correlate action potentials, excitability, and the rate of conduction with the structural features of conducting elements (cf. references in Gasser and Grundfest, 1939; Grundfest 1940). In the study of growth and maturation of normal and regenerating fibres, the range of diameters and the respective amounts of axoplasm and myelin in various portions and at various stages of development of fibres, as well as the length of internodes were investigated (ref. in Young 1950, Hursh 1939, Evans and Vizoso 1951, Whitear 1952, and others). The nodal region has been examined lately in search for quantitative data relevant to the theory of saltatory conduction. (Hess und Young 1952, Gasser 1952). Almost all these investigations were performed on fixed fibres.

For the purpose of the present study it was essential to use isolated fresh fibres in which the physical state of axoplasm and the volume relations were undisturbed. To what extent these conditions are fulfilled in teased fibres may be surmised only indirectly since it is not possible to check the form of teased fibres by comparing it with that of fibres in the nerve trunks in the body. There are several possible sources of distortion of shape of the fibres introduced by our experimental procedure. The main of these seem

to be: the separation from the cell body, the removal of sheaths, the changes of environment of fibres, and the mechanical stresses inflicted by teasing.

The changes due to separation of the axon from the perikaryon develop very slowly and the influence of this factor on the shape of the fibre during the first hour seems to be negligible.

The effects of removal of sheaths have been extensively investigated (Crescitelli 1951, Shanes 1953, 1954 and others). For the question of possible alterations of the shape of the fibres, the observations of Shanes concerning the changes of weight of desheathed nerves are relevant. He found that in those nerves which swell after desheathing, the swelling is due to an increase of interstitial spaces, whereas the volume of fibres remains unchanged, as well as their functional properties.

The modified environment of fibres and conditions of partial asphyxiation prevailing in our experiments lead to enlargement of incisures and demyelination of the nodes. This effect is, however, not instantaneous and care has been taken during the experiments to measure the fibres before the beginning of deterioration.

The deformability of the nerve fibres suggests that serious alterations of their shape might be brought about by the mechanical stresses exerted by teasing .Apart from direct damage caused by the needle, two kinds of deformations are worth mentionning. When a bundle is stretched rapidly and abruptly released, the fibres recoil and become sinuous (Plate I, fig. 8). In the concavities of the fibres the myelin is folded and indents the axoplasm (Plate I, fig. 9).

When a fibre is elongated slowly beyond certain limits, it becomes moniliform (Plate I, fig. 10). Similar distortion of shape takes place in other kinds of protoplasm too if the relation of length to diameter of a protoplasmic thread exceeds a critical value. It was observed by Chambers and Höfler (1931) when a filament of protoplasm was drawn off by a micro-needle from the tonoplast of Allium cepa or from a starfish egg. In nerve fibres, as in these filaments, the deformation was reversible and in about two hours after release of elongation, the contour of the fibre became smooth again (Plate I, fig. 11), showing clearly that the beaded appearance was not due to an incipient Wallerian degeneration. Such distortions were never observed in fibres more than 10 μ in diameter. Either the degree of elongation was insufficient to cause this de-

formation in large fibres or they were more easily broken by traction.

With careful teasing such distortions were very rare and the fibres presented a relatively uniform appearance in spite of diversity of magnitudes and directions of stresses to which they had been subjected. When the fibres were teased in a particular, say proximo-distal, direction, there was statistical predominance of stresses in this direction. If a distortion of nodal asymmetry were brought about by these stresses, it would be revealed by comparison of the results obtained on fibres teased in opposite directions. No such influence could be detected by statistical analysis of our measurements.

On the basis of all these facts it may be assumed that, under conditions prevailing in our experiments, the fibres preserve initially the shape they had in the body. Exception must be made only for the terminal portion of cut internodes which diminishes in diameter when the axoplasm flow out from the cut end (L u b i ń s k a 1955).

Thus a fibre appears subdivided into internodes, each of which begins at the proximal end by a slight bulbous enlargement. Immediately beyond this bulb lies the narrowest portion of the internode. Then the diameter of the fibre increases slowly along the internode and, at its end, a second bulb („proximal" in respect to the adjacent node), larger and longer than the first, is formed. This pattern is repeated at each internode.

The widening of the fibre on the proximal side of each node resembles the deformations obtained by W e i s s and H i s c o e (1948) at any level of the fibre when the nerve had been constricted for several weeks by an arterial sleeve. The asymmetry of the nodal region has been interpreted previously (L u b i ń s k a 1954) similarly to the interpretation Weiss gave to the phenomena observed after constriction of the nerve, as a manifestation of the proximo-distal flow of axoplasm dammed at each node by narrowing of the channel at the level of the „cementing disc". Thus the morphology of a nerve fibre seems to provide an additional confirmation of the fact that flow of axoplasm from the cell body toward periphery is continuously going on in the axon, as suggested by the course of regeneration, by the phenomena of neurosecretion and by Weiss experiments.

The asymmetry of the nodal region being interpreted as a manifestation of flow, it appeared necessary to compare the form and dimensions of the bulbs at levels far apart along the fibre to see whether the departures from cylindrical shape attenuate with the increasing distance from the perikaryon. As it is impossible to isolate the fibres on great lengths, the nodal regions in fibres of similar caliber taken from two levels separated by some 200 mm were compared. A difficulty arose about the justifiability of such comparison, as frequent mentions appear in the literature that the nerve fibres taper peripherally. In such case the fibres at the lower level should be compared with fibres of a larger caliber at the upper level. The search for quantitative data concerning this point in the literature has shown, however, that the fibres really taper, owing to dichotomy near the entry into the muscle or into the skin (Eccles and Sherrington 1930, Cooper 1929), whereas in the nerve trunks the fibres dichotomize rarely. Constant caliber of fibres in undividing trunks over lengths of 50—80 mm, comparable to those used in the present study, were found by Causey (1948), by Evans and Vizoso (1951) and by Quilliam 1956) in rabbits, and by Rexed and Sourander (1959) in dorsal roots of cat. Contrary indications are given by Bjorkman and Wohlfart (1936), on an unbranched portion of the tibial nerve of guinea-pig. These authors found, along a stretch of 3 mm of the nerve, a reduction of mean diameter of fibres at the peripheral end amounting to 16 per cent of the mean diameter at the proximal end, as well as a significant increase of the number of fibres at the distal end. In our material on cats, taken far from the entry into terminal organs, we never observed either branching of fibres or peripheral thinning of consecutive internodes on stretches of teased fibres some 10—15 mm long, although dichotomizing fibres were frequently seen near the muscles in branches of hamstring nerves and in the nerve to gastrocnemius. We found however, that at the level of the ankle the fibres of the largest caliber are much more scarce than in the thigh. This may be explained by the fact that great majority of large fibres has left the trunk at knee level to innervate the muscles of the leg. In the light of these data it seems legitimate to assume that the majority of fibres maintain a constant caliber along the nerve trunk. The results described in the present paper show that the dimensions of the bulbs in fibres

of equal sizes remain invariable and independent from the distance separating them from the cell body. So far as the interpretation of the bulbar asymmetry as a manifestation of axoplasmic flow is correct, this state of affairs indicates that the intensity of flow is constant along unbranched fibres.

The fact that the flow continues undamped through a very long narrow channel with distensible walls, after having traversed hundreds of nodal constrictions, indicates that some local mechanisms acting at each level of the fibre contribute to the propulsion of axoplasm. The existence of such mechanisms has already been postulated by Weiss on the basis of location of deformations in partly constricted fibres. It may also be inferred from the constant rate of nerve regeneration independent both from the level of the lesion Gutmann (1955) and from the time that elapsed after the start of regeneration (Lubińska and Olekiewicz 1950).

The study of the outflow of contents from cut ends of fibres (Lubińska 1956) shows that in a fragment of excised fibre the interaction between intraaxonic pressure and the elasticity of neurilemma gives rise to flow of axoplasm towards the sites of lessened pressure at the cross-sections. The outflow of axoplasm is accompanied by reduction of neurilemmal diameter in the terminal portions of the fibre. As both cross-sections of an excised fibre communicate with atmospheric pressure, the outflow takes place from both ends and is not unidirectional as in the body, where the perikaryon must be the source of increased pressure.

There is yet a very long way to understanding the axodynamics of nerve fibres since the necessary quantitative data concerning intraaxonic pressure, mechanical properties of the neurilemma, and viscosity of axoplasm in vertebrate fibres are lacking. It seems worthwhile, however, to remark that the very existence of the flow may be a factor maintaining the continuity of the axoplasmic thread and counterbalancing the tendency to Wallerian degeneration. This hypothesis is suggested by experiments of Taylor (1935), where the breaking up of fluid cylinders into droplets has been prevented as long as a field of flow was maintained in the medium, and occurred when the flow was brought to a standstill. In the light of this fact it is possible to conjecture that the Wallerian degeneration is due to the arrest of proximo-distal flow in fibres separated from their perikarya.

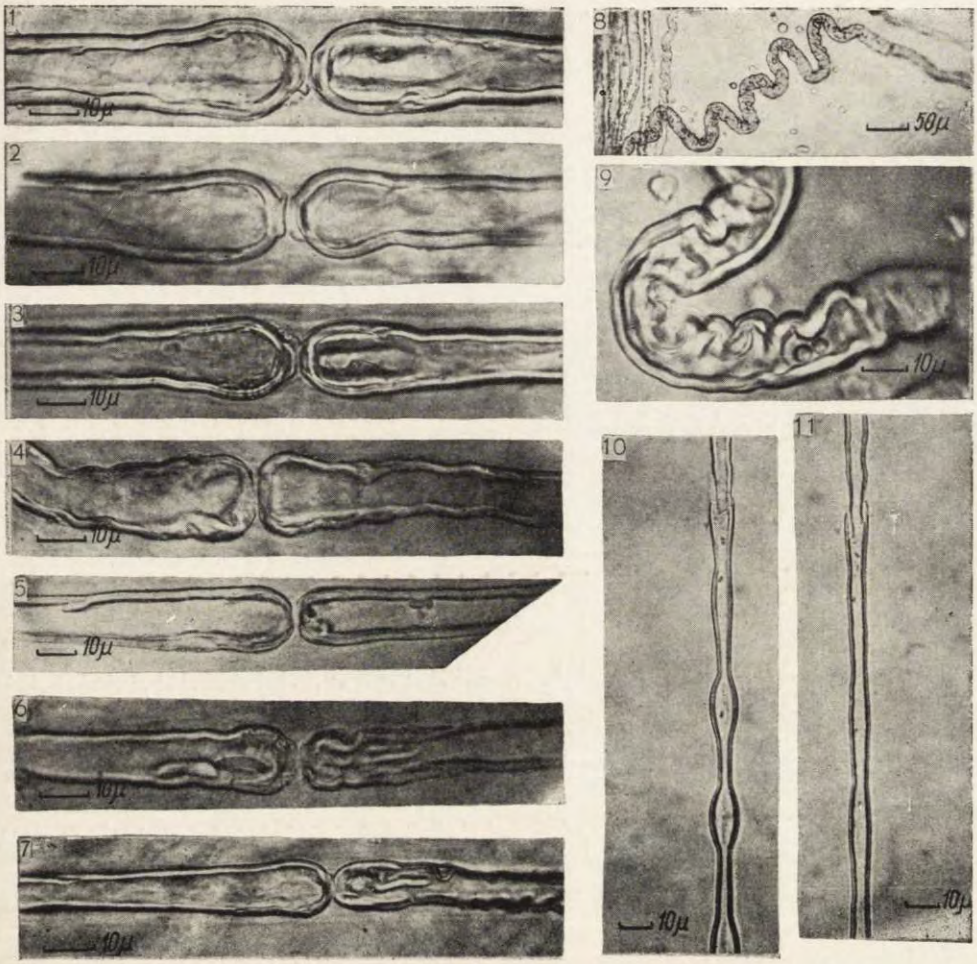

PLATE I

Photomicrographs of living fibres of cats

1—7. Asymmetry of the nodal region. Proximal bulbs of the left, 8. Configuration of a fibre stretched and abruptly released during teasing, 9. A fragment of the same fibre under higher power to show the distortion of myelin, 10. Moniliform appearance of a slowly stretched fibre, 11. The same fibre after release of tension, photographed two hours later. The contour of the fibre is smoothed again

SUMMARY

1. The shape of living myelinated nerve fibres of cat, teased in in blood plasma or Ringer was investigated quantitatively.

2. The bulbous enlargements of the fibre on both sides of a node of Ranvier present a marked asymmetry, the proximal bulb being both larger and longer than the distal one.

3. The quantitative relations between various dimensions of the bulbs and of the nodes proper, according to the caliber of the fibres, were determined.

4. A very slight systematic change of diameter was detected along each internode (outside the nodal region), the narrowest portion lying near the beginning. The mean diameter of the initial half is some 5 per cent thinner than the mean diameter of the terminal half of the internode.

5. The shape and proportions of the nodal region in peripheral fibres of equal sizes are invariable at various distances from the cell bodies.

6. The shape of nerve fibres is discussed in relation to the proximo-distal flow of axoplasm in the axons.

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THE PHYSICAL STATE OF AXOPLASM IN TEASED VERTEBRATE NERVE FIBRES

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Although many features of living nerve fibres, especially the reaction to compression, the Wallerian degeneration and the outflow of contents from the cut ends of the fibres (Calugareanu 1901, Causey and Palmer 1952, Lubińska 1952, 1956, Young 1937, 1944), indicate that the axoplasm is liquid, the examination of isolated nerve fibres frequently reveals gelation of axoplasm (Chambers and Kao 1951, de Renyi 1929, Staempfli 1952, Schneider 1952). These conflicting facts suggest that the axoplasm is liquid under certain conditions and solid under others, and that sol-gel transformations may occur in it under the influence of various factors. Some of these factors will be mentioned briefly but the purpose of the present paper is to show that, according to the physical state of axoplasm, the fibres exhibit marked differences in relations between their components and in the course of deterioration. Those differences which may be inferred from examination of the shape and appearance of various portions of living fibres will be described here.

In carefully teased preparations of vertebrate nerve fibres in Ringer's solution both liquid and gelated axoplasm may be seen.

The proportion of each kind of fibres depends on the degree of mechanical mishandling to which they had been subjected in the course of isolation, on the content of glucose in the bathing medium, and on the species of the animal. Thus the amphibian fibres examined (frog, toad, salamander) are, according to the criteria listed in table I, more fluid than mammalian fibres. Strong mechanical stresses arising during the teasing increase the number of gelated fibres in the preparation, whereas an increased concentration of glucose in Ringer seems to hamper the gelation of axoplasm.

Cut ends of teased fibres. It is at the cut end of a fibre that the physical state of axoplasm is most easily revealed. If the axoplasm is liquid, it forms rapidly a convex meniscus at the cross-section. The myelin spreads on the newly created surface and the borders of the cut myelin tube coalesce, ensheathing the axoplasm completely (Fig. 7, 8). The fluid axoplasm flowing slowly out of the nerve tube forms a spherical droplet at the opening of the fibre. In the initial stages of the outflow this droplet is enclosed in a shell of myelin that is a continuation of the myelin sheath in the fibre (Fig. 9, 10, 11).

In fibres with gelated axoplasm, the cross-section is flat or slightly convex. The myelin does not wet the gelated axoplasm and the cross-section remains naked. The opened myelin tube does not close up (Fig. 1, 2, 3). At the borders of an opened myelin tube the myelin figures appear early whereas in tubes closed over the axoplasm the formation of myelin figures is considerably delayed. Sometimes the axoplasm protrudes from the fibre (Fig. 4, 5, 6). It either retains then its cylindrical shape, or becomes slightly irregular. When the protruding axoplasmic cylinders are long (they may occasionally attain a length of 100—200 μ), the myelin tube, retracted and pleated around the axoplasm, will be seen farther up the fibre (Fig. 5). The appearance of such fibres indicates that not only the new surface of gelated axoplasm is not wetted by myelin, but that the usual relationship between the axoplasm and the myelin on the preexisting cylindrical interface in the fibre is also disturbed, and the myelin slips on the gelated surface of axoplasm it no longer wets.

The naked cylinders of axoplasm, protruding from the sheath were already observed by Schneider (1952) and by Wilska and Kotilainen (1953) in the peripheral fibres broken

by traction, by C h u (1954) in the axons of human motoneurons isolated by shaking, and by H o d g k i n and K a t z (1949) after expression of axoplasm from giant fibres.

The gelation of the axoplasm, like that of many other kinds of protoplasm, is accompanied by an increase of volume indicated by the enlarged diameters of the nodes and by protrusion of axoplasm from the cut end. The poisoning of nerve with cyanure brings about, among other changes, a violent gelation of axoplasm. The nodal region of a poisoned fibre with a swollen and contorted axoplasmic core is shown in fig. 40.

D e t e r i o r a t i o n of f i b r e s i n a g e i n g p r e p a r a t i o n s. The morphological changes become apparent in various fibres of the same preparation at different times. In fibres with gelated axoplasm the alterations set in almost immediately. In liquid fibres the deterioration may begin within the first hour following isolation, but more often a perfectly normal appearance is maintained for long periods of time, sometimes up to 24 hours.

In all fibres the greatest structural instability manifests itself at the nodes which demyelinate before any change can be detected in the internodes.

In fibres with liquid axoplasm the deterioration begins with the swelling of the juxtanodal Schwann cell cytoplasm and possibly, that of some intraaxoplasmic elements. The myelin splits into layers near its insertion at the node and different layers are displaced independently of each other by the swollen nodal material (Fig. 32). Gradually the whole myelin tube is pushed away from the node and the bulbs are filled with swollen Schwann cell cytoplasm surrounding the axoplasmic core (Fig. 33). As the swelling progresses the displaced myelin tube, folded and wrinkled, is seen at some distance from the swollen region. Different stages of such demyelination are shown in fig. 34—39.

The demyelination starts usually earlier in the larger proximal (L u b i ń s k a 1954) than in the distal bulb but this rule is not an absolute one.

In the fibres with gelated axoplasm the demyelination of the nodes occurs abruptly and it is difficult to observe the intermediary stages. The final appearance of the nodal region in these fibres differs from that in liquid fibres, in that the wrinkled myelin can never be seen beneath the swollen region.

The most characteristic alteration of myelin in the internodes

of fibres with gelated axoplasm consists in a rapid and extensive widening of the incisures. The substance of the incisures swells, and presses firmly the external edges of the cylindro-conic segments against the neurilemma, pushing gradually the internal edges toward the interior of the fibre. These edges finally turn at right angles to the axis of the fibre producing a characteristic fenestrated appearance. Fine aggregates of myelin lamellae cross the space of enlarged incisures (Fig. 12, 13, 14). Outside the incisures the myelin tube is straight and regular.

In the fibres with liquid axoplasm the incisures remain narrow for a long time. Ultimately they enlarge slightly and their edges acquire an undulating outline. In some of these fibres a peculiar change of myelin intervenes in advanced stages of deterioration. The myelin tube infolds and indents the axoplasm (Fig. 20, 21, 22), and the amount of myelin in the fibre seems greatly increased. As these fibres do not appear swollen, this increase of the volume of myelin occurs presumably at the expense of water released from axoplasm. Sometimes the whole lumen of the fibre is filled with a mixture of axoplasm and myelin (Fig. 23, 24). When eventually these fibres degenerate, ovoids filled with this mixed material will be observed (Fig. 25), instead of the usual clear axoplasmic drops enclosed in shells of myelin (Fig. 18, 19).

The axoplasm remains homogeneous indefinitely in gelated fibres whereas the liquid axoplasm separates soon into an optically denser core surrounded by a clearer external phase (Fig. 26, 27, 28, 29). Brownian movements may be seen in both phases. Sometimes the denser phase, instead of forming a core, breaks into drops connected by narrow necks as during the Wallerian degeneration. However neither the external phase of axoplasm nor the myelin participate it the formation of these droplets (Fig. 30, 31).

The ultimate fate of both kinds of fibres is different. The gelated fibres retain the cylindrical shape and a continuous column of axoplasm indefinitely. The liquid fibres, on the contrary, soon acquire an unduloid outline, which becomes more pronounced with time, and finally the fibre undergoes the Wallerian degeneration (Fig. 15—19).

The main differences between the fibres with fluid axoplasm and those with gelated axoplasm are summarized in table I.

Table I

Characteristic features of isolated nerve fibres according to the physical state of their axoplasm

Liquid axoplasm	*Gelated axoplasm*
Cut ends of fibres	
1. A convex meniscus appears initially at the cut end and later on the axoplasm flows out from the fibre, forming a spherical drop.	1. The cross-section remains flat. Sometimes axoplasm protrudes from the cut end, retaining the cylindrical shape.
2. Myelin spreads on the axoplasm.	2. Myelin does not wet the axoplasm.
3. Free borders of the myelin tube coalesce over the cross-section of axoplasm. Myelin figures do not appear at such fibres, until advanced stages of deterioration are reached.	3. The myelin tube remains open at the cross-section. Myelin figures appear soon near its end.
Course of deterioration	
4. Demyelination at the nodes begins later. Incisures enlarge slightly.	4. Rapid demyelination at the nodes and strong widening of the incisures.
5. In the internodes the outline of myelin is undulating and folds of myelin indent the axoplasm. Axoplasm looses its optical homogeneity and separates into two phases.	5. Between the incisures the myelin tube remains straight and the axoplasm is homogeneous.
6. The shape of the fibre changes gradually from cylinder to unduloid and finally the content of the fibre breaks into ovoids of Wallerian degeneration.	6. The fibre preserves indefinitely the cylindrical shape.

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PLATE I

Figs 1—6 Cut ends of fibres with gelated axoplasm. Myelin tubes remain open. Axoplasmic cylinders protrude from some fibres (arrows). Fig. 5 — a fibre with a very long axoplasmic cylinder and myelin tube retracted and pleated higher up the fibre (axoplasm is poorly visible, myelin in focus). Figs. 7—11. Cut ends of fibres with liquid axoplasm. Myelin tubes close up on the cross-section. Figs. 7, 8 — convex meniscus of axoplasm. Figs. 9, 10, 11 — droplet of outflowing, axoplasm ensheathed by myelin. Figs. 12—14. Enlarged incisures in deteriorating fibres with gelated axoplasm. Figs. 15—19. Progressive changes of shape and Wallerian degeneration in fibres with liquid axoplasm. Figs. 20—25. Mode of deterioration observed sometimes in fibres with liquid axoplasm. The amount of myelin increases in the fibre. Myelin folds and indents the axoplasm. In late stages of deterioration the fibre may be completely filled with mixed material. Fig. 25. Ovoids formed by mixed axoplasm and myelin.

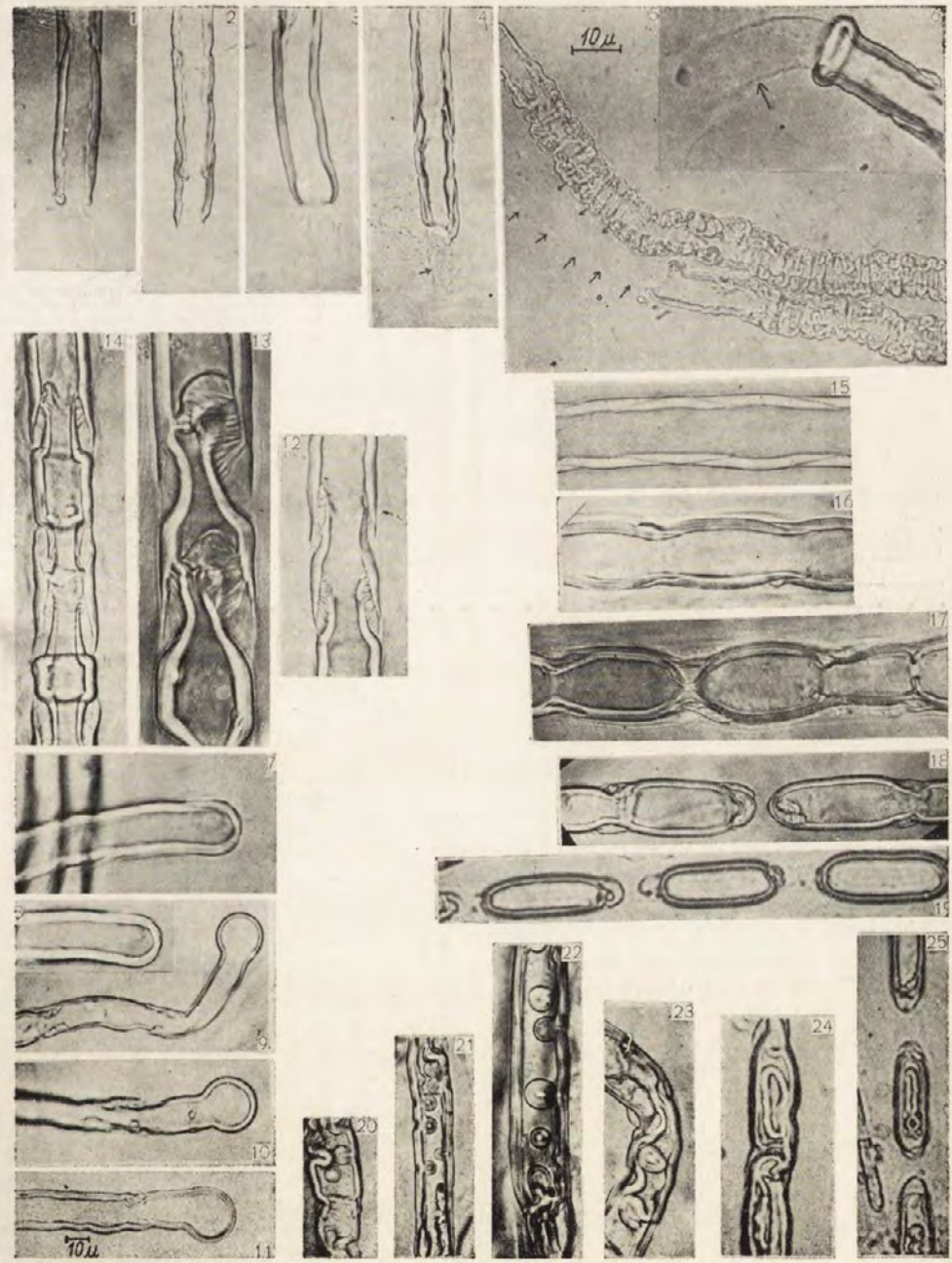

PLATE I

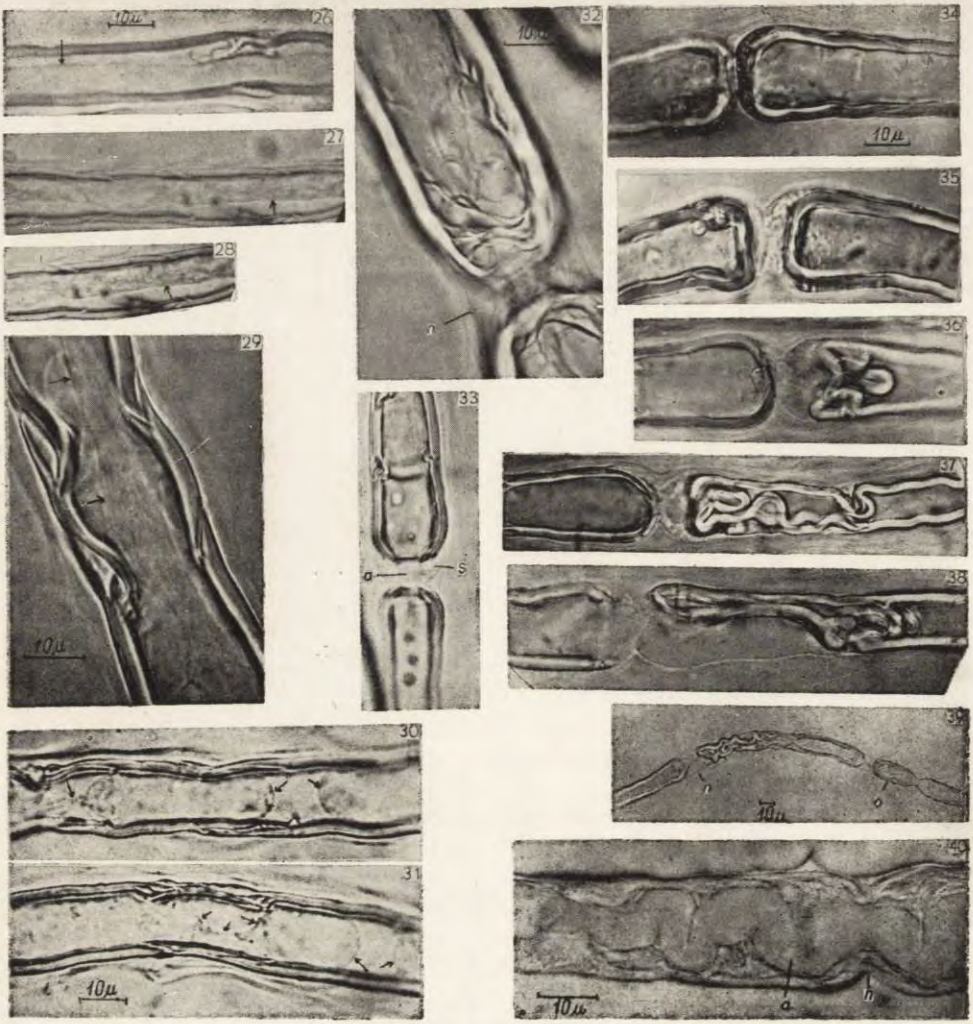

PLATE II

Figs. 26—31. Separation of axoplasm into two phases in deteriorating liquid fibres. Arrows — interfaces. Figs. 30—31. One of the axoplasmic phases forms droplets resembling the ovoids of Wallerian degeneration; the other phase is continuous. The fibre does not collapse between the droplets. Fig. 32. Beginning of demyelination of a node of Ranvier in a fibre with liquid axoplasm. Myelin near the node separates into layers (n — node). Fig. 33. Demyelination of the node. The whole myelin tube is displaced from the node by the swollen Schwann cell cytoplasm (s). A brigde of axoplasm (a) is visible in the demyelinated portion. Figs. 34—39. Progressive stages of demyelination of the nodes in fibres with liquid axoplasm. The myelin tube pushed away from the node is wrinkled. Fig. 39 a low power photograph of a degenerating fibre with wrinkled end of myelin tube beneath the demyelinated node and an incipient ovoid (n— node, o — ovoid). Fig. 40. Nodal region in a fibre poisoned with cyanure. Myelin is altered and crosses the node. The gelated axoplasm is swollen and contorted (a — axoplasm, n — node).

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RECIPROCAL TRANSFORMATIONS OF HETEROGENEOUS CONDITIONED REFLEXES

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In our previous papers of this series (J. Konorski and G. Szwejkowkska 1950, G. Szwejkowska 1950, J. Konorski and G. Szwejkowska 1952, G. Szwejkowska 1952) we were concerned with the analysis of transformations of conditioned reflexes into reflexes of the „opposite sign", i.e. excitatory reflexes were transformed into inhibitory reflexes and vice versa. It has been found that when the conditioned reflex to a given stimulus had been transformed into its opposite, the new conditioned connections established between the respective cortical „centres" are, so to say, superimposed on the old ones, which are by no means destroyed. And so such a transformed conditioned stimulus acquires a „mixed" significance being neither purely excitatory nor purely inhibitory.

In this paper an attempt is being made to throw some light on the properties of the conditioned reflexes which were transformed into „heterogeneous" reflexes (i.e. reflexes reinforced by other unconditioned stimuli), and as the subject of our analysis we took the transformations of alimentary into defensive reflexes and of defensive into alimentary reflexes.

METHODS

The experiments were performed on dogs in an ordinary conditioned reflex chamber.

The technique of experiments with alimentary conditioned reflexes was the same as in our previous papers (K o n o r s k i and S z w e j k o w s k a 1950). The isolated period of conditioned stimuli was 10 or 20 seconds, the intertrial intervals lasted about 5 min. The salivation from the parotid gland fistula was measured by an improved Ganike-Kupalov apparatus.

The defensive conditioned reflexes were reinforced by a single condenser discharge of 2 μ F and 100—180 V, applied to the hindleg of the dog. The isolated period of conditioned stimuli was 3, 5 or 10 seconds, the intertrial intervals lasted about 2—3 min. The movement of the leg was recorded on kymograph. Both the latent period and the amplitude of the reflex were determined from the records.

RESULTS

The experiments were performed on two dogs, which have been kept in the laboratory for a long time and were used in experiments reported in previous papers.

Cygan, a mongrel, was kept in the laboratory since 1948, when he was about 3 years old. Among others he had acquired the following conditioned reflexes: a l i m e n t a r y reflexes to the sound of a Metronome and of a Bell, d e f e n s i v e reflexes to the sound of Splash of water falling into a vessel filled with water, and to the sight of a rotating small Propeller.

The present study included following series of experiments.

S e r i e s I. 16th May — 16th June 1952. Preparatory experiments.

In this series alimentary conditioned stimuli, the Metronome and the Bell, were applied, and, in addition, a new stimulus, a Whistle, was introduced and applied without reinforcement once during each experimental session. Owing to generalisation, this stimulus elicited at the beginning a slight conditioned reaction, which soon dropped almost to zero.

In table I the protocol of one of the experiments of this series is given. It can be seen that salivation to the Whistle is nil, and the conditioned reflexes to the Bell and to the Metronome are of similar magnitude. In the last experiments of this series, after several applications of the alimentary stimuli, we applied also a defensive conditioned stimulus, the Propeller, reinforced after 3 se-

conds by an electric shock to the hindleg. Usually this stimulus applied immediately after alimentary stimuli evoked a very small reaction, which increased only at the second or third defensive trial.

Series II. 17th June — 7th July 1952. The transformation of the excitatory (Metronome) and inhibitory (Whistle) alimentary conditioned stimuli into defensive excitatory stimuli.

Experiments of this series ran as follows: At the beginning of each session an alimentary conditioned stimulus, the Bell, was applied several times with alimentary reinforcement. Then a defensive conditioned stimulus, the Propeller, was applied several times with shock reinforcement. When the defensive conditioned response to this stimulus became normal, the Metronome and the Whistle were successively applied, each day in reverse sequence. Both these stimuli were reinforced after 3 seconds by the electric shock. At the end of each session the Propeller was again applied for control.

Table I

A typical course of experiment of the preperatory series „Cygan", 4th June 1952, No 16

No of trial	Time in min	Conditioned stimulus	Its isolated period in sec.	Salivary conditioned reaction in divis. of scale			Reinforcement	Unconditioned reaction (first 10 sec.)
				first 10 sec.	second 10 sec	total		
1	1	Metronome	20	5	16	21	Food	39
2	6	Bell	20	8	17	25	Food	49
3	11	Metronome	20	6	12	18	Food	42
4	16	Whistle	20	0	0	0	—	—
5	19	Bell	20	3	9	12	Food	41
6	24	Bell	20	2	11	13	Food	45
7	28	Metronome	20	2	10	12	Food	42

In the first two experiments the dog did not raise his hind leg either to Metronome or to the Whistle. On the third day, a slight movement of the leg was observed in response to the Whistle, while to the Metronome the dog remained immobile. From the forth day (20th of June) the defensive reflex to the Whistle becomes as strong as that to the Propeller, while the reflex to the Metronome remains

mostly absent and appears only occasionally. Fig. 1 represents the formation of the defensive reflex to both stimuli, and in Fig. 2 kymographic records of some experiments of this series are given. The difference between the reactions to the Whistle and to the Metronome is clearly seen in both figures.

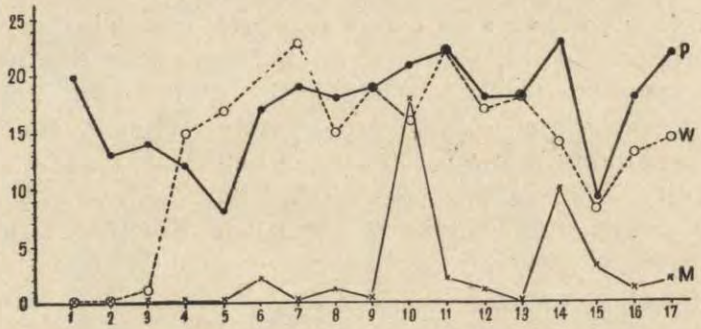

Fig. 1. Transformation of alimentary excitatory (Metronome) and inhibitory (Whistle) stimuli into defensive stimuli, „Cygan", Series II, Abscissae: No. of experiments. Ordinates: amplitude of defensive conditioned reaction in mm of kymographic record. M — defensive reflex to Metronome. W — defensive reflex to Whistle. P — defensive reflex to Propeller (control). The striking difference between the reflex to Metronome and Whistle is clearly seen.

S e r i e s III. 8th July — 4th November 1952. The fixation of defensive reflexes to the Metronome and the Whistle and lengthening of the isolated period of the stimuli to 10 seconds.

As long as the Metronome was applied once daily only, the defensive reflex to it was rather weak or at all absent. But when we began to apply the Metronome more frequently the picture changed rapidly, the reaction to the Metronome becoming as strong as to the other stimuli (Fig. 3a).

Since in the following series of experiments the transformation of defensive reflexes into alimentary reflexes was aimed at, it was necessary to lengthen the isolated period of conditioned stimuli to at least 10 seconds, in order to be able to observe the conditioned salivary reaction. As a result of this change (which was introduced gradually) the reflexes became weak and unstable and their latent periods grew to 5—9 seconds. In these circumstances the weakness of the defensive reflex to the Metronome was again revealed: it became much weaker than the reflex to the Whistle and very frequently did not appear at all (Fig. 3b, c, d.).

Series IV. 5th November 1952 — 23rd March 1953. Introduction of a pair of new stimuli: a Rattle reinforced by shock, and an oscillating Disk applied without reinforcement.

In November 1952 two new stimuli were introduced, the Rattle and the Disk. While first of these stimuli was reinforced by shock,

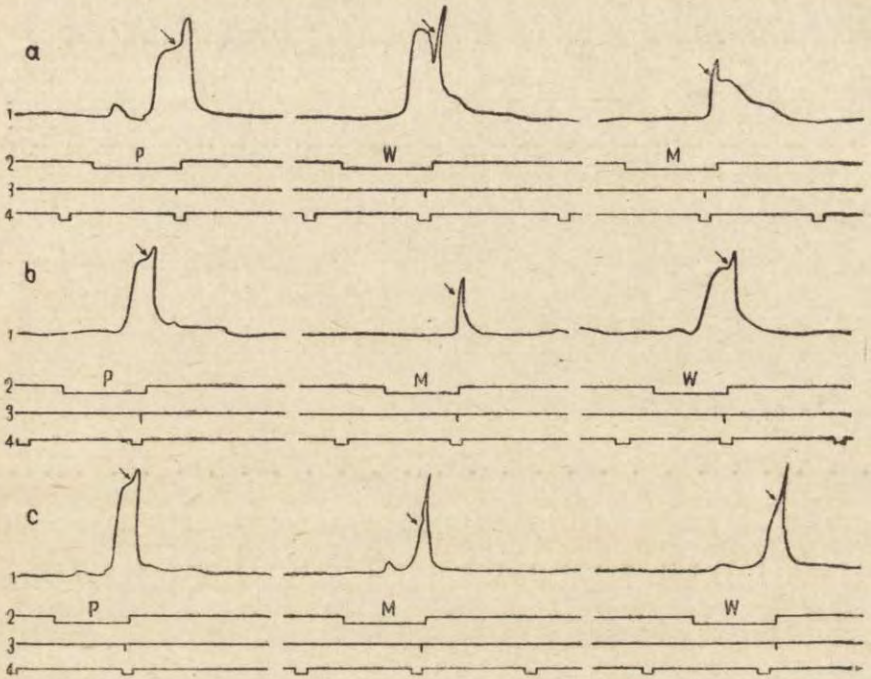

Fig. 2. Kymographic records of some experiments concerning transformation of alimentary into defensive reflex. „Cygan", Series II, 1. Motor reaction of the right hindleg. Arrows denote unconditioned reaction. 2. Conditioned stimulus: P — Propeller, W — Whistle, M — Metronome. 3. Unconditioned stimuli (shock). 4. Time (5 sec). a — 6-th, b — 13-th, c — 15-th experiments of this series. The conditioned reflex to Whistle is as strong as the reflex to Propeller, while the reflex to Metronome is either weaker or absent

the second was not. Alongside with these stimuli the old defensive stimuli, the Propeller and the Splash, were applied as well. The isolated period of all stimuli was 10 seconds.

After a few experimental sessions the reflex to the Disk became inhibited. Representative experiment of this series is presented in Fig. 4.

Series V. 24th March — 27th April 1953. The transformation of defensive conditioned reflexes into alimentary ones.

At the end of March 1953 we returned to the alimentary reflexes. The old alimentary conditioned stimulus, the Bell, was applied again several times in succession with an isolated period

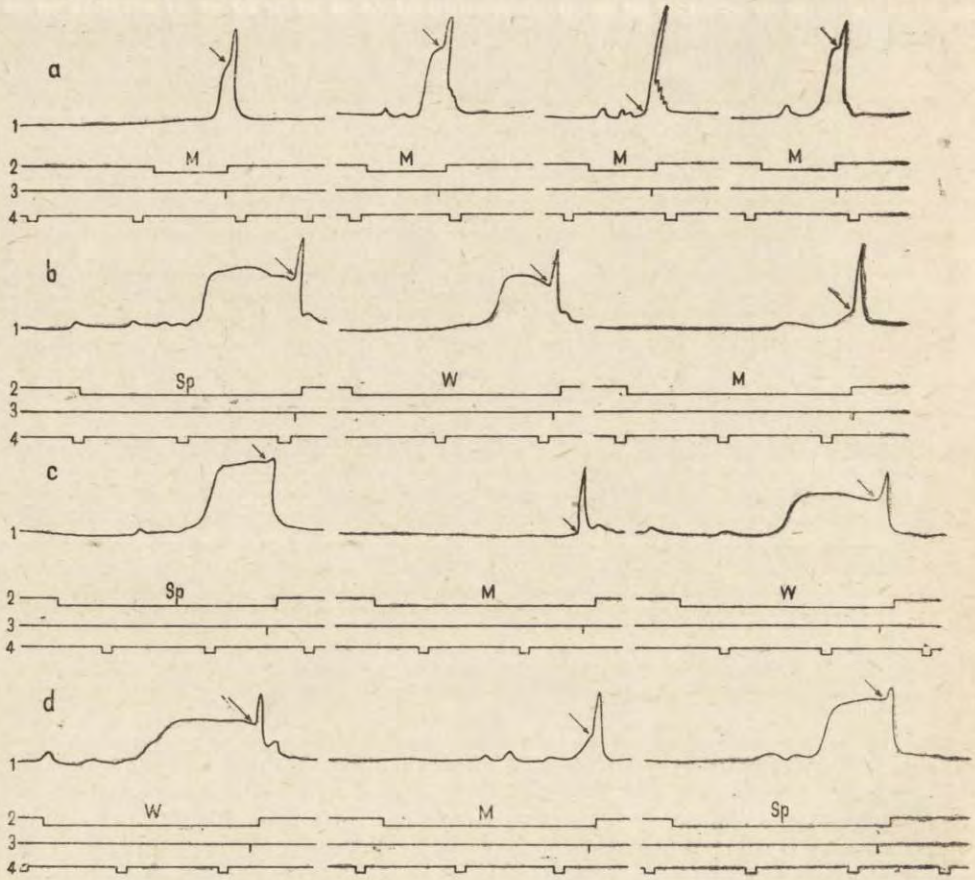

Fig. 3. Further evolution of the reflex to Metronome. „Cygan", Series III. a — Experiment of 8.VII.52, No 44. The repetitive application of Metronome causes its effect to increase, b, c, d — experiments of 15.X.52, No 80, 21.X.52, No 85, 22.X.52, No 86. M — Metronome, Sp — Splash, W — Whistle. The prolongation of isolated period to 10 sec. exhibits the relative weakness of the reflex to Metronome

of 10 seconds. In the third experiment, at the 5th and 7th trial, the Rattle and Disk were respectively applied and after 10 seconds, instead of the chock, they both were reinforced by food. This experiment is presented in table IIa.

It can be seen that there is no alimentary response neither to the Rattle nor to the Disk, and that the unconditioned reflex following these stimuli is much diminished. During the application of the Disk the dog remains calm, while to the Rattle he exhibits a vigourous and lasting defensive reaction which stops after he gets food. On the next day (table IIb) the Disk already elicits a quite significant salivary reaction (9 divisions of the scale), while the Rattle evokes again only a strong defensive reaction.

Table II

Transformation of defensive into alimentary reflexes (series V)

a) „Cygan", 28 th March 1953, No 149/1

No of trial	Time in min.	Conditioned stimulus	Its isolated period in sec.	Defensive conditioned reflex	Alimentary conditioned reflex	Reinforcement	Unconditioned reflex (first 10 sec.)	Remarks
1	2	Bell	10	—	6	Food	34	
2	7	Bell	10	—	15	Food	44	
3	14	Bell	10	—	7	Food	47	
4	19	Bell	10	—	9	Food	43	
5	24	*Rattle*	10	4"—10" very strong —	0	Food	29	He puts the leg down at presentation of food
6	29	Bell	10	—	6	Food	42	
7	34	*Disk*	10		0	Food	25	

b) „Cygan", 29 th March 1953, No 150/2

1	4	Bell	10	—	9	Food	27	
2	9	Bell	10	—	15	Food	41	
3	14	Bell	10	—	11	Food	41	
4	15	*Disk*	10	—	9	Food	39	
5	24	Bell	10	—	12	Food	39	
6	29	*Rattle*	10	3"—10" very strong	0	Food	35	At the presentation of food immediately puts
7	34	Bell	10	—	9	Food	38	the leg down

In further experiments the Rattle and the Disk continued to be applied once daily in reverse sequence. The whole course of this series is given in Fig. 5. It can be seen that the defensive reflex to

the Rattle has disappeared after 4 trials, but the alimentary reflex to this stimulus remained weak to the very end of this series and

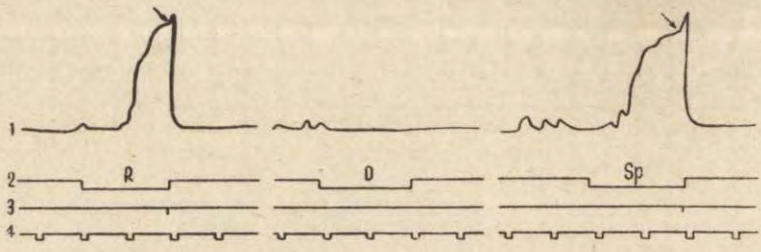

Fig. 4. Elaboration of the excitatory and inhibitory conditioned defensive reflex „Cygan", Series IV. Experiments of 20.XI.52, No 108, and 25.XI.52, No 112 Sp — Splash, R — Rattle, D — Disk. Rattle and Splash evoke defensive reflexes, while Disk does not

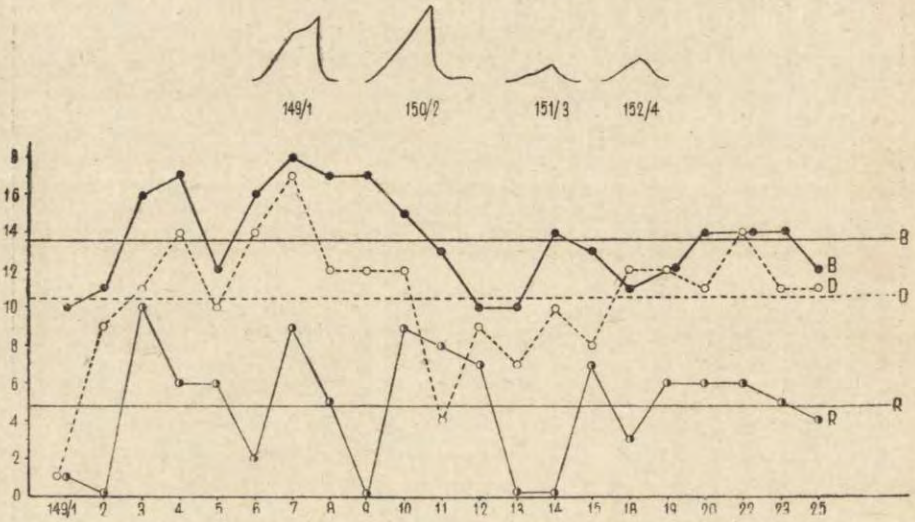

Fig. 5. The course of transformation of excitatory (Rattle) and inhibitory (Disk) defensive stimuli into alimentary stimuli „Cygan", Series V, Abscissae: No of experiments. Ordinates: salivary reflex in divisions of scale. R — Rattle, D — Disk, B — Bell (control). Horizontal lines represent mean values of the respective reflexes. The alimentary reflex to Rattle is much weaker than that to Disk. Above: conditioned defensive reflex to Rattle in the first four days of transformation.

sometimes even dropped to zero. On the contrary, the reflex to the Disk reached very soon the level of 80% of the reflex to the Bell; taking into account the rather moderate strength of the stimulus, this value may be considered as nearly normal.

It must be noticed that the process of transformation of the defensive conditioned reflex into the alimentary one proved rather trying to the animal: the dog, usually very calm, became restless and the salivary reactions to the Bell grew erratic. In these circum-

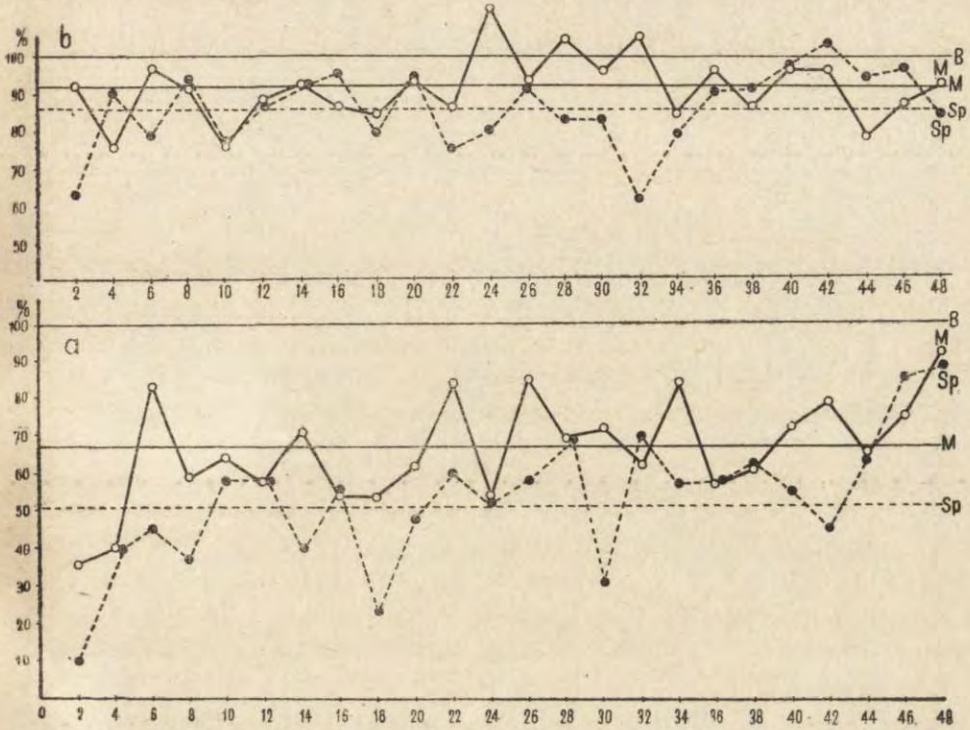

Fig. 6. Transformation of conditioned defensive stimuli (Metronome and Splash) into alimentary stimuli „Cygan", Series VI and VII, Abscissae: No of experiments in the respective series (each point represents two successive experiments). Ordinates: salivary reaction in percentage of that to the control stimuli. a — conditioned reactions, b — unconditioned reactions during first 10 sec. M — Metronome, Sp — Splash. Both reflexes are weaker than the control reflex, but the reflex to Metronome is somewhat stronger than that to Splash.

stances, in order to avoid chronic disorder of the conditioned-reflex activity we had to proceed with great caution and to stop time and again the application of the Rattle and the Disk for several days till the condition of the dog became normal.

When the alimentary reflexes to the Rattle and the Disk became more or less stable, we applied both of them, as previously, along

with the defensive conditioned stimuli. It turned out that against this background the Rattle elicited again a quite considerable defensive response thus proving that it still preserved previous signalling meaning. The Disk on the other hand did not evoke this reaction at all.

S e r i e s VI. 23rd June 1953 — 5th December 1954 and s e r i e s VII, 9th December 1953 — 5th April 1954. The transformation of the Metronome and of the Splash into alimentary conditioned stimuli.

The aim of these two series of experiments was to compare rate and degree of conversion into conditioned alimentary stimuli of the Metronome, a defensive stimulus which had been alimentary before transformation, and of the Splash, so far a purely defensive stimulus. Each of these conversions was accomplished in a separate series of experiments. In these experiments, as usually, between the applications of the Bell (a purely alimentary conditioned stimulus), the Metronome in series VI and the Splash in series VII were applied respectively, once daily, both with food reinforcement. At the end of series VII the two transformed stimuli, the Metronome and the Splash, were applied so that we could compare their respective effects.

The comparison of both series of experiments is presented in Fig. 6. It can be seen that the conditioned reflex to the Bell is generally greater than to the Splash, although it does not attain the „normal" strength, it had before its conversion into the defensive reflex (Fig. 6a). The unconditioned salivary reflex following the converted stimuli was also weaker than that following the control stimulus (Fig. 6b).

The frequent application of the transformed stimuli led (as in series V) to more or less manifest disturbances in animal's behaviour. In order to avoid experimental neuroses we had sometimes to abstain from applying these stimuli till the dog was back to normal.

S e r i e s VIII. 7th April 1954 — 25th May 1954. Analysis of the „conditioned structure" of the transformed stimuli.

At the end of series V we found that when the alimentary conditioned stimulus derived from a defensive one was applied against a purely defensive background, its defensive character came to light again. Presently similar examination of the stimuli transformed in the last series, i.e. the Metronome and the Splash was

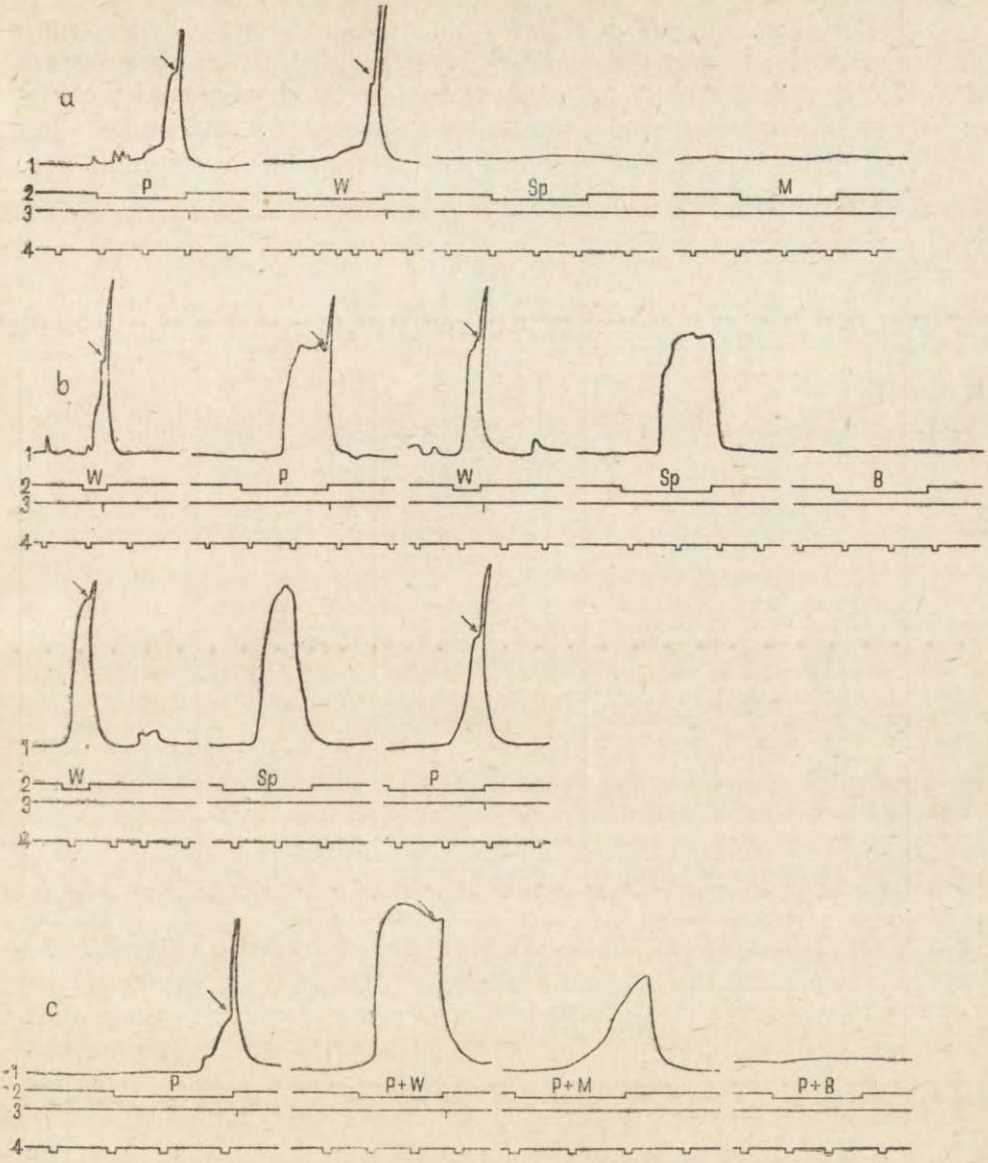

Fig. 7. The various alimentary stimuli applied against the defensive background, „Cygan", Series VIII. a. When all stimuli are applied with 10 seconds isolated period, Metronome and Splash evoke no defensive reaction (exp. 9.IV. 54) b. When defensive stimuli are applied with 3 seconds isolated period, Metronome and Splash evoke a strong defensive reaction, while Bell evokes alimentary reaction (exp. 10.IV.54) c. When Metronome is applied together with Propeller there is a moderate defensive reaction, when Bell is applied with Propeller there is no reaction (exp. 13 and 14.IV.54)

undertaken. To this effect two old defensive conditioned stimuli, the Propeller and the Whistle, were introduced again and were reinforced as usually by a shock. Against this background the Metronome, the Splash and the Bell were tested. In order to keep the defensive character of experiments unperturbed these stimuli were used without any reinforcement.

Table III

Alimentary stimuli applied against the defensive background „Cygan", Series VIII

Background stimuli	Tested stimuli		
	pure defensive stimuli: Propeller, Whistle	mixed stimuli: Metronome, Splash	pure alimentary stimulus: Bell
Defensive stimuli reinforced after 10 seconds	a weak and delayed defensive reaction	no reaction	not tested
Defensive stimuli reinforced after 3 seconds, tested stimuli lasted 10 seconds	very strong and early defensive reaction	strong and early defensive reaction	alimentary reaction, salivation
Simultaneous application of a defensive stimulus with a tested stimulus during 10 seconds.	very strong and early defensive reaction	moderate defensive reaction	no reaction

The first of experiments of this kind is shown in fig. 7a. The Propeller and the Whistle are here reinforced by shock only after the lapse of 10 seconds, and therefore the defensive conditioned reflexes remain rather weak and unstable. The Splash and the Metronome used against such a background evoke neither defensive nor alimentary response.

In order to intensify the defensive conditioned reflexes, in the following experiment (Fig. 7b) the Whistle was reinforced by a shock in the 3rd second of its action, and, after several repetitions of such trials, other stimuli applied for 10 seconds were tested. The result was quite unequivocal. While the Bell produced a slight alimentary response, both the Splash and the Metronome elicited a strong defensive reaction, not less intense than the Propeller.

In other experiments the isolated period of conditioned stimuli was again 10 seconds (due to that the reflexes were rather weak and delayed), and from time to time two stimuli in various combinations were applied s i m u l t a n e o u s l y. In many control experiments it was established that the simultaneous application of two purely defensive conditioned stimuli produces a very strong facilitation: the defensive reflex reaches its maximum and the latent period shortens considerably. Now, if a conditioned defensive stimulus was applied simultaneously with the Metronome or the Splash, the defensive reflex was more or less the same as to a single defensive stimulus, while the combination of a defensive stimulus with the Bell caused complete inhibition of the defensive reaction (Fig. 7c).

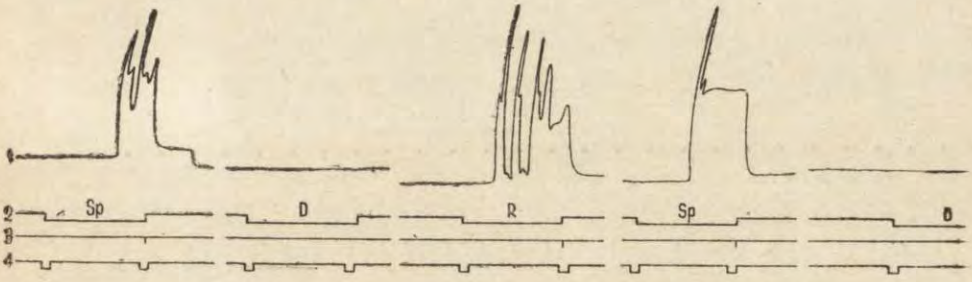

Fig. 8. The elaboration of the conditioned defensive excitatory reflex to Rattle and Splash and of the inhibitory reflex to the oscillating Disk. „Nepek", Series I, Differentiation between Rattle and Disk is established (exp. 28.III.52, No 52)

The results of this series are summarized in table III. The complete sequence of experiments on Cygan is presented in table IV.

The second experimental dog was Nepek, a mongrel who had been living in the laboratory since 1948, when he was 2 years old. This dog had a very copious salivation: in all alimentary experiments he salivated continuously during all intertrial intervals, and did not quite stop even in purely defensive experiments. This gave us the opportunity to study alimentary reactions in more detail than in the case of our other dog, in which the threshold of salivation was much higher. In Nepek a number of conditioned alimentary reflexes had been previously established, to the Bell and the Metronome among others.

S e r i e s I. 22 nd November 1952 — 1 st April 1953. The elaboration of the excitatory conditioned defensive reflex to the Rattle and to the Splash and of the inhibitory reflex to the oscillating Disk.

Defensive conditioned reflexes were formed in this animal without much difficulty, and the dog could easily develop the differentation between the oscillating Rattle and the Disk. The kymographic record of one of the experiments is presented in fig. 8.

S e r i e s II. 2nd April 1953 — 2nd June 1953. The transformation of reflexes to the Rattle and to the Disk into alimentary reflexes.

From the 2nd of April onwards only the alimentary conditioned stimulus, the Bell, with 10 seconds' isolated period was applied, and after a few days the Rattle and the Disk were introduced. Both

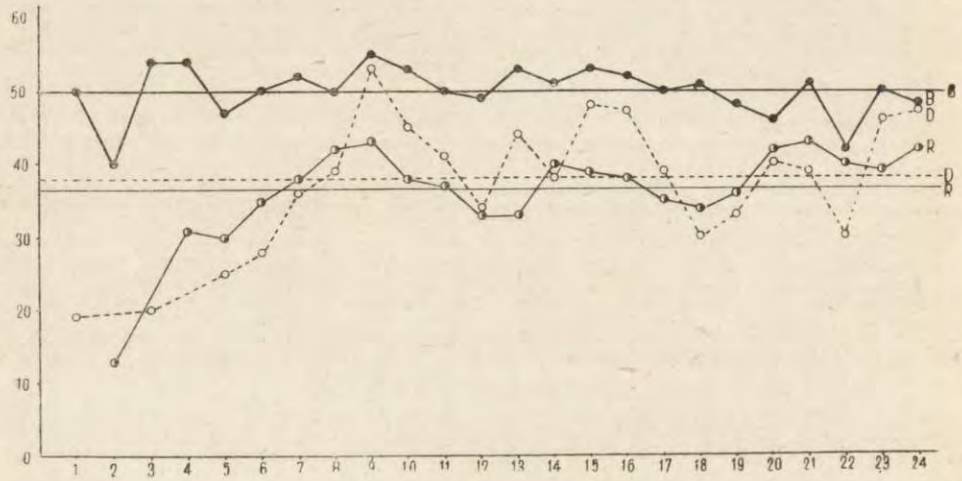

Fig. 9. The transformation of conditioned reflex to Rattle and Disk into alimentary reflexes. „Nepek", Series II. Abscissae: No of experiments. Ordinates: Salivary reaction. B — Bell, D — Disk, R — Rattle. Horizontal lines represent mean values of reflexes to Bell (B), Disk (D) and Rattle (R). There is no significant disparity between the reflex to Rattle and Disk

these stimuli were now (after 10 seconds' action) reinforced by food. The course of these experiments is presented in fig. 9. It can be seen that the conditioned response to both these stimuli reached very soon a stable level amounting to 70% of the reaction to the Bell. In contradistinction to similar experiments on Cygan there

Table IV

The course of experiments on Cygan

Series	Stimuli applied				Results
	Alimentary	Defensive	Transformed from alimentary to defensive	Transformed from defensive to alimentary	
I	Metronome, excitat. Bell, excitatory Whistle, inhibitory	Propeller, excitatory	—	—	
II	Bell, excitatory	Propeller, excitatory	Metronome Whistle	—	The defensive reflex to Whistle was formed readily, that to Metronome very resistantly
III	—	Propeller, excitat Splash, excita. Metronome,excitat Whistle, excitat.	—	—	The prolongation of the isolated period of the reflex to 10 sec. exposes the weakness of the defensive reflex to Metronome
IV	—	Propeller, excitat Splash, excitat. Rattle, excitat. Disk, inhibitory	—	—	Differentiation of Rattle and Disk
V	Bell, excitatory	—	—	Rattle, Disk	The alimentary reflex to Disk was formed easily, that to Rattle very resistantly. Tendency to exp.neurosis
VI	Bell, excitatory	—	—	Metronome	The effect of Metronome did not return to its previous value. Tendency to exper. neurosis
VII	Bell, excitatory Metronome, excitat.	—	—	Splash	The effect of Splash was even less than that of Metronome. Tendency to exper. neurosis
VIII	Bell, excitatory Metronome, excitat. Splash, excitatory	Propeller, excitat Whistle, excitat.	—	—	Against the defensive background Metronome and Splash elicited defensive response, Bell did not

was no significant disparity between the reflex to the Rattle and to the Disk. This may be due to the relative weakness of the Disk in comparison to the Rattle and the Bell, and consequently to its lower conditioned reaction according to „the law of effect of conditioned stimuli". Thus while the alimentary reflex to the Rattle was lowered owing to its previous excitatory defensive character, the magnitude of the reflex to the Disk was probably that it would reach in „normal" training.

Table V

The effect of repeated application of Rattle on conditioned-reflex activity „Nepek", Series II

No of trial	13. V. 53 Bell 10 sec	12. 5. 53 Rattle 10 sec	13. V. 53 Bell 10 sec	14. V. 53 Bell 10 sec	15. X. 53 Bell 10 sec	19. V. 53 Bell 10 sec
1	50	36	33	52	47	44
2	44	43	32	42	45	44
3	49	42	Refused to eat	34	45	45
4	43	38	Very excited	36	46	45
5	46	44		Refused to eat	25	38
6	46	37		Very excited	30	45
	Mean: 46	Mean: 40			Mean: 40	Mean: 44

As in experiments on Cygan the transformation of defensive into alimentary reflexes apparently presented a very difficult task for the dog. He was very excited during the experiments, barked vehemently and tried to tear away the salivary capsule. Such behaviour never occurred previously.

In one of the last experiments of this series the Rattle was applied six times in succession instead of once as hitherto (table V). Conditioned reflexes to this stimulus were relatively strong, reaching nearly the values obtained to the control stimulus, but on the

following day a complete collapse of the conditioned-reflex activity took place. The conditioned reflex to the Bell dropped and the dog refused to take food on the stand. This happened for the first time in the entire conditioned-reflex „career" of this dog. On the next day conditioned reflexes were somewhat stronger and the dog stopped eating only at the fifth trial. In the following experiments he gradually returned to normal.

Table VI

Alimentary stimuli tested against defensive background

„Nepek", 29 th May 1953, No 97/1

No of trial	Time in min.	Conditioned stimulus	Its isolated period in sec.	Conditioned reflex: motor defensive reaction: latent period in sec.	Conditioned reflex: motor defensive reaction: strength of reaction	Conditioned reflex: salivary reaction in divisions of scale
7	23	Splash	10	4	+ +	13
8	26	Splash	10	8	+ +	6
9	29	Bell	10	—	0	43
10	34	Bell	10	—	0	47

„Nepek", 30 th May 1953, No 98/2

5	16	Splash	10	5	+ +	6
6	19	Splash	10	7	+ +	10
7	22	*Rattle*	10	5	+ + +	15
8	27	Bell	10	—	0	47
9	32	Bell	10	—	0	56

„Nepek", 2 nd June 1953, No 100/4

5	14	Splash	10	6	+ +	0
6	17	Splash	10	5	+ + +	11
7	20	*Disk*	10	—	0	25
8	25	Bell	10	—	0	44
9	30	Bell	10	—	0	38

S e r i e s III. 29th May 1953 — 17th June 1953. The analysis of the „conditioned structure" of the transformed stimuli.

After the transformation of defensive into alimentary reflexes, the defensive conditioned stimulus (Splash) was introduced again and after a series of defensive trials the Bell, the Rattle and the

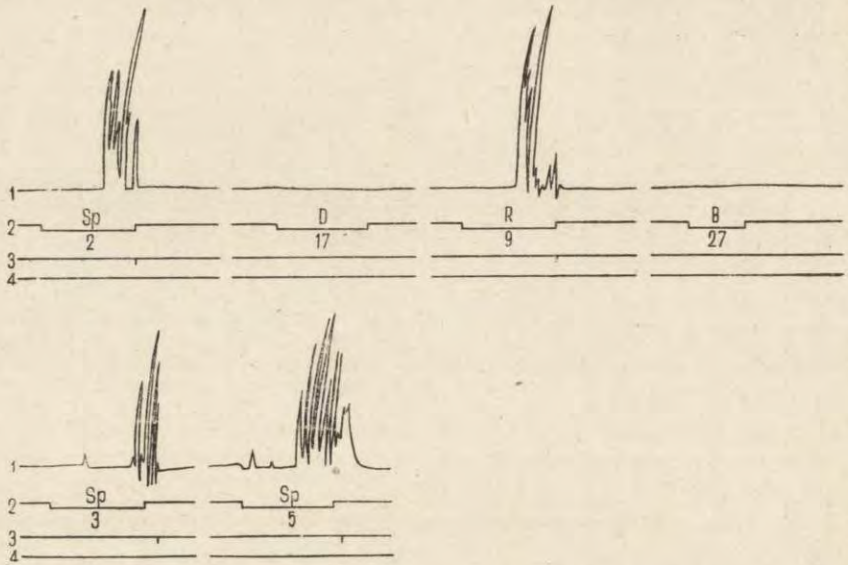

Fig. 10. Alimentary stimuli tested against defensive background. „Nepek". Series III. Exp. of 17.XI.53 Sp — Splash, defensive stimulus. D — Disk, alimentary stimulus transformed from defensive inhibitory stimulus. R — Rattle, alimentary stimulus transformed from defensive excitatory stimulus. B — Bell, "purely" alimentary stimulus. Numbers denote the salivary reactions to respective stimuli

Disk were tested (table VI). In nearly all these experiments the old nature of the Rattle was fully brought to ligth: in contradistinction to the Bell and to the Disk this stimulus evoked a more or less vigourous defensive response, while its salivary effect was much diminished.

In another kind of experiments the Rattle, the Disk and the Bell were applied without reinforcement among defensive trials (Fig. 10). Again, the Rattle evoked only a slight salivary reaction along with a conspicuous defensive response, the Disk evoked a stronger salivary reaction without any trace of a defensive one, and the Bell caused a copious salivation also without defensive reaction.

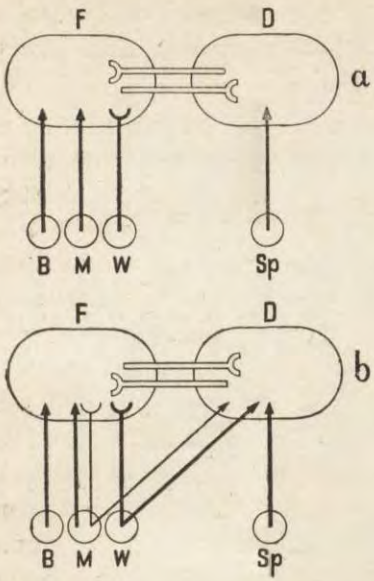

Fig. 11, The schematic presentation of the transformations of an alimentary conditioned reflex into the defensive reflex. F — alimentary centre, D — defensive centre. B — the centre of control alimentary stimulus. M — the centre of excitatory alimentary stimulus transformed into the defensive stimulus. W — the centre of inhibitory alimentary stimulus transformed into the defensive stimulus. Sp — the centre of control defensive stimulus. Double lines represent unconditioned connections; heavy lines represent strong conditioned connections; thin lines represent weak conditioned connections; ↑ - represent excitatory connections; ⊤represent inhibitory connections; a — the connections between centres before transformation; b — the connections between centres after transformation

DISCUSSION

In the previous papers of this series it has been found that when a „primarily inhibitory" stimulus (i. e. a stimulus which was not reinforced from the very beginning of its application) is transformed into an excitatory conditioned stimulus the corresponding positive conditioned reflex does not attain the „normal" value it would certainly achieve if it were reinforced from the beginning. It was concluded that the original inhibitory connections, which had been formed between the „centre" of the conditioned stimulus and the „centre" of the unconditioned stimulus are not destroyed by the formation of new excitatory connections between the centres, but are being preserved for a long time, and, maybe, for ever. In accordance with that we might a priori suppose that with the transformations of conditioned reflexes into reflexes of another kind (e. g. of alimentary into defensive reflexes, or vice versa), this preservation of old connections will also take place and even become more plain. For, when the inhibitory reflex is transformed into the excitatory reflex, the preservation of the old inhibitory connections may be inferred only indirectly from the „dwarfish-

ness" of the respective excitatory reflex, whereas in the case of transformation of reflexes into heterogeneous ones, each of these reflexes can be measured directly.

The preservation of old connections after the transformation of a reflex into heterogeneous one is indicated in the experiments of Frideman (Pavlov 1940, p. 227, Pawłow 1952, p. 192), Rikman (Pavlovian Wednesdays 1949, I, p. 313) and Vatsuro (1949). Pavlov, in his description of Frideman's experiments, draws attention to the fact that when a conditioned alimentary reflex was transformed into the acid conditioned reflex and then back into the alimentary reflex, this last conversion was very rapid. In Rikman's experiments an analogous fact was observed: the transformation of a defensive conditioned reflex into an alimentary reflex required much time and was very difficult to achieve, whereas the transformation in the opposite direction occured immediately. Vatsuro's experiments proved that after the transformation of the defensive into the alimentary reflex the defensive nature of the stimulus can, under certain circumstances, be disclosed again.

The facts presented in this paper confirm and extend the above findings. To begin with, we have found that the alimentary conditioned stimulus which has been transformed from a defensive stimulus evokes a much smaller alimentary reaction than a control stimulus (which was from the very beginning reinforced by food), and conversely, the defensive conditioned stimulus transformed from an alimentary stimulus remains less effective than its control counterpart. Then we have established that when an alimentary reflex has been transformed into a defensive reflex, and then back into the alimentary, it does not reach the magnitude it had before the first transformation. It was also found that an inhibitory alimentary stimulus is converted into the defensive stimulus much easier than an excitatory alimentary stimulus, and conversely, an inhibitory defensive stimulus is transformed into the alimentary much easier than an excitatory defensive stimulus. Finally, we have established that the alimentary conditioned stimulus transformed from a defensive stimulus and placed once more against the defensive background elicits a defensive reaction thus proving that its original nature has by no means been destroyed.

On the basis of these data let us now try to explain the physiological mechanisms underlying the heterogeneous transformations of conditioned reflexes.

As a starting point of our consideration we take the generally accepted view that the physiological basis of the formation of a conditioned reflex is the establishment of neural connections between the cortical „centre" of the conditioned and the unconditioned stimulus, considering both the concept of „centre" and that of „connections" purely physiologically without precising their morphological meaning.

Let us suppose now that a well established defensive conditioned stimulus is not any more reinforced by an electric shock but instead reinforced by food. We are justified in assuming, that in such a case: 1^0 between the centre of the conditioned stimulus and the defensive centre the inhibitory connections are formed, just as they would be formed if this stimulus were simply no more reinforced by a shock, and 2^0 between the centre of the stimulus and the alimentary centre excitatory connections are formed.

It follows from our previous papers (J. Konorski and G. Szwejkowska 1950, G. Szwejkowska 1950, J. Konorski and G. Szwejkowska 1952, G. Szwejkowska 1952) that when a well established conditioned stimulus ceases to be reinforced, the inhibitory connections which are formed between the respective centres are generally poor, as the existing excitatory connections hinder their development. This explains why the inhibitory reflex transformed from the excitatory reflex is generally weak and unstable. As to the establishment of new excitatory connections between the centre of the stimulus and the centre of the new reinforcing stimulus (in our case food centre) they too have a poor chance of normal development, since the two unconditioned centres concerned, defensive and alimentary, are antagonistic to each other, and therefore the excitation of the defensive centre, owing to the existing connections between the respective centres, inhibits continually the alimentary centre. For this reason the conversion of the defensive stimulus into the alimentary one is difficult and deficient. That the previous connections between the centre of the conditioned stimulus and the defensive centre are preserved for a long time is substantiated by the fact that this stimulus applied against the defensive background elicits the defensive response. On the other hand, the assumption that these

very connections hamper the full development of the alimentary conditioned reflex is substantiated by the fact that an i n h i b i t o r y defensive stimulus is converted into an alimentary stimulus without such a difficulty. Indeed, as this stimulus does not excite the defensive centre, the alimentary centre is free from inhibition and there is nothing that would obstruct the formation of the alimentary conditioned reflex to this stimulus.

In our experiments the overt defensive reaction consisting in leg lifting to the conditioned stimulus disappeared very promptly in the course of its transformation into the alimentary stimulus. This was due to the following circumstances: 1^0 the defensive reinforcement was deliberately kept on a rather low level in order not to complicate too much the process of transformation, and 2^0 the stimulus in the course of transformation was always applied between the alimentary and never between the defensive stimuli. The fact that in spite of disappearance of the motor defensive reaction the alimentary conditioned reflex to the converted stimulus was dwarfed proves that excitation of the defensive centre was still sufficiently strong to exert the inhibitory influence upon the alimentary centre. This is not surprising at all if we take into account that various effects of excitation of the defensive centre may have different threshold values and the threshold of the motor reaction in our type of experiments may be considered as relatively high. This is well proved by the fact that even in normal conditions the conditioned defensive reflex, evaluated in terms of the specific motor response, was often subthreshold (especially when the isolated period of the stimulus was long) which did not mean that it was absent (cf. facilitation of two subthreshold reflexes).

Precisely the same sort of considerations may be applied to the mechanism of transformation of the alimentary into the defensive reflex (fig. 11). Here too, the new conditioned reaction (in this case defensive) was below normal owing to the fact that excitation of the alimentary centre produced by old conditioned connections inhibited partly the defensive centre and did not permit the formation of the defensive connections. This again is proved by the fact that the i n h i b i t o r y stimulus was easily transformed into the defensive stimulus, because it did not excite the alimentary centre. Just as in the case of the previously described transformation, the excitation of the alimentary centre, sufficient to hamper the deve-

lopment of the defensive reflex, may be subthreshold when measured by salivation.

Let us consider for a moment the case in which the alimentary conditioned reflex was transformed into the defensive reflex, and then again into the alimentary one. It has been shown that it did not reach its previous value, although it was a little stronger than the alimentary reflex derived from a „purely" defensive reflex. This shows that, although the connections of the stimulus with the defensive centre built up in the course of the first transformation were not so strong as in the case of a normal defensive reflex, they were nevertheless sufficiently powerful to prevent the alimentary reflex to attain its previous level in the course of the second transformation of the stimulus. The preservation of the connections with the defensive centre has been proved also by the fact that the stimulus applied against the defensive background elicited a defensive response. It may be recalled that in the case of a simple extinction and restoration of a conditioned reflex (when only inhibitory but not heterogeneous connections intervene) the return to normal is practically complete.

In the course of heterogeneous transformations animals are very prone to develop experimental neuroses. In particular this was observed during conversion of a defensive into an alimentary conditioned stimulus and consisted in greater or lesser disturbances of the general behaviour of the animal. This fact can be easily explained if we take into account that the excitatory defensive conditioned stimulus, owing to the antagonism between the alimentary and defensive centres, has a strongly inhibitory effect on the alimentary centre, and therefore its repeated reinforcement by food produces in this centre a violent „clash" between the excitatory and inhibitory processes.

SUMMARY AND CONCLUSIONS

1. When a conditioned defensive stimulus is transformed into a conditioned alimentary stimulus the resulting alimentary reflex is weak and unstable, since the connections established earlier between the cortical centre of the stimulus and the defensive centre prevent the formation of the connections between the centre of that stimulus and the alimentary centre. There is no such obstacle in

the case of inhibitory defensive conditioned stimulus transformed into the excitatory alimentary conditioned stimulus.

2. When a conditioned alimentary stimulus is transformed into conditioned defensive stimulus the resulting defensive reflex is weak and unstable, since the connections established between the cortical centre of the stimulus and the alimentary centre prevent the formation of the connections between the centre of this stimulus and the defensive centre. As in the previous case the process is not hindered when the inhibitory alimentary conditioned stimulus is transformed into the excitatory defensive stimulus.

3. When an alimentary conditioned stimulus is transformed into a defensive and then back into the alimentary stimulus, its effect is smaller than it was originally.

4. When the alimentary conditioned stimulus transformed from a defensive conditioned stimulus is applied against the defensive background (i. e. between conditioned stimuli reinforced by shock) it evokes a more or less prominent defensive response, which proves that the old connections established between the centre of this stimulus and the defensive centre have not been destroyed by the formation of new connections between these centres.

5. The transformation of a defensive conditioned reflex into the alimentary conditioned reflex is a very difficult task for the animal. Very often it leads to more or less serious experimental neuroses evoked by the clash between excitatory and inhibitory processes in the alimentary centre.

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THE EFFECT OF THE REMOVAL OF FRONTAL POLES OF THE CEREBRAL CORTEX ON MOTOR CONDITIONED REFLEXES

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The functions of the frontal lobes of the cerebral cortex studied by the ablation technique have been for many years subjected to extensive investigations, and many theories and assumptions have been put forward to account for the vast experimental material collected by numerous authors. Of course, there are differences in data obtained by various research workers depending chiefly on the animals used for experimentation and on the extent of ablations. Nevertheless, nearly all authors working in this field agree that frontal ablations do not produce any s p e c i f i c changes in cortical activity, such as disorders of either afferent or efferent functions, or impairment of learning ability. Even if there are some data pointing to such changes (e. g. autonomic disturbances caused by frontal ablations, cf. F u l t o n 1946, or specific impairment of olfactory discrimination, A l l e n 1940) the majority of authors do not consider these results as the most specific and essential for our understanding of the rôle played by frontal lobes. According to the overwhelming amount of evidence beginning from the classical works of F e r r i e r (1876), B i a n c h i (1895), H i t-

z i g (1904), B e c h t e r e v (1907) etc., the frontal lobes are endowed with some more general and less concrete functions, and their ablations are usually described under such headings as „lack of ability to synthesis, impairment of intelligence, changes of personality", and so on. All such conclusions are drawn either on the basis of general observation of the behaviour of animals deprived of frontal lobes or on specific psychological tests in which the disability of operated animals to cope with some complicated experimental tasks was manifested. There are, however, only few works which approach the whole problem from the standpoint of the physiology of higher nervous activity and tend to formulate the results of ablations directly in physiological terms. (For references see B r u t k o w s k i et al. 1955).

The present work is an attempt to fill this gap and to show that such an approach, although certainly not unique in dealing with the problem, is fruitful and leads to a better understanding of the data obtained by other methods.

MATERIAL AND METHODS

The work was performed on dogs in an ordinary conditioned-reflex chamber. Six dogs were taken for these experiments, but as the work on this problem is still going on in our laboratory, the experiments reported here are fully confirmed on about ten other dogs.

In the present paper only experiments with motor alimentary conditioned reflexes are dealt with. These reflexes thoroughly studied by one of us in previous papers (K o n o r s k i and M i l l e r 1933) have been called conditioned reflexes of the second type to distinguish them from classical Pavlovian conditioned reflexes. They are now called instrumental conditioned responses by the majority of American authors (cf. H i l g a r d and M a r q u i s 1940). To put it briefly and quite generally, this type of reflexes consists in that the animal learns to perform a certain movement if its performance is reinforced by a positive unconditioned stimulus (as the food), or if an opposite movement is reinforced by a negative (noxious) unconditioned stimulus.

The course of experiments reported here is as follows.

First, a number of conditioned motor alimentary reflexes (both excitatory and inhibitory) were established, and after a more or less prolonged training one of the two cortical ablations was made in which either the frontal poles or parts of parietal lobes were bilaterally removed.

Following post-operative recovery which took usually from 2 to 6 days, the conditioner-reflex experiments were resumed and the status of the dog was thoroughly tested. After a lapse of time the second of the two operations was made (or the frontal ablation was enlarged), and the conditioned-reflex

activity was studied again. The experiments on one dog lasted about 2—3 years.

Now we shall describe in more detail the conditioned-reflex training and the surgical procedure.

Conditioned reflex training

The dog was first habituated to the general experimental procedure and environment, in particular to stand quietly on the stand in the experimental chamber, to obtain food in small morsels from the foodtray placed before him, etc. Then the actual training began. The dog was urged to lift his right foreleg and to put it on the foodtray at the sound or sight of a given conditioned signal, the performance of this movement (passive or active) being reinforced by the presentation of food (one morsel of bread of about 8 cm^3). After several days each application of the signal almost immediately elicited the learnt movement and was followed by the presentation of food. Thus, each trial lasted about 5 sec., and the intertrial intervals were 1—2 min. Then some other auditory or visual conditioned stimuli were also introduced.

The excitatory conditioned reflexes having been established, we proceeded to the formation of inhibitory reflexes. We made use of two kinds of internal inhibition, namely differentiation and conditioned inhibition.

In order to establish differentiation, a stimulus similar to the one applied as a conditioned stimulus was introduced and applied (among other stimuli) without reinforcement. Owing to the generalization this stimulus elicited at first the learnt movement but after a number of unreinforced trials differentiation developed and the conditioned response to the stimulus became inhibited.

The elaboration of conditioned inhibition was carried out as follows: A new stimulus differing from conditioned stimuli hitherto applied was chosen and it was applied just before a particular conditioned stimulus. The conditioned stimulus when preceded by the extra stimulus (conditioned inhibitor) was not reinforced. After a number of such trials the inhibitory reflex to this compound was elaborated and the conditioned stimulus applied after the conditioned inhibitor did not elicit the learnt movement. This being done, we have gradually prolonged the interval between both components of the compound until the dog was still capable to unfailingly inhibit the motor reaction to the conditioned stimulus following the conditioned inhibitor. The duration of this interval varied in different dogs, depending also on the strength of the conditioned inhibitor. Usually in our experiments it amounted to 10—15 seconds.

In some dogs we also used a kind of conditioned inhibition which we call „alternation". In these experiments one and the same stimulus was applied throughout each session, alternatively reinforced and not reinforced by food. In special experiments it was shown that in this case the termination of the act of eating plays the rôle of the conditioned inhibitor conditioning the inhibitory reaction to the next stimulus.

Surgigal procedure

a. Frontal lobes ablations. After the incision of skin in the medial line and pushing aside the temporal muscles the frontal and partly the parietal bones were trephined and the sinus frontalis opened. The hiati to the sinuses were tightly packed with wax, the bones covering the frontal lobes were removed, the dura mater was divided and the cortex of the frontal lobes was destroyed by subpial suction. The lesion always included gyri prorei and greater parts of gyri orbitales. The posterior border of the lesion ran usually in sulci praesylvici, but in some operations the rostral parts of gyri sigmoidei anteriores (a few mm. before sulcus cruciatus) were also removed. The dura mater, the muscles, the subcutaneous tissue, and the skin were then sutured.

b. The parietal ablation. After the medial incision of the skin, the temporal muscles were pushed aside and the parietal bone was trephined without opening the sinus frontalis. After dividing the dura mater the cortex of gyri suprasplenialis, gyri entolaterales and the rostral parts of gyri ectolaterales, were bilaterally removed by subpial suction. The lesion situated about 20 mm behind the sulcus ansatus formed usually an irregular quadrangle 20 mm long and 11 mm wide.

At the end of the experiments the animals were sacrificed and the lesions carefully verified macroscopically. The microscopic study of the material will be given separately.

RESULTS

A detailed description of the results obtained on all six dogs used in this investigation is given elsewhere (Brutkowski et al., 1955). As the results obtained in each dog were very alike and fully confirmed by our further experimental material, we feel entitled to present here an extensive report of experiments performed on one dog, which may be considered as typical, supplementing it by some additional data obtained from experiments on other dogs.

A one-year-old mongrel weighing 12 kg. was kept in the laboratory from 1949. He accustomed himself to the experimental conditions very easily, and quickly developed all the excitatory and inhibitory conditioned reflexes. He was always very quiet and matter-of-fact and his conditioned-reflex activity was excellent.

In the preoperational training which lasted about one year alimentary conditioned reflexes to the sound of a Metronome, Bell and Bubbling of water were formed and firmly established. The differential inhibitory reflexes were formed to another timbre of the Bell and Bubbling. They were established after 12 and 4 repe-

titions of these stimuli, respectively. A rotating Propeller suspended before the dog was used as a conditioned inhibitor in combination with the Metronome. The inhibitory reflex to this compound was formed after 24 repetitions and then the interval between these stimuli was gradually prolonged to 10 seconds. The kymographic record of the last experiment before operation is given in Fig. 1a, its protocol in Table I.

First operation (frontal)

On October 26th, 1950, the first operation was performed during which the cortex and white matter of gyrus proreus were bilaterally removed. The ventral parts of the frontal poles lying on the tractus and bulbus olfactorius were spared. The posterior limit of the lesion was sulcus praesylvicus.

The recovery of the dog was uneventful.

A. General behaviour of the dog after operation. During the first two days the dog was quite apathetic, lay coiled and did not react to external stimuli. Then he recovered very quickly and after a few days it ways very difficult to find in his general behaviour any difference with the preoperative status. In particular no hypermotility reported by some authors after frontal ablations was seen, and the motor efficiency was quite normal. Only the act of eating was in the first days somewhat protracted.

B. Conditioned-reflex activity. The first experiment after operation was performed on October 31st. The protocol of this experiment (Table II) and the kymographic record (Fig. 1b) are presented. As we see the conditioned-reflex activity differs from normal in the following essential points:

1-o: the dog often lifts his leg and puts it on the foodtray in intertrial intervals. Such behaviour never occured before the operation (except, of course, at the very beginning of experiments, more than one year ago).

2-o: the inhibitory reflex to the differentiated Bell and to the Metronome following immediately after the Propeller is disinhibited; this also did not occur for many month.

3-o: contrarily, the excitatory conditioned reflexes do not differ in any regard from those before the operation.

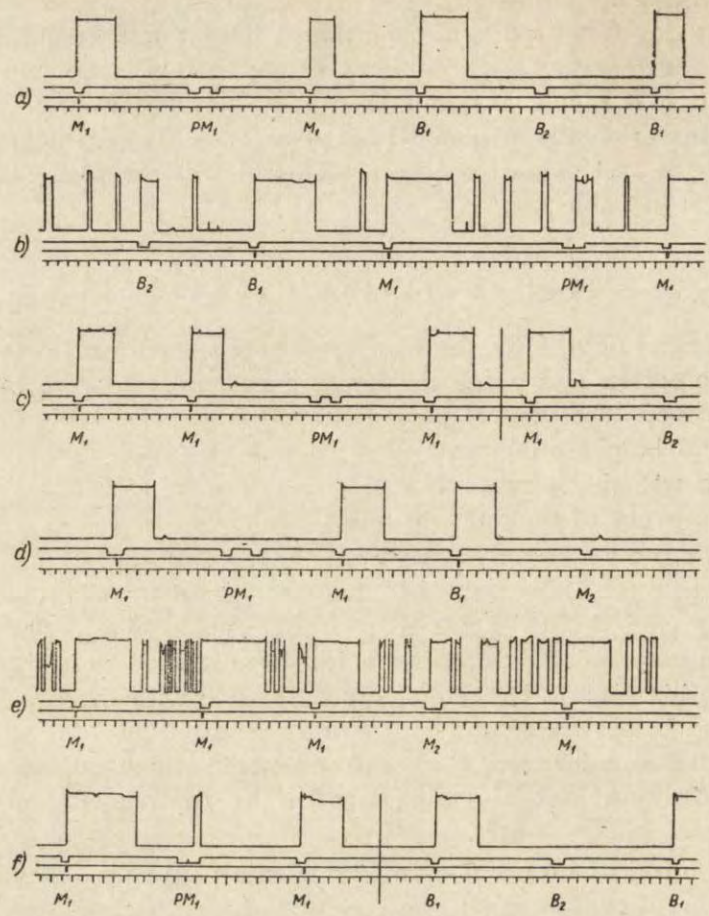

Fig. 1. Kymographic records of parts of some representative experiments in normal condition of the dog and in various periods after successive operations. Each record comprises from top to bottom: lifting the right foreleg and putting it on the foodtray; conditioned stimuli; the moment of food presentation; time (5 sec.); symbols of stimuli: M_1, Metronome, M_2, differential Metronome, B_1, Bell, B_2, differential Bell, PM_1, Propeller — Metronome (inhibitory compound). a — portion of the last experiment before first frontal ablation (No 90, 24.X.1950); b — portion of the first experiment after first frontal ablation (No 91/1, 31.X.1950); c — portion of an experiment $2^1/_2$ months after first frontal ablation (No 136/46, 10.I.1951); d — portion of an experiment after parietal ablation (No 231/8, 21.VI. 1951); e — portion of an experiment shortly after second frontal ablation (No 264/5, 22.X.1951); f — portion of an experiment a month after second frontal ablation (No 284/25, 15.XI.1951).

In the following days the inhibitory capacity of the dog gradually improved (Fig. 2a): In the next experiment the intertrial responses disappeared, but the differentiated stimulus was still disinhibited; it became again inhibitory after 3 repetitions. As to the inhibitory reflexes to the combination of the Propeller and Metronome they failed to appear for a much longer period: even a month after operation the dog was not able to inhibit the response to the Metronome following immediately the Propeller. When he succeded at last to do so, it was sufficient to prolong the interval between the stimuli to 3 seconds to produce a dramatic disinhibition. When in the following days this task was mastered, another prolongation of

Table I

The protocol of the last experiment before first frontal ablation
24 th October 1950, No 90

No of trial	Time of successive trials	Conditioned stimulus	Its isolated period in sec	Latent period of conditioned reaction in sec	Reinforcement	Intertrial reactions
1	1 min	$Metronome_1$	2	1	reinforced	
2	2 min	$Metronome_1$	2	1	reinforced	
3	3 min	Propeller	5	—		
	3m 10 sec	$Metronome_1$	5	—	not reinforced	
4	4 min	$Metronome_1$	2	1	reinforced	
5	5 min	$Bell_1$	2	1	reinforced	
6	6 min	$Bell_2$	5	—	not reinforced	
7	7 min	$Bell_1$	2	1	reinforced	
8	8 min	$Metronome_1$	2	1	reinforced	
9	9 min	Propeller	5	—		
	9m 10 sec	$Metronome_1$	5	—	not reinforced	
10	10 min	$Metronome_1$	2	1	reinforced	
11	11 min	$Metronome_1$	2	1	reinforced	

the interval to 5 seconds again caused disinhibition. Only after two and a half months all the inhibitory reflexes returned to normal and the behaviour of the dog did not differ from his preoperative behaviour (Fig. 1c). The elaboration of a new differentiation (another timbre of Metronome) in this period was quite easy.

C. Neurotic disorders following the operation. Besides the above described disturbances of the inhibitory capacity of the dog, another type of disorder of his behaviour was also observed.

As we noticed before, this dog was before the operation exceedingly well-blanced and quiet, and his behaviour was always quite adequate and matter-of-fact. In the first experiments after operation, in spite of the disturbance of his inhibitory capacity, the general picture of the dog's behaviour was the same. But after

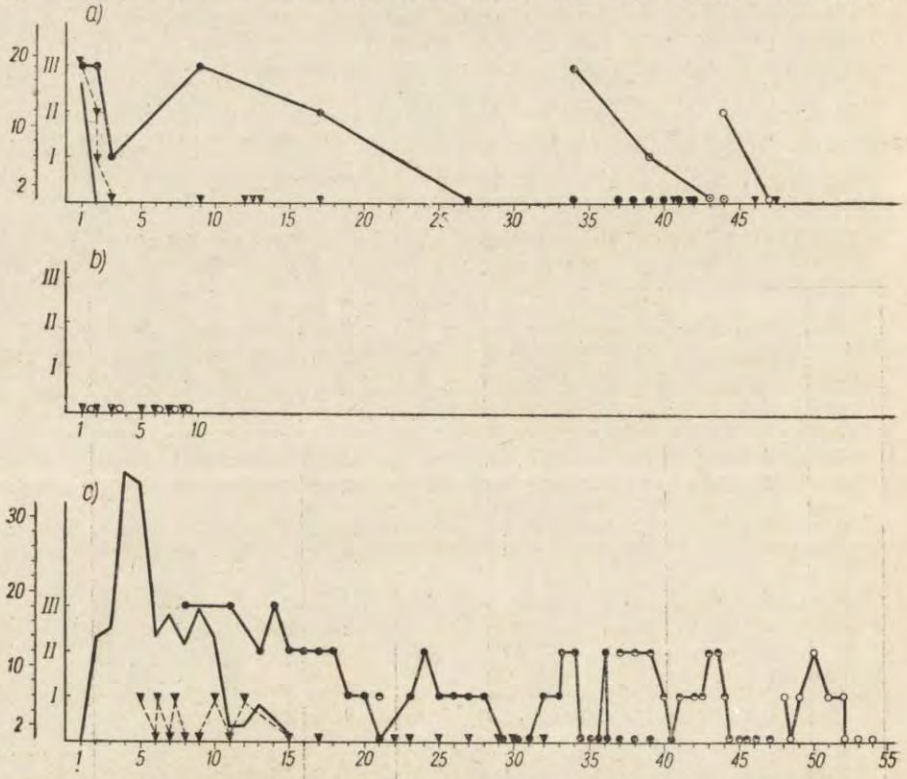

Fig. 2. The disturbances of the inhibitory capacity of the dog after successive operations. Abscissae denote the successive numbers of experiments after each operation. Ordinates denote number of intertrial reactions (Arabic numerals), and the degree of disinhibition of inhibitory reflexes (Roman numerals). I — weak disinhibition, the dog holds his leg on the foodtray for no more than 2 seconds, II — moderate disinhibition, the dog holds his leg on the foodtray for no more than 5 seconds; III — strong disinhibition, the dog holds his leg on the foodtray for more than 5 seconds. Dashed line — number of intertrial reactions. Triangles — differential inhibition. Circles — conditioned inhibition: full circles — interval between conditioned inhibitor and conditioned stimulus O seconds; half-filled circles — interval 2 seconds; dotted circles — interval 3 seconds; hollow circles — interval 5 seconds and more. a — disturbance of inhibitory reflexes after first frontal ablation, b — inhibitory reflexes after parietal ablation, c — disturbance of inhibitory reflexes after second frontal ablation.

a number of experiments in which inhibitory stimuli were repeatedly applied, the behaviour of the animal changed: he was reluctant to enter the experimental chamber, and when put on the stand he was restless, whined and tried to escape. Often he would leave some pieces of food untouched, what never happened previously.

Table II

The protocol of the first experiment after first frontal ablation
31 st October 1950, No 91/1

No of trial	Time	Conditioned stimulus	Its isolated period in sec	Latent period of conditioned reaction in sec	Reinforcement	Intertrial reactions
1	1 min	$Metronome_1$	2	1	reinforced	1
2	2 min 30 sec	$Metronome_1$	2	1	reinforced	—
3	4 min 30 sec	$Bell_1$	2	1	reinforced	3
4	6 min	$Bell_1$	2	1	reinforced	3
5	8 min	$Bell_2$	5	2 (to 10)	not reinforced	1
6	9 min	$Bell_1$	2	1	reinforced	1
7	10 min	$Metronome_1$	2	1	reinforced	3
8	11 min 35 sec	Propeller	5	—		
	11 min 40 sec	$Metronome_1$	5	1 (to 8)	not reinforced	1
9	12 min 35 sec	$Metronome_1$	2	1	reinforced	2
10	14 min	$Metronome_1$	2	1	reinforced	

All these disorders were unmistakenly symptomatic of experimental neurosis. The task required was now too difficult for the animal. When all inhibitory stimuli were excluded for several days the dog recovered rapidly and completely, while a renewal of too frequent applications of inhibitory stimuli led again to more or less express disorders.

For the sake of illustration we quote one of such incidents.

22nd November 1950. Only excitatory stimuli are applied. The dog behaves excellently and is quite calm.

24th November 1950. Condition unchanged.

25th November 1950. The differential Bell and the inhibitory compound of the Propeller with Metronome (in immediate se-

quence) are applied. The Metronome following the Propeller is disinhibited.

27th November 1950. The dog refuses to enter the experimental chamber. During the whole experiment he whines, is very excited, turns towards the door, leaves some pieces of food untouched. The experiment was shortened and only excitatory stimuli were applied.

28th November 1950. Is quieter but still whines after each trial. Only excitatory stimuli are applied.

30th November 1950. Condition unchanged.

2nd December 1950. More or less quiet but refuses to take food in last trials.

4th December 1950. Calm and matter-of-fact.

5th, 6th, 7th December 1950. Condition good. Only excitatory stimuli applied.

9th December 1950. At the beginning of the experiment the dog is quite calm. In the fifth trial the inhibitory compound was applied. The dog displayed a perfect inhibitory response. Nevertheless in the further course of the experiment he becomes excited, whines, gets entangled in the registration lines. The latent period of the motor reaction is protracted and in some trials the conditioned response fails to appear. This n e v e r occured in normal state.

The above most conspicuous intolerance to inhibitory stimuli, and chiefly to the inhibitory compound, compelled us not to apply these stimuli too often and to intersperse the experiments in which the inhibitory stimuli were used with purely excitatory experiments. After a lapse of time this state of „hypersensitivity" to inhibitory stimuli receded and it was possible to return to a quite normal experimental procedure.

Second operation (parietal)

On May 22nd 1951, when the general state of the animal did not differ from his preoperative state, the second operation was performed in which gyri entolaterales, supraspleniales and ectolaterales were bilaterally removed. The frontal limit of the ablation ran ca. 3 cm behind the sulcus cruciatus. On the following day after operation the dog was able to run around the room without any sensory

or motor deficiencies. On the sixth day after operation the experiments were resumed. It was found that the dog behaved in every respect quite normally and his inhibitory capacity was absolutely not disturbed: even the inhibitory response to the Metronome preceded by the Propeller with an interval of 10 seconds (the maximum interval used with this dog) was fully preserved (Fig. 1d and 2b).

Third operation (frontal)

On October 11th, 1951 the third operation was performed in which frontal poles were again exposed. It was found that the dura mater was much thickened and attached to the cortex. The rest of the gyri prorei, orbitales and the anterior parts of gyri sigmoidei anteriores were removed.

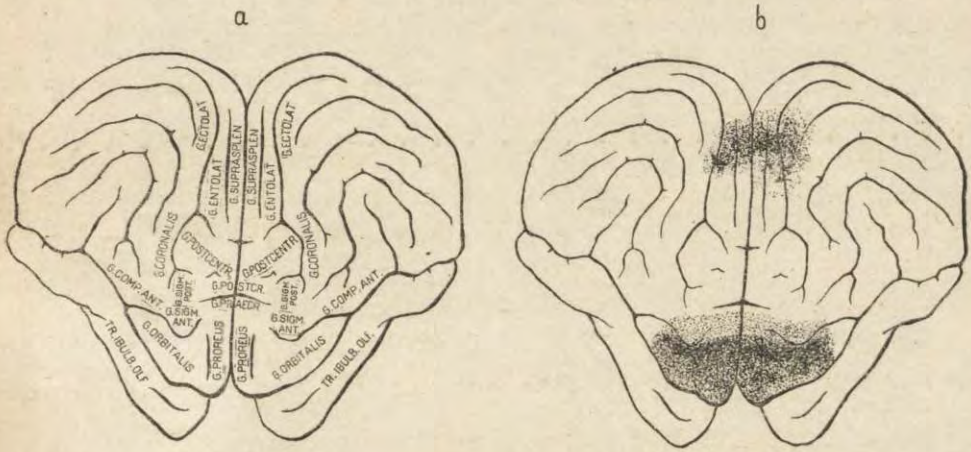

Fig. 3. a — cerebral cortex of dog, designations of gyri used in our laboratory. Both the superior and the lateral surface of the cortex are shown on one plane. b — the cortical lesions in the dog described in text; destroyed areas are marked by dots, the heavier the dotted surface, the deeper the lesion.

On the third day after operation the dog could stand and walk, but he was strongly disoriented in the familiar surroundings and failed even to find his way to the laboratory (he ran it through hundreds of times). This state gradually receded within 10 days.

In the conditioned reflex activity the same disorders as after the first operation were revealed, but this time they were much more

pronounced and lasted longer. And so, in the first experiments, the dog put on the stand performed the learned movement incessantly, so that it was almost impossible to find a quiet moment to apply a conditioned stimulus (Fig. 1e). Such a state lasted through 9 experiments and then gradually subsided (Fig. 2c). In the same time the differential inhibition also became normal.

As to the conditioned inhibition, it was disturbed for a much longer time and did not recover fully up to the end of the experiments (Fig. 1f). The dog died on 31st January of 1952 after a severe epileptic attack.

The results of the autopsy are represented in fig. 3.

The data obtained in other dogs were so similar to those reported above that only a brief account of them is needed.

In some dogs the frontal ablation was limited and did not extend beyond the sulcus praesylvicus, in others it encroached more or less upon gyrus sigmoideus anterior. According to the extent of ablations the results were somewhat different and resembled respectively those obtained after the two ablations described above.

Thus after the more limited frontal lesions there was no impairment of excitatory conditioned reflexes and the only disturbance concerned the inhibitory reflexes, the degree of disinhibition and its duration being similar to that shown in fig. 2a.

The larger lesions, on the contrary, resulted in more severe disorders, namely: disorientation in the familiar surroundings, hypermotility consisting in incessant circling around the room, and entering all narrow corners without being able to get out of them. These symptoms lasted generally no more than one or two weeks. In this period the dogs, when put into the experimental situation, seemed also to be disoriented and often did not react to conditioned stimuli.

When this first period was over and the dog was again fit for conditioned-reflex experiments he performed the learnt movement incessantly hundreds of times, at first even without any attention to conditioned stimuli. Later these intertrial movements gradually subsided, but disinhibition of inhibitory reflexes was still visible and lasted for a long time.

The sequence of the recovery of inhibitory processes was usually the same as described above. Thus, the occurence of the learnt movements in intertrial intervals would disappear earliest. Then the

differential inhibition returned usually to normal and finally, conditioned inhibition gradually improved, first with the immediate or overlapping sequence of both components of the inhibitory compound, and afterwards with the more and more protracted interval

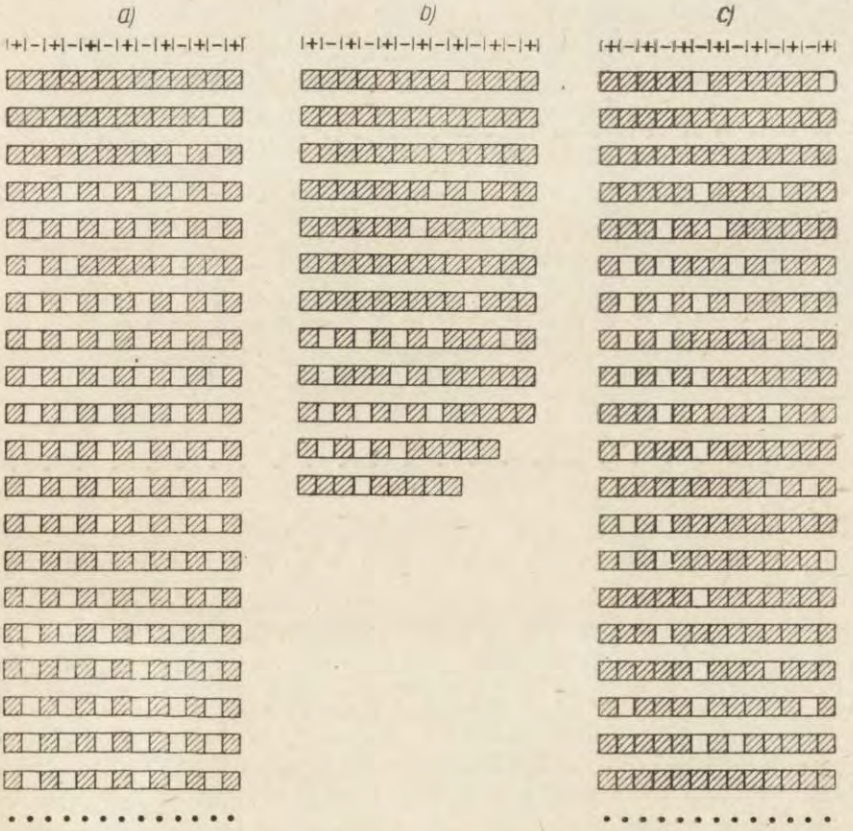

Fig. 4. The elaboration of „alternation" before operation (a), and after two successive frontal ablations (b and c). Only the first 20 experiments in series a and c are given. Series b was discontinued because of experimental neurosis. Black squares denote the positive reaction to the conditioned stimulus, hollow squares denote inhibitory reaction to the conditioned stimulus. + denotes reinforced trials, — unreinforced trials.

between them. But it must be noted that in one dog in which conditioned inhibition was very firmly established before the operation and differentiation was relatively difficult and not well fixed, the latter form of inhibition was more disturbed than the former one.

As to the conditioned inhibitor itself, it did not, as a rule, produce a positive response, except in some dogs in the first experiments after operation, when the animal performed the learnt movement almost incesantly without the least attention to the applied stimuli.

In one dog „alternation" was elaborated, the same stimulus being reinforced only on its every second application. After frontal operation the ability to perform this task was totally lost and in spite of prolonged training it was not recovered (Fig. 4). As the dog became very excited and developed symptoms of experimental neurosis, these experiments had to be abandoned.

As far as the parietal ablation is concerned, irrespectively of whether it was performed before or after frontal ablation, in none of the dogs even the slightest disturbance of inhibitory reflexes occured.

DISCUSSION

When we start to train a dog to perform a given movement in response to a given stimulus by means of food reinforcement, he ordinarily begins with performing this movement immediately he is put on the stand, quite independently of the stimulus applied. Only gradually, as these interval movements are not reinforced, they become inhibited, and the dog stands quietly performing the movement at the conditioned signal exclusively. The same happens when a stimulus similar to that evoking the movement is applied without reinforcement. Owing to the existence of generalization, the dog performs initially the learnt movement to this stimulus, and stops doing so only after a number of its unreinforced repetitions. The same applies to the unreinforced conditioned stimulus succeeding the so-called conditioned inhibitor. On the contrary, the conditioned inhibitor itself, which is not similar to any of the conditioned stimuli, does not evoke a trained movement even at the beginning of its application. We have called such a stimulus a „primarily" or „purely" inhibitory stimulus in contradistinction to the differentiated stimuli, or conditioned stimuli succeeding the conditioned inhibitor; such stimuli always contain some excitatory elements alongside with the inhibitory ones (K o n o r s k i and S z w e j k o w s k a 1952).

Putting this in terms of conditioned connections we would say that the „centre" of a primarily inhibitory stimulus is linked up

with the „centre" of the unconditioned stimulus only by means of inhibitory connections, while the „centre" of a „mixed" excitatory-inhibitory stimulus is linked up with it both by excitatory and inhibitory connections.

The present experiments have shown that after frontal bilateral ablations the cortical inhibitory process is greatly impaired while the excitatory process remains more or less unchanged. And so the dog put in the experimental situation performs without any difficulty the learnt movement, proving thus that the conditioned excitatory connections formed between the „centre" of this situation and the respective motor centre are left intact. As to the inhibitory connections they are destroyed, and leave the field to the action of excitatory connections only. As a result of this the dog performs the learnt movement not only to the conditioned excitatory stimuli, but also to those stimuli which have a mixed character possessing both the excitatory and inhibitory elements. Such a stimulus is first of all represented by the experimental situation itself, which— as we have just said — elicited a conditioned response at the beginning of the training and afterwards lost this power only by way of inhibition. That the repetitive performance of the trained movement is not due to general overactivity of the dog but to the specific connections established between respective cortical „centres" is best proved by the fact that the dog performs this movement o n l y in the experimental situation and n e v e r in other circumstances. Furthermore in other experiments carried out by one of us (Ł a w i c ka 1956), in which the dogs were trained to perform two different movements in two distinct experimental situations, the frontal ablations led to a strong disinhibition of both these responses but the dog never performed in one of these situations the movement proper to the other one.

As the experiments proceed, the inhibitory power of the dog is gradually restored. The conditioned reactions displayed in the intertrial intervals disappear first inasmuch as owing to the prolonged training the experimental situation itself has become a strong inhibitory stimulus with a rather poor admixture of excitatory elements; then the inhibition to differential stimuli returns to normal, the more subtle the differentiation, the more retarded is the recovery. Usually the restitution of the conditioned inhibition comes last, especially when the interval between the two components of the inhibitory compound attains a dozen or so seconds. This is easily

explained by the fact that here the conditioned stimulus itself is turned inhibitory when preceded by the conditioned inhibitor, and so the excitatory process called forth by this stimulus is not easily overcome. However, the conditioned inhibitor itself very rarely calls forth the conditioned response; this fact is again easily explicable as this stimulus not being similar to any of the conditioned stimuli is a „pure" inhibitor and has a very poor excitatory admixture if any.

The problem is bound to arise as to whether the recovery of the inhibitory cortical process progresses „spontaneously" after some time, or whether it is due to the post-operative training. Being unable to answer this question definitely, we are nevertheless inclined to favour the latter of these alternatives. This view is substantiated by Ławicka's experiments in two experimental situations: after frontal ablation the retraining of the inhibitory reflexes in one situation had scarcely any effect on the disturbed inhibitory reflexes in the other.

Further experiments of this series (to be published elsewhere) proved that disinhibition of inhibitory conditioned reflexes takes place not only when the dog is trained to put his leg on the foodtray (this movement might be considered as closely connected with the very act of eating) but also with any other learnt movements, such as barking, standing up on hind legs, etc. (Ł a w i c k a). Disinhibition occurs also in quite the same form if instead of motor reflexes classical salivary reflexes are used (B r u t k o w s k i). And so we are compelled to conclude that frontal ablations produce a general impairment of cortical inhibitory processes, while excitatory processes are left undisturbed.

As in some of our dogs the posterior border of the ablation involved rostral parts of gyrus sigmoideus anterior, the disorders ensuing from these lesions should be briefly commented upon.

We saw that these dogs differ from those with the more limited frontal lesions in two respects. First, just after the operation they display a great deal of general disorientation connected with more or less complete disappearance of conditioned reflexes. Secondly, they are much more disinhibited than the dogs with limited frontal lesions.

The first of these disorders was very clearly shown in I. S t ę p i e ń ' s experiments on cats, performed in this laboratory. In these experiments the animals were trained to find their way in

a simple maze, and then they were subjected to large frontal ablations extending to sulcus cruciatus. After the operation the cats were fully disoriented and had to be trained in the maze from the very beginning. The symptom is now subjected to more thorough experimental analysis.

The second disorder (a very strong disinhibition) also deserves a more careful consideration. As previously noticed, the dogs, after a short period of disappearance of conditioned reflexes, show an opposite disorder consisting in the incessant performance of the trained movement. Besides, these dogs reveal a g e n e r a l tendency to stereotyped, repeated movements, such as: going to and from an empty bowl, climbing the foodtray, persistent gnawing the edge of the foodtray, and so on. We think that incessant pacing often observed after large frontal ablations belongs to the same set of symptoms.

What sort of lesions is responsible for these symptoms (nucleus caudatus?), and whether or not they should be considered as a disturbance sui generis not reducible to the impairment of conditioned inhibitory reflexes is a matter demanding further elucidation.

In order to ascertain whether the syndrome of disinhibition described here may have resulted from any cortical lesion as an expression of „general weakening of cortical processes", a control lesion in the parietal region was carried out in each dog either before or after the frontal operation. The fact that after this operation even the most „strained" inhibitory reflexes i. e. those with the greatest admixture of excitatory elements were fully preserved, refutes this supposition and confirms the view that disinhibition obtained in our experiments is closely bound with the frontal ablations. This does not, however, exclude the possibility that lesions in some other regions of the cerebral cortex may lead to the same or similar disorders.

The problem arises what may be the mechanism of the above described disorders.

There is now a growing body of evidence to show that the process of inhibition evoked in a neurone by impulses impinging from another neurone do not depend on the form or location of synapses but on the transmitter produced by the first of these neurones (cf. E c c l e s, F a t t and L a n d g r e n, 1954; E c c l e s, F a t t and K o k e t s u, 1954; F l o r e y and M c L e n n a n, 1955 and others). In keeping with this fact is the evidence that here and

there in the central nervous system there are some definite groups of neurones with an overt inhibitory action, such as the inhibitory respiratory centre in the medulla (cf. P i t t s, 1947), the inhibitory alimentary centre in the hypothalamus (cf. B r o b e c k, 1946, 1955), the central gray inhibitory system in the midbrain (cf. M a g o u n, 1950), etc. Whether or not the well-known and much disputed supressor areas in the cortex belong to such inhibitory centres is a matter for discussion.

Consequently we may assume that all a c q u i r e d inhibitory reflexes also operate through certain intermediary inhibitory centres, one of which is situated somewhere in the frontal lobes. When such a centre is destroyed, the inhibitory conditioned reflexes become much impaired while excitatory conditioned reflexes remain unchanged. This impairment is gradually more or less compensated as other inhibitory centres take over the function of the destroyed one. It is easy to conceive that this compensation must occur by way of additional inhibitory training in order that the new inhibitory connections in place of the destroyed ones might be established. It is also obvious that the more „strained" is the given inhibitory reflex, i. e. the stronger excitatory reflex it must oppose, the less successful will be the compensation.

Future researches will prove whether the line of explantation of the described phenomena is, or is not true. Meanwhile we should like to show that, irrespectively of this, many experimental and clinical data concerning the effects of frontal ablations on the behaviour of animals and man can be easily interpreted as symptoms of the more or less manifest impairment of the conditioned inhibitory reflexes. Such a view had been put forward long ago by K a l i s c h e r (1911) and A f a n a s j e w (1913). Recently it has been amply documented in S t a n l e y and J a y n e s's paper concerning animal experiments (1949) and J a r v i e's paper on clinical material (1954). S t a n l e y and J a y n e s have shown that such frontal symptoms as hypermotility, difficulty in habit reversal, the impairment of the performance of seriatim problems, etc. can be explained as ensuing from disinhibition. In the same way may be explained the results of S h u m i l i n a's experiments from A n o c h i n's laboratory with double reinforcement (1949) and S h u s t i n's experiments with trace conditioned reflexes (1953). I. S t ę p i e ń (1956) in our laboratory has convincingly shown on cats that some forms of hypermotility as well

as the difficulty of reversal learning reported by some authors (Harlow and Dagnon, 1943; Harlow and Settlage 1948) are also easily explained by the impairment of inhibitory processes.

Concluding this discussion we should like to comment briefly on the experimental neuroses often observed in animals after frontal ablations and caused by such experimental procedures which were for the animal quite harmless before the operation. Two factors may be responsible for this. First, it must be noted that owing to the impairment of inhibitory processes the task, which before the operation was quite easy for the animal, now becomes very difficult or even impossible to solve. We know very well from experimental practice that in quite normal animals endowed with the great prevalence of excitatory processes and relative weakness of inhibition all inhibitory tasks (such as difficult differentiations, conditioned inhibitions, etc.) are performed with great difficulties and very often lead to neurosis. And so the „organic" impairment of inhibitory processes by destruction of frontal poles makes a previously quite well-balanced animal overexcitable and more prone than before to experimental neurosis. Secondly, it may be possible that a gross damage to cortical tissue may generally impair the higher nervous activity of a dog and lower his ability to cope with difficult tasks. Irrespectively of which of these factors plays a major rôle in producing experimental neurosis in our dogs, it must be stressed that the detection and proper evaluation of neurotic functional states in such experiments as described in the present paper is of paramount importance lest we should confuse two quite different sets of symptoms and mistake one of them for the other.

SUMMARY

1. The present paper is concerned with the effect of the removal of frontal lobes of the cerebral cortex on the excitatory and inhibitory instrumental conditioned reflexes.

2. It has been found that after bilateral frontal ablations limited to gyrus proreus and gyrus orbitalis excitatory conditioned reflexes are unimpaired while inhibitory reflexes are disinhibited: in consequence the dog performs the learnt movement not only to the excitatory conditioned stimuli but also in intertrial intervals, to

differentiated stimuli and to the conditioned stimulus preceded by conditioned inhibitor.

3. The inhibitory capacity of the dog returns to normal gradually in the following sequence: a) disappearance of intertrial conditioned reactions, b) inhibition of reactions to differential stimuli, c) inhibition of reactions to the conditioned stimulus immediately preceded by conditioned inhibitor, d) inhibition of reactions to the conditioned stimulus preceded by conditioned inhibitor with an interval of a few seconds.

4. When frontal ablations are more extensive, encroaching upon gyrus sigmoideus anterior, the following disorders of animals behaviour are found: a) early symptoms: disorientation in familiar surrounding, incessant pacing or running, partial or total disappearance of conditioned reflexes; b) later symptoms: great tendency to perseverative movements, strong impairment of inhibitory reflexes.

5. After frontal ablations the dogs are more prone to develop experimental neuroses than dogs with intact cortex.

6. After control parietal ablations involving gyrus entolateralis and suparsplenialis no disturbance of either excitatory or inhibitory conditioned reflexes is observed.

7. The physiological mechanism of the impairment of inhibitory processes after frontal ablations is discussed.

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STUDIES ON MOTOR CONDITIONED REFLEXES. 6. ON THE EFFECT OF EXPERIMENTAL SITUATION UPON THE COURSE OF MOTOR CONDITIONED REFLEXES

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In the studies on motor conditioned reflexes the following interesting facts were found K o n o r s k i and M i l l e r (1928, 1933, see also K o n o r s k i 1939). 1) An alimentary motor conditioned reflex of lifting the foreleg to the light and another alimentary conditioned motor reflex of lifting the hindleg to a tone were trained in a dog; each reflex was trained during separate experimental session and a cuff to record the movements on the kymograph was tied round that leg which had to be lifted. The authors observed that the dog always raised that leg on which the cuff was fastened, independently of whatever stimulus was applied; e. g. when the cuff was on the hindleg, the light as well as the tone evoked the lifting of that leg only. 2) An alimentary motor conditioned reflex of barking was established to the sound of a metronome in a dog moving freely about the room. Afterwards the situation was changed, the dog was put into a stand and its movements were restricted. In these new conditions another alimentary conditioned reflex of lifting the foreleg to a tone was established. Then the old stimulus, metronome, was applied; it evoked, however, the lifting of the leg instead of the previous, well established, reaction of barking.

These findings directed attention to the rôle of experimental situation against the background of which a conditioned stimulus is applied.

Recently these phenomena, which appeared very important for explanation of many facts concerning the conditioned reflexes, have been object of renewed investigations.

In a series of papers, an attempt was made to analyse experimentally the rôle of experimental situation in connection with such problems, as 1) elaboration of alimentary motor conditioned reflexes of various motor effects (W y r w i c k a 1953), 2) relations between alimentary motor conditioned reflexes of various motor effects in the course of acute extinction of one of them (W y r w i c k a 1955 a) and 3) elaboration of various motor alimentary reactions to the same conditioned stimulus (W y r w i c k a 1955 b).

The main results obtained are described below.

EXPERIMENTAL PART

Experiments were performed on 6 dogs (mongrels, males aged 2—6 years), in which only alimentary motor conditioned reflexes were elaborated. Experiments were conducted daily, at the same time of the day. In each experiment from time to time, at intervals of about 1—2 minutes, a conditioned stimulus (visual, acoustic or tactile) was applied, evoking a trained movement, which was immediately reinforced by food (bread).

The trained movements were the following: 1) the putting of the right foreleg on the food-tray, 2) the lifting of the right hindleg, 3) the lifting of the left foreleg and 4) the begging. In each dog at least 2 movements were elaborated.

The movements were recorded on the kymograph. The cuffs were attached to all four legs of the dog.

Three groups of experiments were performed. In each group at least 2 dogs were used. In the first group of experiments all conditioned reflexes were elaborated and trained in each dog in one and the same experimental chamber. In the second group of experiments each of these reflexes was elaborated and trained in another experimental chamber, similar to the first. In the third group of experiments each reflex was elaborated and trained also in se-

parate experimental chambers, but these chambers were quite different from one another.

The results of experiments of each group are given below.

The same experimental situation

In each of 2 dogs a motor conditioned reflex of the lifting of the right hindleg to the sound of the rattle was established, and then another motor conditioned reflex of the putting of the right foreleg on the food-tray to the sight of a rotating object was also established in the same experimental situation. Afterwards several experimental series (each lasting about a fortnight) with each of these stimuli were performed in alternating sequence.

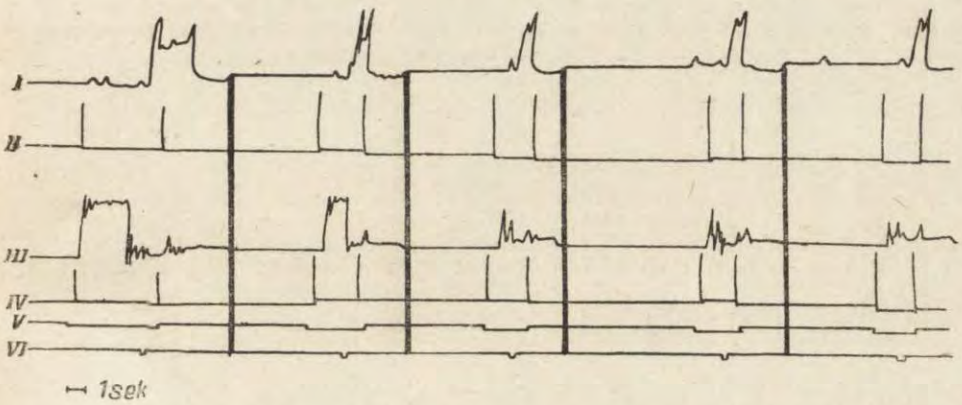

Fig. 1. The beginning of a new series of experiments of the first group (kymographic record) I — movements of the foreleg; II and IV — lines to show the isolated periods of the action of the conditioned stimulus; III — movements of the hind-leg; V — action of the conditioned stimulus; VI — food reinforcement. Double vertical lines denote the 1—2 min. pauses between the trials. In the first trial a hind-leg movement appears, which lasts about 3 sec., and afterwards — the foreleg movement, which is reinforcer by food. In the next trials the hind-leg movement (which is not reinforced by food) is lasting shorter and shorter and it diminishes progressively; on the other hand the foreleg movement (which is reinforced by food) becomes quicker in each successive trial

It was found that at the beginning of each series the dogs always performed the movement which they had performed in the previous series, although the „new" stimulus applied was connected formerly with the other reaction; e. g. when after the training of the

reflex: the rattle → the lifting of the hindleg, on the next day the rotating object was applied (to which the reflex of the foreleg was trained), the movement of the hindleg, instead of that of the foreleg, was made. Only, when a „wrong" movement was not reinforced by food, the proper movement was performed (Fig. 1). Then it was necessary in the course of several days to train anew the reflex in each series. However, in successive series of experiments, the initial period of the appearance of the „wrong' reflex grew gradually shorter. In spite of this, in the course of this group of experiments (which lasted about 7 months) a correct beginning of any new series was never observed; movements from a previous series always appeared at the beginning of the new one.

Protocol No 1

Dog No 3 — 10 IV 1952

Time of successive trials	Conditioned stimulus	Latent period (in sec.) of the motor reaction		Reinforcement by food
		of the foreleg (the „proper" movement)	of the hindleg (the „second" movement)	
1st min	Rotating object for 5 sec	1—3*	3,5, 5, 8	—
2nd min 30 sec	„	1—2, 4—6	6, 7	—
4th min	„	1—3	5	—
5th min 30 sec	„	2—5	6	—
7th min	„	2—3	5, 6	—
8th min 30 sec	„	3—6	1, 2 (small movements)	—
11th min	„	1—6	2, 3 (very small movements)	—
12th min 30 sec	„	2—6	—	—
14th min	„	2—4	—	—
15th min 30 sec	„	—	—	—

*The movement appears in the 1 st second and lasts another 2 seconds.

Although the mutual interchanges of movements were usually found at the beginning of each series, they were observed also sometimes in the further course of series; e. g. the „wrong" move-

ment might be evoked by the external unexpected stimuli, such as the noise in the corridor, the entrance of experimenter to the experimental chamber and other disturbances.

After some months of training, the acute extinction of both reflexes was performed. Each reflex was always extinguished in the middle of the respective series of experiments, when the initial interchanges of movements were over. In such extinction-experiment the conditioned stimulus was applied every 1—2 minutes for 5 seconds and — in spite of appearance of the trained movement — it was never reinforced by food. Protocol Nr 1 shows one of these experiments.

Protocol No 2
(Extract from protocol)

Dog No 4 8. XI. 1952

Time of successive trials	Conditioned stimulus	Latent period (in sec.) of the motor reaction		Reinforcement by food
		of the hindleg (the „proper" movement)	of the foreleg (the „second" movement)	
15th min	Rattle for 5 sec	—	—	—
16th min				F o o d
17th min				F o o d
18th min	Rattle for 4 sec	1,5 , 3	2	+
19th min 30 sec	Rattle for 4,5 sec	1,5 , 3, 4	2 , 3.5	+
21st min	Rattle for 4 sec	1,5 , 2,5 , 3,5	1 , 2 , 3	+

As is shown in this protocol, when the „proper" movement (of the foreleg) is not reinforced by food, the „second" („wrong") movement (of the hindleg) is performed. It was found that: 1) the „second" movement appears in most cases only after some „proper" movements have been made; 2) after several trials the second movement disappears, while the „proper" movement is still evoked; 3) the „proper" movements are more numerous than the „second" movements.

The course of other experiments with the acute extinction was similar in both dogs; detailed numeral data concerning these experiments are given elsewhere (W y r w i c k a 1955 a).

It might be added that, when after the extinction, the food was given to restore the reflex, and the conditioned stimulus was again applied, both movements („proper" and „second") often appeared in the same trials (see protocol No 2).

Two similar experimental situations

The second group of experiments was performed on two other dogs. In each dog a motor conditioned reflex of putting the foreleg on the food-tray to the sight of the rotating object was established in one experimental chamber and a motor conditioned reflex of lifting the hindleg to the sound of the rattle was established in another experimental chamber. These two experimental chambers were situated in the same corridor; the interior of each chamber was similar (a similar stand with an automatic food-tray on the left side of the chamber, the same arrangement of various objects etc.). The difference between these experimental chambers (as the preponderance of light colours in one chamber in comparison with the other) were rather small, and therefore both chambers might be called „similar".

The elaborated reflexes were afterwards trained alternatively in separate series of experiments in those chambers, in which they had been elaborated.

In the course of experiments the same phenomenon of mutual interchanges of two motor reactions was observed in the initial period of each series of experiments just as in the first group of experiments performed in the same chamber.

It may be mentioned however that initial period of interchanges of movements in each series were shorter than in the first group of experiments. There were even some experimental series with a „correct" beginning (without „wrong" movement). However, any unexpected stimulus might immediately evoke a „wrong" movement (as in the first group of experiments).

The acute extinction of each of these reflexes showed aproximately the same results as in the first group of experiments. Namely, in the course of extinction, the „second" movement might be evoked besides the „proper" movement, in the same manner as in experiments conducted in the same chamber.

Two different experimental situations

A. The elaboration of various motor conditioned reflexes to two different conditioned stimuli. Experiments of the third group were performed on the same 2 dogs which were used previously in experiments of the second group. In each of these dogs a motor conditioned reflex of putting the right foreleg on the food-tray to the sight of a rotating object in the usual experimental chamber (designed as the situation I) was established.

Afterwards the experiments were transferred to another place, in another building. This new place was quite different from the usual experimental chamber. There was neither stand nor food-tray. During the experiment the dog was standing on the floor and food (bread) was thrown to it by the experimenter. In this situation (designed as the situation II) a motor conditioned reflex of lifting the left foreleg in one dog, and of begging in another dog to the stimulus called „the noise" was established.

It was found that the elaboration of the reflex in the situation II was very easy, because of the absence of the movement connected with the situation I. When afterwards both reflexes were trained alternatively in separate series of experiments (in those chambers, in which they had been respectively elaborated), each movement appeared in a „correct" form. A movement proper to one situation was never evoked in the other situation neither in the beginning of experimental series nor to unexpected external stimuli.

Likewise, the „second" („wrong") movement did not appear in the course of the acute extinction of each of these reflexes and merely the „proper" movement was evoked.

B. Elaboration of 2 various motor conditioned reflexes to one and the same conditioned stimulus. When it was ascertained that each of the above mentioned motor conditioned reactions always appeared in the correct form, even when experiments were performed successively on the same day, the action of one and the same stimulus was tried in both situations.

For this purpose the sound of the metronome (200 strokes to a minute) was used. At first the reflex of putting the right foreleg on the food-tray to this stimulus was established in the situation I.

Afterwards experiments were transferred to the situation II and then the metronome 200/min. was applied.

It was found that not the movement of the right foreleg (as in the situation I) but the movement of lifting the left foreleg in one dog and the movement of begging in the other dog was evoked.

The same result was obtained when a similar experiment was performed with a tactile stimulus.

However, the first application of the metronome or the tactile stimulus in the situation II caused at first a more or less marked orientation reflex and only after this reflex was over the trained motor conditioned reaction appeared; the trained movement therefore was delayed and diminished (see protocol No 3).

Protocol No 3

a) The situation I. The tactile stimulus is used for the last time in this situation before the series of experiments in the situation II

Dog No 2 13. XI. 1952

Time of successive trials	Conditioned stimulus	Motor reaction	Food reinforcement
6th min.	tactile stimulus	right hind-leg*, immediately	+**
7th min 15 sec	rattle	„ „ „	+
8th min 30 sec	tactile stimuls	„ „ „	+

b) The situation. The tactile stimulus is used for the first time in this situation

Dog No 2 14. XI.1952

6th min 30 sec	noise	left foreleg***, immediately	+
7th min 30 sec	tactile stimulus	left foreleg, the movement is performed in 9th sec.; before the reaction an orientation reflex is observed	+
9th min	tactile stimulus	left foreleg, in 2nd sec.	+
10th min	tactile stimulus	left foreleg, in 1st sec.	+
11th min	noise	left foreleg. immediately	+

* Immediate lifting of the right hind-leg.

** The food was given immediately after the movement had been performed.

*** The immediate lifting of the left foreleg.

The motor reaction reached its normal value only when the stimulus was repeated in the situation II.

After this initial period each reflex might be obtained in the correct and fullsized form even when the experiments in both situations were performed one after another on the same day.

It was interesting however, to examine the cause of the initial delay and diminution of reaction in the situation II. The problem arose whether a change of situation or a change of movement joined with this new situation, was the cause of diminished magnitude of reaction.

For this purpose a control series of experiments was undertaken. In two other dogs a conditioned reflex of lifting the right hindleg was established in both situations. In situation I the sound of the metronome (200/min.) and in situation II — the sound of the rattle were used as conditioned stimuli.

During the experiment in situation I the rattle (which had not been formerly used in this situation) was applied, and a distinct delay and diminution of the motor reaction was shown in both dogs. On the next day the same experiment was performed in situation II, where the metronome (which had not been formerly used here) was applied. The result was the same as in the case of the rattle, namely the motor reaction to metronome was delayed and diminished (the protocols of these experiments are cited elsewhere, W y r w i c k a 1955 b).

These experiments have shown that the reduction of the reaction at the first application of the conditioned stimulus in the new situation (different from the situation in which the stimulus was formerly used), appears both when a different movement or the same movement was trained in the new situation. Therefore it may be concluded that it is not the change of movement but the change of situation which is the cause of the phenomenon.

DISCUSSION

The experiments performed have shown that:

1. When 2 alimentary motor conditioned reflexes with 2 different motor effects are elaborated and trained in a dog in the same experimental situation or in 2 similar experimental situations, then a) mutual interchanges between 2 different motor conditioned reactions take place during the elaboration of these reflexes and b) the acute extinction of one of these reactions evokes the appearance of the other motor reaction;

2. When the above mentioned reflexes are elaborated in 2 different experimental situations, then a) there are no interchanges between the motor conditioned reactions, and these reactions are always evoked in the „correct" form, even if the same conditioned stimulus is used to evoke each reaction, and b) the acute extinction of one of these motor reactions does not evoke the other.

It was also found that:

3. If a conditioned stimulus, which had been formerly used only in one definite experimental situation, is applied for the first time in a new experimental situation (different from the first), it evokes a delayed and diminished reaction, which is preceeded by an orientation reflex.

Let us consider these facts.

As is known, the training of a motor conditioned reaction in a definite situation causes a formation of conditioned connections not only with the conditioned stimulus but also with the experimental situation. Owing to this the experimental situation alone causes „a readiness" to the appearance of the motor reaction, before

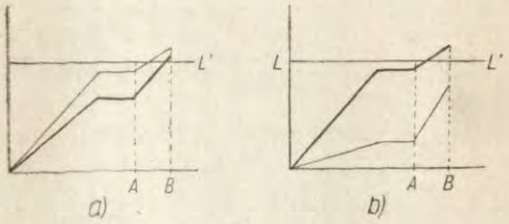

Fig. 2. The relations between the excitations levels in the centres of various movements in the initial period of a new series of experiments, conducted in one and the same situation (or in two similar situations) (a), and in two different situations (b). Ordinates — the height of motor excitation level; abscissae — time. LL' — the level of liminal excitation. A — the moment of the application of the conditioned stimulus. B — the moment of the appearance of the reaction. Thin line — the excitation of the centre of recently trained movement; thick line — the excitation of the centre of movement which begins to be trained in the current series of experiments. a. In experiments conducted in the same (or in two similar situations), the excitation level of the recently trained movement is higher than that of the other movement, and then the former is evoked even to the stimulus joined with the latter. b. In experiments conducted in two different situations, the excitation level of the movement, which was recently trained in another situation, is much lower than that of the movement proper to the situation; therefore this latter movement is evoked even to the stimulus formerly used in the connection with another movement. More detailed explanation is given in the text.

the application of the conditioned stimulus (which finally evokes that reaction) (see K o n o r s k i 1939, 1948). Otherwise, the experimental situation alone causes the subliminal excitation of the centre of the motor conditioned reaction, the application of a conditioned stimulus increases this excitation to a supraliminal level and the motor reaction appears (W y r w i c k a 1952).

When 2 different motor conditioned reflexes are trained in successive series in the same experimental situation, then — owing to the connections that have been formed — the situation alone may increase the excitation in the centres of both movements. However, one of these movements (which is not reinforced by food) is partly inhibited; therefore the subliminal excitation in its centre does not reach as high a level as in the centre of a trained movement (which is always reinforced by food).

Now, when the conditioned stimulus is applied, it causes the further increase of excitation in the centres of both movements; in the centre of the trained movement the excitation increases owing to direct connections of this centre with the centre of the stimulus, as well as with the feeding centre (see W y r w i c k a 1952); in centre of the second movement the excitation increases only through the feeding centre.

Now, when the other conditioned stimulus, associated with the second movement, is applied, it is insufficient to cause any change in the pattern of subliminal excitation in both centres, evoked by experimental situation. The situation continue to evoke a higher excitation in the centre of the formerly trained movement, than in the centre of the second movement associated with the new stimulus.

Therefore the new stimulus, which excites the centres of both movements through the feeding centre (as mentioned above) may evoke the previously trained movement (which is never joined with it) (Fig. 2 a). This is the explanation of the interchanges of movements in the initial period of each series of experiments. This phenomenon will disappear only when, following the non-reinforcement of the „wrong" movement, inhibitory connections will arise (or will be restored) between this movement and the situation, and when, owing to reinforcement of the „proper" movement, excitatory connections will arise (or will be restored) bettween this latter movement and the situation.

Now, how can it be explained that the „second" movement appears in the course of the acute extinction of one of the reflexes,

established in the same situation? It must be kept in mind that the excitation in the centre of the „proper" movement, caused by a situation, is higher than in the centre of the „second" movement, but this difference is not a great one. When the conditioned stimulus is applied and the „proper" movement, evoked by this stimulus, is not reinforced, the excitation in the centre of this movement will be diminished after some seconds. Then — with the continually lasting excitation of the feeding centre — the excitation of the centre of the „second" movement will gain a temporary preponderance over the centre of the „proper" movement and the „second" movement will be performed (Fig. 3a).

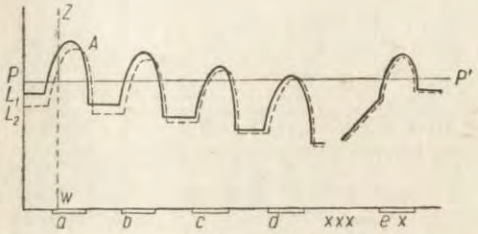

Fig. 3. Scheme of the supposed changes in the level of excitation in the centre of the „proper" movement and of the „second" movement in the course of the acute extinction of a motor conditioned reflex. Ordinates indicate the level of motor excitation, abscissae — time. The successive periods of the action of the conditioned stimulus (a, b, c, d, e) are indicated on the x-axis. X — denotes the moment of food reinforcement. The line PP' denotes the liminal level of excitation. The surve l_1 (continuous line) indicates the changing level of excitation in the centre of the „proper" movement, and the curve l_2 (dotted line) — the changing level of excitation in the centre of the „second" movement in the course of the acute extinction. The vertical dotted line WZ indicates the moment in which the reinforcement was usually given. A point A denotes the moment in which the excitation of the „second" movement begins to predominate over the excitation of the centre of the „proper" movement. Fuller explanation is included in the text.

The same is repeated in several successive trials; however the level of excitation in the both centres decreases gradually, together with the advancing process of the extinction. At last, the moment approaches when the acting stimulus evokes only the „proper" movement, while the „second" movement cannot be evoked, because the level of excitation in its centre it too low (Fig. 3 d).

As is known, the more a movement is repeated and is not reinforced the stronger is the decrease of excitation in its centre (see

Konorski and Wyrwicka 1952). Therefore the excitation in the centre of the „proper" movement was more reduced, than in the centre of the „second" movement (because the latter appeared a smaller number of times in the course of acute extinction than the „proper" movement; see experimental part). Then, together with the extinction of the reflex a gradual equalization of excitation levels in the centres of both movements may occur. This state remains probably also in the farther part of the experiment, after the restoration of the reflex by the food (see Fig. 3d, e); then the conditioned stimulus often evokes both movements during the same trial (see protocol No 2).

The above explanation is valid also for the facts which were found in the second group of experiments, performed in two similar situations. In both situations the same elements of environment, being connected with both movements, cause the phenomenon of mutual interchanges of these movements, as well as the appearance of the „second" movement in the course of the acute extinction (just as in the same situation).

In the third group of experiments two different situations had no common elements. Each situation was connected only with one movement (with that movement which had been trained in it) and therefore each situation excited only the centre of one movement. This caused the absence of the mutual interchanges or the appearance of the „second" movement during the acute extinction.

However it must be remembered that each situation always excites the feeding centre, and through it the centres of all movements connected with it. Therefore we must assume that each situation excited not only the centre of the „proper" movement, but also to some degree the centre of the „second" movement. It is however obvious that the excitation level of the centre of the „proper" movement (connected with this situation) was much higher than that of the centre of the „second" movement (which was not connected with the situation) (see Fig. 2b).

When the conditioned stimulus, previously always used only in another situation, was applied, the excitation in the centres of both movements arose (as it was above explained). However, as the subliminal excitation in the centre of the movement connected with the situation was much higher than in the centre of the other movement, the movement trained in this situation appeared and not the other movement which was previously associated with this

conditioned stimulus in other, different situation. This is the cause of the appearance of two different motor reactions to the same stimulus.

It is necessary to explain also the appearance of an orientation reflex and of the delay of motor reaction at the first application of the conditioned stimulus in a new situation. The control experiments have shown that this phenomenon took place also when the same motor reaction was trained in both situations; it is not caused therefore by the interference between both movements. It may be supposed that this phenomenon is a result of the lack of the connections between the conditioned stimulus and the new situation, in which it was for the first time applied. Owing to these connections, which arise at the repeated application of the stimulus in the definite situation, this situation leads to an increase of excitation in the centre of the trained movement. As a result, the dog „expects", so to say, the stimulus, which is usually applied and reacts to it immediately, without any orientation reflex.

When a conditioned stimulus is never applied in this situation, it is not „anticipated", i. e. its centre is not excited by this situation, because of the lack of connections between the stimulus and the situation. Then the first application of the stimulus in the new situation causes a more or less marked orientation reflex and afterwards — a delayed motor reaction.

SUMMARY

Two alimentary motor conditioned reflexes of different motor effects were elaborated in each of 6 dogs. When these two reflexes were elaborated and trained in one and the same experimental situation or in two similar situations respectively, then the mutual interchanges of motor reactions were observed, and in the course of the acute extinction of one of these reactions the other reaction often appeared. When both reflexes were elaborated respectively in two different situations, the mutual interchanges of motor reaction and the appearance of the other motor reaction in the course of the acute extinction was never observed; each movement appeared in a „correct" form even when the same conditioned stimulus was used in both situations to evoke the motor reaction. The mechanism of these phenomena has been discussed.

The author wishes to thank Professor J. K o n o r s k i for valuable discussion of this paper.

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ACTIVITY OF SOME RESPIRATORY ENZYMES DURING THE DEVELOPMENT OF SILKWORM, *BOMBYX MORI* L.

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In previous investigations (L. W o j t c z a k 1952 b) some characteristic changes in the respiratory enzyme systems during the development and metamorphosis of the waxmoth, *Galleria mellonella*, has been observed; an increase in the activity of phenoloxidase before pupation, a U-shaped curve of the endogenous oxygen uptake of homogenates during the course of metamorphosis, and a gradual increase in the succinoxidase activity during pupal growth are the chief phenomena that has been noticed. It is interesting to investigate whether analogous changes are also characteristic for other insects. For this reason the silkworm was taken as the object for present experiments.

MATERIAL AND METHODS

The larvae of the silkworm were bred at 20°—25°, the pupae at 23°—25°. The experiments were performed during four succesive years. The larval stage was about 40 days long, the pupal period lasted in different years from 12 to 18 days. The age of pupae was expressed not in days but in percentages of the whole pupal stage. Oxygen uptake of pupae and adults was measured in Warburg apparatus at 25°, in special vessels of about 100 ml. capacity. The activity of respiratory enzymes was determined also in the Warburg apparatus at 30°, in ordinary vessels of about 15 ml. capacity. Homogenates were

prepared as described previously (W o j t c z a k 1952 a). Phosphate buffer of pH 6.6 was used in all experiments. Endogenous oxygen uptake of homogenates was measured during a 60 minute period in intervals of 15 minutes, and then substrates were added from the side arms of the vessels. The following substrates were used: 2 per cent phenol or 1 per cent catechol for phenoloxidase essey and 0,2 M sodium succinate for succinoxidase. The activity of both enzymes was measured by the degree of the increase of oxygen uptake of homogenates when substrates had been added. Following quantities of solutions were usually placed in each Warburg vessel: 2.0 ml. of homogenate in main compartment, 0.8 ml. of substrate solution in side arm and 0.1 ml. of 10 per cent KOH in central well for CO_2 absorption. Oxygen consumption was calculated as microliters O_2 per mg. fresh weight of insects per hour (QO_2).

EXPERIMENTAL

T h e l a r v a l s t a g e was studied from the third day on after hatching of larva. Table I presents the endogenous oxygen uptake of homogenates and the activity of phenoloxidase as well as of succinoxidase in various stages of larval growth. It is obvious that the endogenous oxygen consumption of homogenates and the activity of phenoloxidase are considerably higher during all four periods of moulting and before pupation in comparison to feeding periods (Fig. 1). Immediately after ecdysis both oxygen uptake of homogenates and the activity of phenoloxidase decrease abruptly and rise again before the approaching moulting. The activity of succinoxidase, as measured in these experiments, proved very low.

P u p a l s t a g e . The pupae were examined throughout the whole period of metamorphosis (Table II). It appears that the endogenous oxygen uptake of homogenates decreases in the first period of pupal development till the age of 10 per cent of pupal average lifetime, then it rises till the age of about 50 per cent. In the second half of metamorphosis a decrease of the rate of oxygen uptake of homogenates takes place again. The changes in the activity of phenoloxidase, as measured by way of catechol oxidation, are very similar to the changes in the endogenous oxygen consumption of homogenates (Table II, Fig. 2a and 2b). The activity of succinoxidase, however, being on a rather low level immediately after pupation rises gradually during the pupal stage and attains its maximum in the last few days of pupal life.

The changes of the oxygen uptake of homogenates of pupae described above seem rather striking since for other insect species

examined so far the oxygen consumption of homogenates in the course of pupal life follows a U-shaped curve similar to the respiration curves of living pupae (H e l l e r 1947; W o j t c z a k 1952b).

Table I

Oxygen uptake of homogenates of silkworm larvae and the activities of phenoloxidase and of succinoxidase. All values expressed in ml. O_2 per gram fresh weight per hour

Instar	Days after hatching or moulting	Number of experiments	Endogenous O_2 uptake in consecutive 15 min. intervals				Oxidation of substrates: phenol O_2 uptake	Oxidation of substrates: phenol increase of O_2 uptake	Oxidation of substrates: succinate O_2 uptake	Oxidation of substrates: succinate increase of O_2 uptake
			±SD							
I	3	3	0.91 0.31	0.60	0.33	0.23	0.49	0.26	0.30	0,07
	5 (moulting)	11	1.21 0.22	0.80	0.40	0.26	0.59	0.33	0.42	0.16
II	0	3	0.81 0.17	0.53	0.27	0.18	0 28	0.10	0.21	0.03
	2—4	10	0.70 0.23	0.40	0.26	0.18	0.34	0.16	0.18	0.00
	5 (moulting)	4	1.05 0.22	0.62	0.38	0.22	0.54	0.32	0.33	0.11
III	0—1	4	0.64 0.13	0.40	0.27	0.13	0.39	0.25	0.22	0.08
	2—5	8	0.45 0.14	0.29	0.18	0.14	0.58	0.45	0.17	0.04
	6 (moulting)	5	0.88 0.19	0.32	0.14	0.12	0.88	0.76	0.16	0.04
IV	0	4	0.60 0.02	0.30	0.18	0.14	0.45	0.31	0.19	0.05
	3—5	5	0.38 0.07	0.19	0.10	0.09	0.65	0.56	0.09	0 00
	7 (moulting)	5	0.80 0.16	0.22	0.14	0.14	1.02	0.88	0.18	0.04
V	0—1	5	0.34 0.11	0.17	0.09	0.06	0 42	0.34	0.07	0.01
	4—6	7	0.31 0.09	0.19	0.13	0.09	0.21	0.12	0.07	0 00
	9—12	3	0.15 0.02	0.08	0.05	0.05	0.40	0.35	0.05	0.00
	13—15 (spinning)	5	0.41 0.16	0.26	0.18	0.14	0.40	0.26	0.09	0.00
	16 (prepupa)	5	1.03 0.31	0.39	0.18	0.13	1.00	0.87	0.12	0.00

SD — standard deviation

In order to find out what is the position in regard to the silkworm the oxygen consumption of living silkworm pupae in the course of metamorphosis was measured. The results of these experiments, similar to those obtained by B i a ł a s z e w i c z (1937), are shown in Fig. 3. As can be seen, the oxygen consumption of pupa decreases

after pupation, its minimum coincides with the age of about 10 to 20 per cent of pupal average life length. Later on, the rate of oxygen consumption increases, and during the last days of pupal life remains on a rather high constant level. Thus it is obvious that the oxygen uptake of homogenates and the activity of phenoloxidase run parallel to the respiration of living pupae only during the first half of pupal life, while in the second half the enzymatic activity and the respiration take a very different course.

I m a g i n a l s t a g e. The oxygen consumption of living adults and of homogenates of adults as well as the activity of

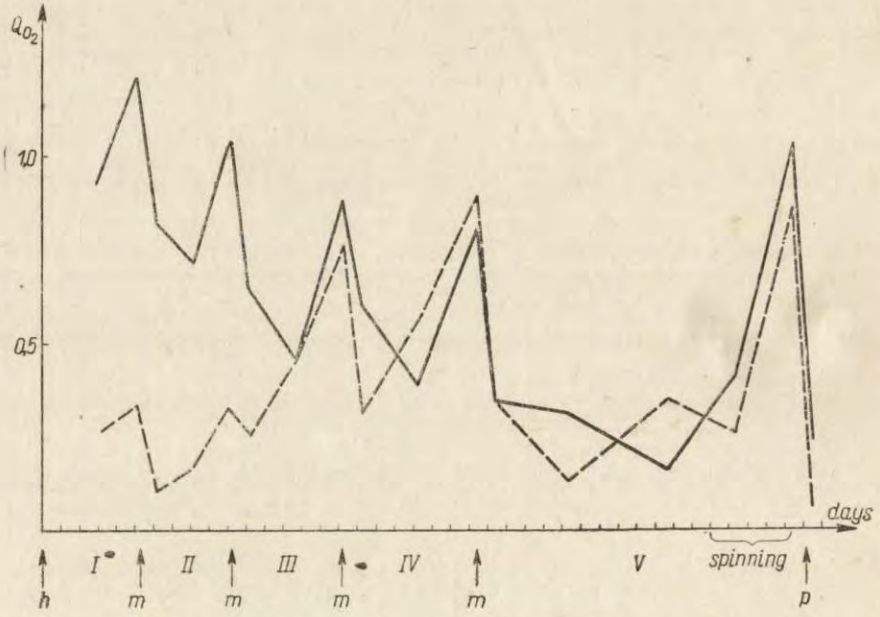

Fig. 1. Endogenous oxygen uptake of homogenates (———) and activity of phenoloxidase (- - -) in the course of larval development of *Bombyx mori*. Endogenous oxygen consumption expressed in microliters per mg. fresh weight per hour (Q_{02}), during the first 15 minutes of measurement; phenoloxidase activity expressed as the increase in Q_{02} when phenol has been added. h — hatching of larva, m — moulting, p — pupation, I, II, III, IV, and V — consecutive instars. Temperature 30°.

phenoloxidase and of succinoxidase were measured (Table III). The oxygen uptake of adult insects proves to be higher than that of pupae just before emergence. Male adults consume twice as much

Table II

Endogenous oxygen uptake and the activities of phenoloxidase and of succinoxidase during pupal development of silkworm. Endogenous oxygen uptake of homogenates during the first 15 minutes of experiment; substrate oxidation expressed as the increase of O_2 consumption when substrates are added to the homogenate. All values expressed in ml. O_2 per gram fresh weight per hour

Pupal age in per cent	Number of experiments	Endogenous O_2 uptake of homogenates (first 15 minutes of experiment)	Oxidation of substrates	
			catechol	succinate
prepupa	5	1.03	3.15	0.00
2	4	0.77	0.90	0.00
4	4	0.47	0.94	0.03
9	3	0.24	0.77	0.02
12	6	0.45	1.27	0.06
19	7	0.48	1.43	0.04
27	6	0.60	2.04	0.05
35	8	0.68	2.43	0.08
42	7	0.67	2.42	0.07
51	6	0.84	2.61	0.07
58	5	0.71	2.84	0.10
65	5	0.63	2.99	0.19
72	7	0.31	0.39	0.24
81	7	0.50	1.04	0.15
89	5	0.48	1.28	0.22
96	8	0.25	0.31	0.24

oxygen as female ones; the endogenous oxygen uptake of homogenates and the activity of succinoxidase are also higher in males than in females. Contrary to this, the activity of phenoloxidase is higher in female insects than in male ones.

DISCUSSION

The most interesting phenomenon noticed in the course of larval growth of the silkworm was a considerable increase in the endogenous oxygen uptake of homogenates during the periods of larval moulting. Also an increased level of phenoloxidase activity, as compared to feeding periods, was observed during moulting. This is quite surprising since contrary to that a decrease in the respiration of the whole larva during the periods of moulting had been described so far (B i a ł a s z e w i c z, 1937; Y a m a f u j i 1937). Thus it is obvious that the oxygen uptake of homogenates and the

activity of phenoloxidase do not run parallel to the changes in the respiration of larvae during their feeding and moulting periods. The increased activity of some respiratory enzymes during moulting periods must be connected with the intensity of some metabolic processes significant for those periods (W. Niemierko, Kąkol and Załuska 1954; S. Niemierko, P. Włodawer and A. Wojtczak 1956). It is likely that the increased activity of phenoloxidase during the periods of moulting and in the prepupal stage may be due to a special role which this enzyme plays in the moulting and pupation processes of insect larvae (Sussman 1949; Wojtczak 1954).

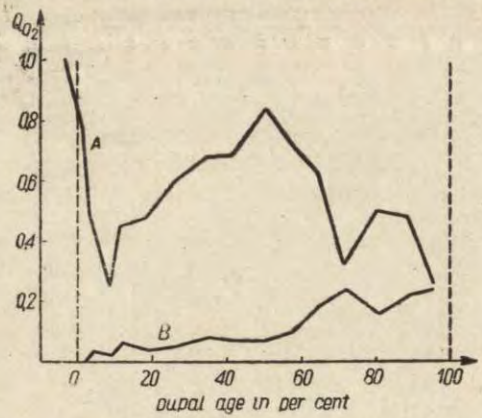

Fig. 2a. Endogenous oxygen uptake of homogenates of silkworm pupae (A) and the activity of succinoxidase (B). Pupal age expressed in percentages of pupal average lifetime, endogenous oxygen uptake in microliters per mg. fresh weight per hour (Q_{O_2}), during the first 15 minutes of measurement, and succinoxidase activity as the increase in Q_{O_2} when succinate has been added. Temperature 30°

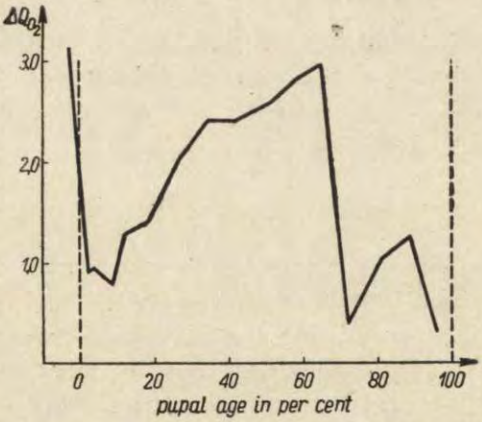

Fig. 2b. Activity of phenoloxidase during pupal development of silkworm, expressed as the increase in Q_{O_2} when catechol has been added

It is known that the oxygen uptake of homogenates of pupae of *Celerio euphorbiae* (Heller 1947) and of *Galleria mellonella* (Wojtczak 1952b) follows a U-shaped curve similar to the respiration curve of living pupae in the course of metamorphosis. It was worth while to find out what was the position as regards the silkworm. Present experiments have

shown that changes in the „respiratory" activity of homogenates of pupae are similar to the changes in pupal respiration only during the first half of metamorphosis. Later on, however, the oxygen uptake of homogenates and the activity of phenoloxidase drop while the respiration of pupae remains on a constant high level. Since the activity of the succinoxidase system is in the second half of metamorphosis rather intense and the activity of phenoloxidase shows a marked decrease, it seems likely that the cytochrome system rather than the phenoloxidase is the terminal link in the respiratory chain of pupa during that period of metamorphosis.

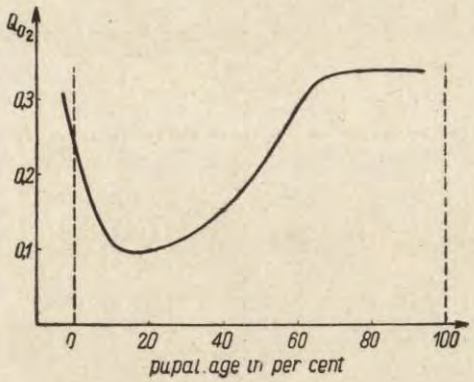

Fig. 3. Oxygen consumption of silkworm pupae, in microliters per mg. fresh weight per hour (Q_{O_2}). Temperature 25°, pupal age expressed in percentages of pupal average lifetime

A gradul increase in the succinoxidase activity during the pupal development of silkworm is analogous to the corresponding increase concerning *Calliphora erythrocephalla* (A g r e l l 1949) and *Galleria mellonella* (W o j t c z a k 1952b).

Table III

Oxygen uptake of adult silkworms, of their homogenates, and activities of phenoloxidase and of succinoxidase. Endogenous oxygen uptake of homogenates during the first 15 minutes of experiment; oxidation of substrates expressed as the increase of O_2 consumption when substrates are added to the homogenate. All values expressed in ml. O_2 per gram fresh weight per hour

Sex	O_2 uptake of living insect	Endogenous O_2 uptake of homogenates (first 15 minutes of experiment)	Oxidation of substrates	
			catechol	succinate
♂	0.83	1.15	0.87	0.79
♀	0.42	0.39	1.71	0.32

The respiration, the oxygen uptake of homogenates, and the activity of succinoxidase are considerably higher in adult males than in females. Higher activity of succinoxidase and higher level of

tissue respiration had been also found in males as compared with females of other insect species (Barron and Tahmisian 1948; Harvey and Beck 1953; McShan et al. 1954; Sacktor and Thomas 1955).

SUMMARY

During the entire development period of the silkworm, *Bombyx mori*, the oxygen consumption of homogenates and the activities of phenoloxidase and succinoxidase were measured, as well as the oxygen uptake of living pupae and adults. During all four periods of larval moults and before pupation a considerable increase, as compared with the periods between moultings, in oxygen uptake of homogenates and in the activity of phenoloxidase was observed. The succinoxidase activity was weak throughout the whole larval stage.

During the first days after pupation, mean oxygen uptake of living pupae decrease, and later on it rises, remaining on a constant high level during the last days of metamorphosis. Similarly, both the endogenous oxygen consumption of homogenates of pupae and the activity of phenoloxidase decrease after pupation and rise later on; they show, however, a new decrease in the second half of the pupal stage. The succinoxidase activity increases gradualy in the course of metamorphosis.

The oxygen consumption of male adults is twice as high as that of females. Considerably higher in adult males than in females are also the endogenous oxygen uptake of homogenates and the activity of succinoxidase.

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THE EXTERNAL SEXUAL CHARACTERS OF *BOMBYX MORI* L. AND *GALLERIA MELLONELLA* L. LARVAE

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In the course of studies on the biochemistry of the waxmoth and silk-worm in this Institute it became necessary to distinguish males and females through their external sexual characters in different stages of development.

While the external differences between male and female imago of the silk worm are very distinct and commonly known — insufficient data are available in literature concerning the waxmoth, although the E c k s t e i n key (1933) contains some indications which facilitate distinction of the sexes of some species of the *Galleriinae* family by the colour of their wings or length of their palps.

B o r c h e r t (1936) provided photographs of male and female waxmoth without, however, supplying any detailed descriptions. The photographs show the differences in size, in thickness of abdomen and length of the antennae of the male and female specimen. These characters however, do not prove to be sufficiently definite in practice. Thus: 1) the size of each specimen varies considerably depending on breeding conditions, 2) the thickness of the abdomen of females depends on the quantity of eggs they laid during their lifetime, 3) the length of the antennae is not at all characteristic,

since they break easily. Only in exceptional cases does an imago bred in captivity, keep undamaged antennae.

Differences in the length of labial palps of the imago of *Aphomia sociella* L. *(Galleriinae)* as indicated by E c k s t e i n, led to the idea that such differences might also exist in the case of *Galleria mellonella*. Our observations of this phenomenon led to positive results. The female waxmoth posesses well-developed labial palps which extend forward, giving the head of the imago a triangular shape. The labial palps of the male on the other hand are bent and convergent, making the head nearly round. These differences can be easily noticed by the naked eye, even while watching the moth through the glass walls of the container (Fig. 1).

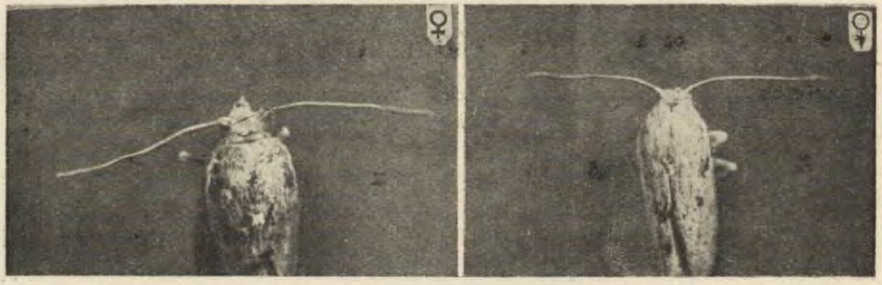

Fig. 1. *Galleria mellonella* L. imago

The correct sex identification of the imago according to the shape of the head has been confirmed by many dissections.

The pupae of some Lepidoptera families show distinct differences between the posterior abdominal segments, depending on their sex (I m m s, P o r t i e r, B o u r g o g n e). Female pupae have two openings on the 8th or 9th segment, or in rare instances a single common opening on the 8th segment. These are the orifices of the bursa copulatrix and the vagina. Male pupae always have a single genital aperture located on the 9th segment. There is no difficulty distinguishing the differences in the external form of the silkworm and waxmoth pupae (Fig. 2).

It is most difficult, however, to find external characters by which female larvae are distinguished from the male. B o u r g o g n e (Traité de Zoologie) provided few indications concerning

some species or families of Lepidoptera. The female larvae of *Lasiocampidae* are larger than the male, the larvae of *Lymantriidae* differ in colour, *Bombyx mori* L. *(Bombycidae)* female larvae show during their last instar on the 8-th and 9-th segments four small abdominal spots (Ishiwata spots), which the males lack. Sometimes it is possible to distinguish the sex of larvae during the last instar by observing the gonads visible through the cuticle, as in *Ephestia kuehniella* Z. *(Pyralididae)*. B o u r g o g n e maintains that it is either very difficult or even impossible to distinguish the external

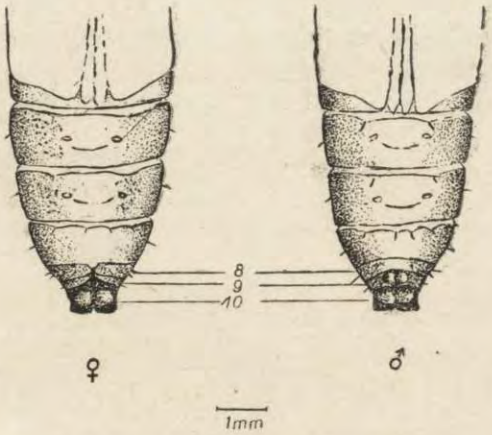

Fig. 2. *Galleria mellonella* L., pupae

sex characters of larvae in most instances. Like B o u r g o g n e, M i c h a j ł o w (1950) indicates the presence of buds of accessory glands (discs of Ishiwata) in *Bombyx mori* females during their last larval instar which are clearly distinguishable from the outside and appear only in female specimen.

There are no major difficulties distinguishing the sex of female silkworm larvae during their fifth instar. According with what we said above one can recognize as male those specimen, which lack Ishiwata discs. Detailed examination enabled us however to observe in male larvae on the ventral side of the 8-th segment, nearest to the median line, a characteristic object clearly showing through in the form of two whitish tiny stripes. To ascertain the nature of the organ which is visible through the cuticle and is characteristic of the male specimen, we separated it. On the basis of the studies of V e r s o n and B i s s o n (1896), Z a n d e r (1903) and

Z i c k (1911) concerning the development of the reproductive organs and accessory sexual glands of various insect species, we have defined it as the Herold organ (Fig. 4). We found it to be identical with the buds of male reproductive organs described by H e r o l d in 1915 and named later as the „Herold organ". In the above-mentioned works one can find detailed data on the distribution of buds of accessory glands in female and male specimen

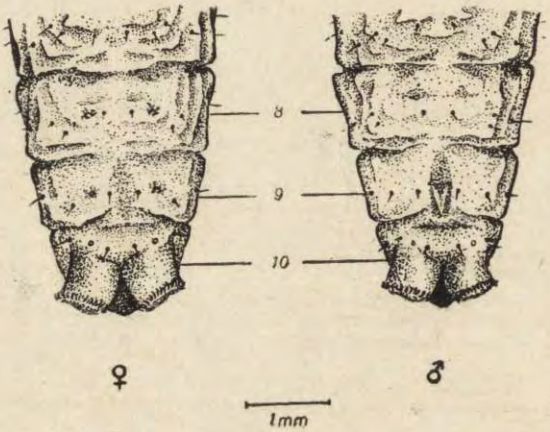

Fig. 3. *Galleria mellonella* L., larvae, last larval instar

during their larval life. None of the above authors has, however, provided any practical indications as to how to distinguish sexes of larvae by their external appearance.

In our study thanks to systematical observations we found it possible to ascertain that sex can be determined by external characters during the whole period of growth, beginnig with the first moulting. Difficulties in the sex determination of larvae arise in the moulting periods, when the new cuticle is formed and also at the last larval instar, just before spinning begins.

According to V e r s o n ' s work (1896), towards the end of the last instar the buds of female accessory glands approach the median line at the same point between the 8-th and 9-th segments where we noticed the Herold organ showing through in males. The assumption is justified, that this causes the blurring of the external differences between males and females.

It is much more difficult, however, to find external characters which would enable us to determine the sex of the waxmoth larvae. Piepho (1940) stated, that the size of heads of the wax moth larvae, while in the same instar, varies according to sex. Due to individual variations the same author does not consider this character as sufficient for the purpose of determining sex. It has therefore been necessary to seek other external sexual characters of the waxmoth larvae. By transmitting light through larvae we can distinguish the characteristic shape of gonads, which are specific for each sex. This is possible, only in the case of larvae with digestive tracts empty, since full digestive tracts do not let enough light through, thus obscuring the picture. Towards the end of the last instar masses of the fat body also obscure the structure of gonads.

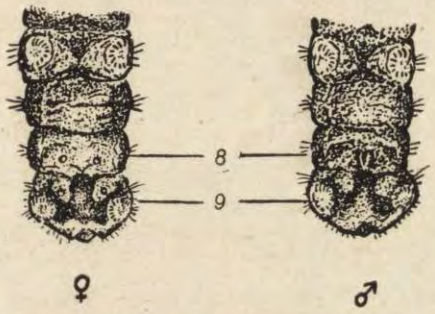

Fig. 4. *Bombyx mori* L., larvae, third instar

The search for organs of wax moth larvae corresponding to Ishiwata discs or Herold organs of other insects has been successful. We have been able to discern through the thin cuticle of the wax moth larvae outlines of the buds of male accessory glands in the form of two tiny stripes. The triangular Herold organ on the ventral side of the 9-th segment is attached with one end between the 9-th and 10-th segments, and the other two ends point forward. In female specimen of the waxmoth larvae buds of female accessory glands have been found. These buds do not however present the shape of distinct spots like in the case of the silkworm, but only just touch the cuticle; while on the side of the abdominal cavity they are covered with a muscular layer. The transparency of the discs and their very small size do not allow them to be seen even with magnification. One can distinguish very clearly however the

net of tracheae, which envelops this constellation of embryonic cells. The tracheae are found on both sides of the median line of the 8-th and 9-th abdominal segments of the female waxmoth larvae, and are frequently grouped in the shape of a horse-shoe (Fig. 3). These observations have been confirmed by dissecting the larvae and by direct inspection of the gonads. As in the case of the silk worm, it is very difficult to determine the sex of the waxmoth during moulting and just before spinning begins.

The author wishes to express her thanks to Doc. S. Niemierko for ~~his~~ her kind interest in this work.

SUMMARY

1. The external sexual characters of larvae, pupae and imago of *Galleria mellonella* L. are described.

2. A description of the appearance of external sexual characters of male larvae has been added to data on the external sexual characters of female *Bombyx mori* L. larvae which are generally known.

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STUDIES ON THE BIOCHEMISTRY OF WAXMOTH (*GALLERIA MELLONELLA* L.) 13. ROLE OF PHOSPHOLIPIDS IN THE UTILIZATION OF WAX

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In previous papers Niemierko and Włodawer (1950, 1952a) have shown that the digestion and assimilation of wax by *Galleria mellonella* larvae is connected with hydrolysis of the wax in the gut and with a partial oxidation of unsaponifiable substances into fatty acids.

Further investigation (Niemierko and Włodawer 1952b) has indicated that there is a large quantity of phospholipids in the intestinal wall of the feeding larvae, while in the gut of starved larvae the amount of phospholipids is 2 or 3 times smaller. These results suggested that lipids are absorbed from the intestine in a phosphorylated form.

In view of these observations it appeared to be of some interest to investigate more carefully the assimilation of wax by the larvae and to elucidate the role of phospholipids in these processes.

MATERIAL AND METHODS

As the subject of the study full grown waxmoth larvae, *Galleria mellonella*, having a body weight of 150—200 mg have been used. The experiments were made on feeding and on starving larvae. The period of starvation lasted

from one to twenty days. When duration of the fasting was longer than 24 hours the larvae were ligated just behind the head (Niemierko and Wojtczak 1950).

The digestive tract dissected from the body of the larvae under ether narcosis was analysed immediately or after 2—3 hours incubation in a moist chamber at a temperature of 37°.

For each experiment digestive tracts from 10 to 20 larvae were taken simultaneously. Each series of experiments was repeated several times. In some experiments the intestine tissue and the contents of the gut were analysed separately.

In addition several experiments have been carried out on homogenized intestines in a buffer solution (0.15 M glycine and 0.15 M $NaHCO_3$; pH = 8.3). For a group of 20—30 digestive tracts 5 ml of buffer solution were used. The homogenized samples were analysed either immediately or after 2—3 hours of incubation.

In several cases the whole larva, the fat body, the muscles and the blood were subjected to analysis.

The total content of lipids was estimated according to Niemierko et al. (1952), the lipid phosphorus was determined by the method of Fiske and Subbarow (1925) after oxidation of a sample of lipid extract with concentrated nitric acid and a small quantity of sulphuric acid.

The amount of phospholipids was calculated by multiplication of the value obtained for phosphorus by 25. In some experiments phospholipids were determined after their precipitation by acetone using the method of Borgstroem (1952a).

The acetal phosphatides were determined according to Feulgen (1951) applying fuchsin solution prepared after Christl (1953). The extinction was measured in Coleman junior spectrophotometer at the wave length 550 mμ. Standard solutions of acetal phosphatides not being available the determinations gave only the possibility to measure the relative amounts under different physiological conditions.

RESULTS

The amount of lipids and phospholipids in the digestive tract of the feeding larvae is shown in table I. In this series of experiments the digestive tract was examined together with its contents. The weight of one intestine was 30 to 40 mg. It follows from the data in this table that the phospholipids form about 20% of the total amount of lipids.

It was known from the previous investigation (Niemierko and Włodawer 1952-a) that the digestive tract of the feeding larvae is abundantly filled with food and contains large amounts of emulgated and partially hydrolized wax. The presence of this substance made difficult the interpretation of the data obtained. In

view of this in the next series of experiments the tissue and the contents of the intestine were examined separately. The results obtained, shown in table II, indicate that only a very small amount of phospholipids can be found in the contents of the intestine, the

Table I

Lipids and phosholipids in the intestine of waxmoth larvae
(Mean values, calculated for one gut)

Experiment No	Lipids mg	Phospholipids	
		mg	% in lipids
1	2.72	0.525	19.5
2	1.90	0.415	21.8
3	1.61	0.405	25.2
4	3.00	0.475	15.8
5	3.02	0.500	16.8
6	2.90	0.550	19.0
7	2.22	0.525	24.0
8	3.05	0.600	20.0
9	2.73	0.575	21.3
10	2.62	0.450	17.5
Mean	2.62	0.502	20.1

Table II

Lipids and phospholipids in intestinal tissue and lumen
(calculated for one gut)

Experiment No	Lipids		Phospholipids			
	in tissue mg	in lumen mg	in tissue		in lumen	
			mg	% in lipids	mg	% in lipids
1	0.57	1.42	0.245	43	0.088	6.3
2	0.83	1.70	0.420	51	0.085	5.0
3	0.95	1.73	0.400	42	0.105	6.2
4	0.65	1.32	0.335	51	0.095	7.3
5	0.84	1.51	0.395	47	0.070	4.7
6	0.70	1.40	0.345	49	0.130	9.3
Mean	0.76	1.51	0.357	47	0.096	6.4

majority of them being present in the intestinal wall and amounting to approximately 50% of total lipids.

A comparative analysis of other tissues and organs of the larvae has shown that they contain a considerably smaller percentage of phospholipids. This is illustrated by the following data where the percentage of phospholipids in lipids in different tissues is shown.

Total larva	— 5 %
Fat body	— 1,5%
Muscles	— 4 %
Blood	— 10 %
Intestinal tissue	— 47 %

As we can see from the above the phospholipids are present in the lipids of the intestinal tissue in considerably larger quantities than in other tissues. This should be given special consideration as, according to the analyses, the honeycomb on which the larvae were fed contains no phospholipids.

Tentative analyses of the three divisions of the intestine have shown that phospholipids are mainly present in the fore- and midgut, whereas their content in the hindgut is considerably smaller.

To make it clear whether large quantities of phospholipids in the intestine are associated with the ingestion of food and assimilation of wax, experiments were carried out with the starving larvae. The results are shown in table III. We can see that the average content of phospholipids in one digestive tract of feeding larvae amounts to 400—500 μg, and already after 24 hours of inanition rapidly falls to 180 μg. After 48 hours of hunger this content amounts only to 88 μg, it is to about 20% of the initial value. However, in the case of more prolonged starvation, further falling of phospholipid level is quite insignificant.

The results obtained suggested that the rapid fall of the phospholipid level at the beginning of the inanition period corresponds to the amount of phospholipids accumulated in the digestive tract during feeding. Further experiments, as it seems, have confirmed this supposition. The larvae were deprived of food for 24 hours and then some of them were refed for 24 hours. The results are shown in table IV. It can be seen that after the initial rapid fall in the level of phospholipids in starvation, there is a sudden rise of it in

the digestive tract of larvae when they are refed. The rapid disappearance of phospholipids from the gut observed in the short period of time after the cessation of feeding led to the assumption that very active phosphatases able to split the phospholipids are present in the digestive tract.

Table III

Lipids and phospholipids in the gut during starvation (mean values, calculated for one gut)

Period of starvation days	Lipids mg	Phospholipids	
		mg	% in lipids
0	1.92	0.420	22
0	2.53	0.520	21
1	0.70	0.180	26
2	0.43	0.088	22
7	0.51	0.075	15
11	0.40	0.065	16
20	0.32	0.062	21

In order to investigate this problem, experiments were carried out with the isolated guts which were kept for 2—3 hours in a moist chamber at 37°. The results of these experiments are shown in table V. They demonstrate that during the period of incubation 30 to 50% of phospholipids is broken down.

Analogous results have been obtained in experiments with homogenized digestive tracts. We see from the data in table VI that after 2—3 hours of incubation of a homogenized intestine the level of phospholipids falls rapidly. In some experiments as much as 40% of them is broken down during the incubation.

The two latter experiments were carried out in vitro: the first one with the gut with its unimpaired histological structure, the second one with the homogenized gut in which not only the histological but also the cell structure had been destroyed. Both of these experiments indicate the presence in the gut cells of some very active enzymes hydrolysing the phospholipids.

DISCUSSION

There are many papers on the digestion and absorption of lipids indicating that the formation of phospholipids plays an important

role in it. However, this problem has not been solved yet. The earlier literature is given by Verzar and Mc Dougall (1936) and by Bloor (1943), the recent data can be found in the papers of Bergstroem et al. (1952) and Borgstroem (1952 b, 1952 c).

Table IV

Lipids and phospholipids in the gut of starved and refed larvae (calculated for one gut)

Experiment No	Material	Lipids		Phospholipids	
		mg	% of initial value	mg	% of initial value
I	feeding larva	3.0 3.3	100	0.48 0.55	100
	starving larva (24 hours)	0.73 0.75	23	0.17 0.18	34
	refed larva	2.5 2.0	72	0.39 0.36	73
II	feeding larva	3.0 3.3	100	0.59 0.61	100
	starving larva (24 hours)	0.95 0.90	29	0.22 0.21	35
	refed larva	2.7 2.8	87	0.46 0.45	77
III	feeding larva	2.8 2.8	100	0.59 0.57	100
	starving larva (24 hours)	0.92 0.95	33	0.22 0.21	37
	refed larva	2.5 2.0	84	0.48 0.49	82

Almost all experiments in this field have been carried out on mammals. Digestion and absorption of lipids by insects, and in par-

ticular the role of phospholipids in these processes, as it appears, has not yet been investigated.

The results of the present work dealing with this problem indicate that in the intestinal wall of feeding *Galleria* larvae almost half the quantity of all the lipids consists of phospholipids. The amount of phospholipids in the gut of larvae deprived of food falls rapidly, but rises immediately after the larvae are refed. These data indicate that the accumulation of phospholipids in the gut cells is associated with the process of ingestion.

Table V

Lipid phosphorus in isolated guts during incubation
(mean values calculated for one gut)

Experiment No	Time of incubation hours	Lipid — P in controls µg	Lipid — P in incubated guts µg	Difference %
1	3	33	22	— 33
2	3	33	17	— 48
3	3	27	18	— 35

The analysis of the honeycomb on which the larvae feed has proved that it is entirely devoid of phospholipids. These data suggest that the accumulation of phospholipids observed during feeding depends entirely on their formation in the gut cells.

As it has been mentioned above the lipids of different tissues and organs of the larvae contain a considerably smaller quantity of phospholipids than the tissue of the gut, and especially of the midgut. Lipids of the haemolymph are the only ones relatively rich in phospholipids which amount to as much as 10% of all the lipids contained therein. This fact, it seems, is another proof that the role of phospholipids in the process of assimilation of wax is considerable, and that the products of hydrolytic breakdown of wax which are formed during its digestion penetrate the gut wall in a phosphorylated form. Experiments with incubation of isolated gut in a moist chamber, as well as the experiments with homogenates prove the presence of some very active enzymes which split phospholipids in the cells of the digestive tract. It seems that the majority of phospholipids formed during the absorption of wax undergoes a prompt dephosphorylation in the gut cells, while the re-

maining part enters the haemolymph in an unchanged or slightly changed form.

As mentioned above some amount of phospholipids, though small, has been found in the lumen of the gut of feeding larvae.

Table VI

Lipid phosphorus in homogenized guts during incubation

Exp. No	Time of incubation hours	Lipid — P in controls µg	Lipid — P in incubated samples µg	Difference %
1	1¾	67	55	— 19
		68	58	— 15
			59	— 13
2	2¼	71	45	— 39
		76	39	— 46
			47	— 36
3	2	127	90	— 31
		134	93	— 29
			98	— 25
4	3	147	114	— 24
		153	114	— 24
			99	— 34

The data obtained in our experiments do not permit to localize the exact site where the phospholipids are synthesized. The cytochemical investigation of P r z e ł ę c k a (1956) indicates that phospholipids are formed in the epithelial cells of the midgut of feeding larvae. It seems quite probable that a certain quantity of phospholipids formed in the intestine tissue penetrates from the epithelial cells to the lumen of the intestine. One can suppose that their presence in the contents of the intestine is one of the factors facilitating the emulgation of wax, which had been observed by N i e m i e r k o anod W ł o d a w e r (1952 a). The role of phospholipids in the processes of emulgation of lipids in connexion with their digestion and absorption has been often assumed (F r a z e r 1949; A u g u r et al. 1947).

The results of the experiments presented in this work point to a considerable physiological activity of phospholipids in the digest-

ive tract. It seems that the intestinal tissue of Galleria larvae may offer an excellent material not only for the examination of enzymatic splitting of phospholipids, but also of their enzymatic synthesis.

Experiments which have been carried out so far do not give us any data concernig the chemical structure of phospholipids formed in the gut tissue of the Galleria larvae. We do not know whether they are compounds of the type of generally known phospholipids or whether their chemical structure is entirely different, due to the fact that they are synthesized from the products of the breakdown of wax. This problem is going to be examined further. The only characteristic feature of phospholipids which has been observed in this work is that they contain a certain amount of acetal phospholipids. However, it is quite possible that the chemical structure of these acetalphosphatides is entirely specific.

Experiments carried out in the present investigation indicate that simultaneously with the disappearance of phospholipids during fasting and their reappearance in the gut after feeding the acetal phosphatides show quite analogous changes. In the gut of the larvae deprived of food the quantity of acetal phosphatides falls after 24 hours to 50% of the initial value, and rises again as soon as the larvae begin to feed.

The physiological role of acetal phosphatides in the animal body is still obscure. However, their appearance in the gut of *Galleria mellonella* can be understood more easily. In previous papers (N i e m i e r k o and W ł o d a w e r 1950, 1952) it was shown that during digestion and utilization of wax by Galleria higher alcohols and perhaps hydrocarbons too are oxidised to fatty acids. The formation of acetal phosphatides which, as it is known, contain higher aldehydes, could be regarded as an intermediate step in these processes.

SUMMARY

About half of all the lipids in the intestine tissue of the feeding *Galleria* larvae consists of phospholipids. During the period of starvation the quantity of phospholipids in the gut falls considerably, but rises again quickly, as soon as the larvae begin to feed.

A considerable part of phospholipids is broken down during 2—3 hours of incubation of the whole gut and of homogenized gut.

It seems that during the utilization of wax by *Galleria* larvae the formation of phospholipids plays an important role.

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STUDIES ON THE BIOCHEMISTRY OF WAXMOTH (*GALLERIA MELLONELLA* L.). 14. CYTOCHEMICAL STUDY OF PHOSPHOLIPIDS IN THE INTESTINAL TRACT OF WAXMOTH LARVAE

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The results of some previous investigations from this laboratory (Niemierko and Włodawer 1950, Niemierko and Włodawer 1952a, Niemierko and Włodawer 1952b, Włodawer 1955) concerned with the digestion and utilization of wax by *Galleria mellonella* larvae seem to indicate that the synthesis of phospholipids in the intestine is an important step in wax assimilation.

The present work was undertaken in order to get cytochemical information which could contribute to the elucidation of the mechanism of wax absorption.

MATERIAL AND METHODS

In all experiments the full grown larvae of wax moth, *Galleria mellonella* L. were used.

The intestines were studied under following conditions:

1. In feeding larvae
   a. immediately after the removal from the body

b. after three hours of incubation in a moist chamber at 36°C

2. in starved larvae

3. in larvae which had ceased to feed before they began to spin the cocoon.

In order to study the changes occurring during the incubation the experiments were performed in the following way: the intestinal tract was cut lengthvise into two parts and one was incubated in the moist chamber at the temuperature 36°C, whereas the other half was fixed immediately and served afterwards as a control.

The larvae subjected to experimental starvation were ligated in order to prevent metamorphosis (W. Niemierko and L. Wojtczak 1950).

Phospholipids were detected by the use of Baker's acid haematein test, the control sections were extracted with pyridine. The test was performed either on frozen sections or on paraffin embedded material, which, according to Rennels (1953) may be employed too.

OBSERVATIONS

The epithelia of all parts of the intestinal tract were investigated by Baker's acid haematein test. In the foregut numerous unicellular hillocks protrude into the lumen of the intestine. They are coated by a thick coarse cuticle with sharp spicules. No phospholipids could be detected in these cells (Fig. 1 and 2). The midgut epithelium cells on the contrary were stained heavily. In the epithelium of mesenteron aboundant small granulations were visible, giving blue or blue-black colour with Baker's haematein (Fig. 3 and 4). After pyridine extraction these granules disappeared (Fig. 5). In the cells of the hindgut only a few phospholipids granules could be detected.

It was observed that the quantity of phospholipid inclusions in the midgut epithelium varies considerably according to the physiological state of the intestine under observation. These fluctuations were so conspicuous that they could be revealed even by the qualitative test used here. A distinct decrease in the quantity of phospholipid granules was observed after incubation of the intestinal tract in moist chamber at 36°C for three hours (Fig. 6). This decrease did not occur simultaneously in all cells. In some sections the quantity of phospholipid granules did not differ essentially from that in the controls; in other sections there were cells in which the phospholipids were almost completely lacking.

In the midgut epithelium of experimentally starved larvae no phospholipid inclusions could be detected by Baker's test (Fig. 8).

Investigation of the midgut of larvae which have ceased to feed and began to spin their cocoon has shown either a total disappearance of phospholipids, or the persistence of some granules at the basal part of cells only (Fig. 7).

DISCUSSION

It results from our observations that the appearance of phospholipid granulations in the cytoplasm of midgut epithelial cells depends on the physiological state of the larvae. In the epithelial cells of every zone of the midgut of the feeding larvae phospholipids can be detected in quantity. Incubation of excised intestines brings about a considerable decrease in phospholipids. The disappearance of these compounds is also observed during starvation, it is immaterial whether the starvation of larvae is a physiological one (in the period of cocoon spinning) or whether it is induced experimentally. In the empty intestines of starved larvae the phospholipids cannot be detected by Baker's test.

There is a good agreement between the results described here and those obtained earlier by Niemierko and Włodawer. These authors have shown using biochemical methods that the quantity of phospholipids in the intestinal tract of *Galleria mellonella* depends on the physiological state of the animal (Niemierko and Włodawer 1952b, Włodawer 1955, Niemierko, Włodawer and Przełęcka 1955).

It is noteworthy that whereas no phospholipids can be detected by Baker's test after five days of starvation the biochemical analysis shows that 16—17% of the previous amount of these compounds still persist. This discrepancy can be explained by the impossibility to detect the so called masked phospholipids which are structural components of the protoplasm by the method used here.

The morphology and histology of the foregut indicates that its role consists mainly in purely mechanical grinding of food. It is in the midgut that the main processes of digestion and absorption go on.

The larvae of *Galleria mellonella* feed — as is well known — on honey comb. The appearance of phospholipids in the cells of midgut epithelium of the larvae suggests that phospholipids play an important part in the processes of wax absorption.

I am indebted to Prof. W. Niemierko for his kind interest in this work and his advice and suggestions.

SUMMARY

When Baker's acid haematein test was applied to the intestinal tract of feeding waxmoth larvae, phospholipid granules in their midgut epithelial cells were shown in quantity. The number of the inclusions in the cytoplasm depends on the physiological state of the animal.

After 3 hrs of incubation in moist chamber the quantity of phospholipid granules decreased.

During the experimental, as well as during physiological starvation the phospholipid granules could no more be detected.

The correlation between the absorption of wax by the larvae and the appearance of phospholipids in their midgut epithelial cells seems to confirm the hypothesis that phospolipids constitute an important step in utilization of wax by waxmoth larvae.

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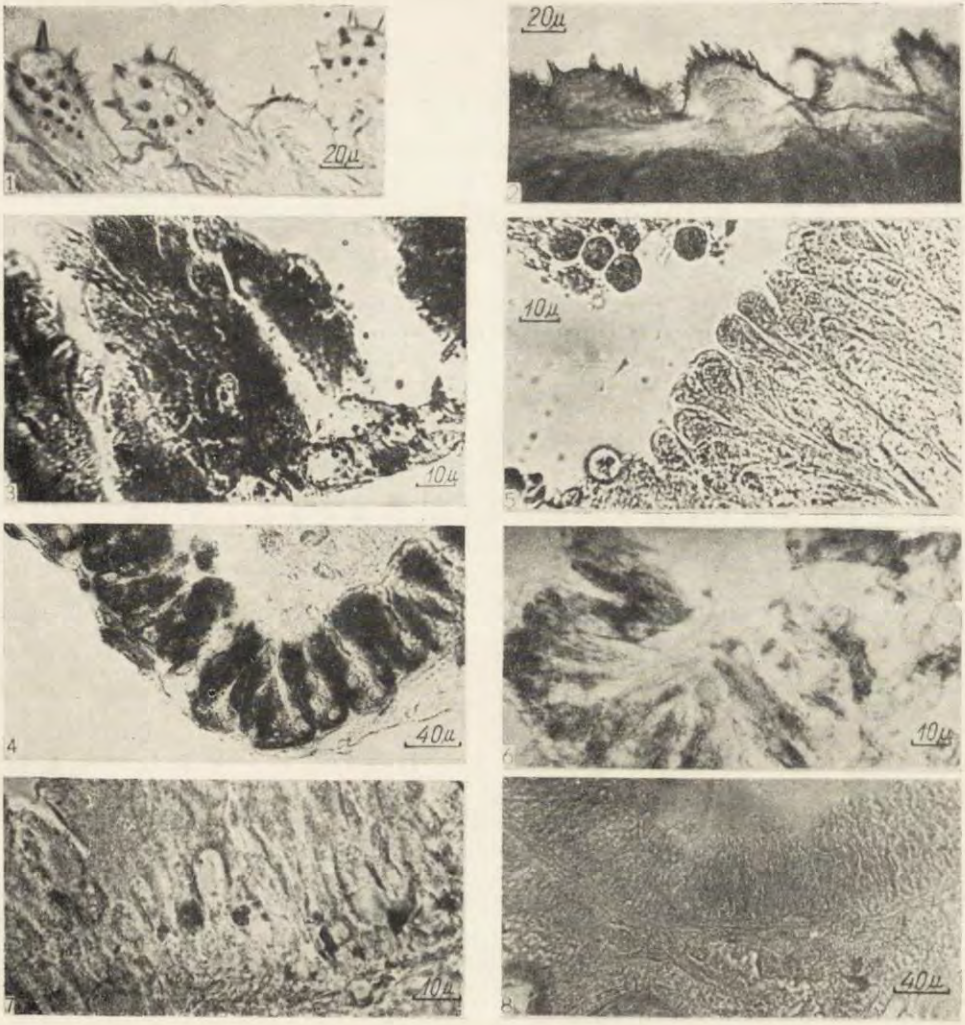

PLATE I

Frozen sections (10 micron thick) of the alimentary tract of wax moth larvae. Baker's acid haematein test for phospholipids. 1 and 2. Proventriculus of a feeding larva; several unicellular spicular hillocks protruding into the lumen of the foregut. No phospholipids are seen (× 200). 3 and 4. Midgut of a feeding larva. The epithelial cells are filled with a great quantity of phospholipid inclusions. Fig. 3 (× 600). fig. 4 (× 200). 5. Control experiment pyridine extraction. Midgut of a feeding larva. No phospholipid granulations are seen (× 600). 6. Midgut epithelium of a feeding larva, after 3h^{rs} of incubation of the excised intestine in a moist chamber at 36^{0}C. The number of phospholipid granulations is decreased in comparison with non incubated intestines (× 600). 7. Midgut epithelium of a larva just before the spinning. Only a few phospholipid granulations are seen at the basal part of cells (× 600). 8. Midgut epithelium of an experimentally starved larva. The 5th day of starvation. No phospholipid inclusions seen (× 200)

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STUDIES ON THE BIOCHEMISTRY OF THE WAXMOTH (*GALLERIA MELLONELLA* L.). 15. PYRO- AND POLYPHOSPHATES OF THE EXCRETA OF LARVAE AND THEIR ENZYMATIC HYDROLYSIS

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It has been ascertained (N i e m i e r k o and N i e m i e r k o 1949, 1950a, 1950b) that the wax moth larvae excrete not only orthophosphate but also a considerable amount of labile phosphorus compounds which hydrolyze to orthophosphate after heating in 1 N HCl at 100° for 7 minutes (this labile fraction was denoted as „P_7"). A part of this fraction has been identified as metaphosphate by means of the metachromatic reaction with toluidine blue and protein precipitation. S. N i e m i e r k o and W. N i e m i e r k o (1950) made some attempts at a quantitative determination of the P_7-fraction and they proved that about a half of it could be precipitated with lead acetate at pH 2, this precipitate being most likely metaphosphate. They also observed that the P_7-fraction was hydrolyzed when incubated with homogenates of wax moth larvae.

In recent years meta- and polyphosphates have been found in moulds (M a n n 1944), in yeast and in some bacteria (W i a m e 1949, E b e l 1952b, 1952c). The metaphosphate of *Aspergillus niger* was similar to the macromolecular synthetic polymer, called Graham's salt (I n g e l m a n and M a l m g r e n 1950). As con-

cerns animals meta- and pyrophosphates have been found only in insects, namely in *Celerio euphorbiae* (Heller, Karpiak and Zubikowa 1950) and in *Galleria mellonella* (Niemierko and Niemierko 1950a, b, Niemierko and Wojtczak 1952). A great number of polymeric varieties which resemble each other as regards their chemical and physical properties makes the separation, the indentification and the determination of poly- and metaphosphates rather difficult.

The aim of the present studies is to investigate in more details the nature of metaphosphate present in the excreta of wax moth larvae and to identify other components of the P_7-fraction. The following procedures of separation and identification of meta- and polyphosphates were used: 1) precipitation as Mn and Ba salts, 2) chromatography, 3) dialysis, 4) enzymatic identification.

The investigations of the enzymatic hydrolysis of pyro- and polyphosphate from the excreta and the properties pyrophosphatase of the wax moth are being described.

METHODS

Phosphorus compounds were extracted from the excreta of the wax moth larvae by means of ice-cold water. In most cases the excreta of starving larvae were used as the source of polyphosphates, since they contain less lipid material and are easier to extract. In addition to the phosphorus compounds, the extracts contained other constituents soluble in water as well, especially great quantities of uric acid. Phosphorus was determined by the method of Fiske and Subbarow (Umbreit and others 1945). Labile phosphorus compounds (P_7) were hydrolyzed in 1 N HCl at 100° for 7 minutes. The pyrophosphate was determined according to Kornberg (1950), and precipitated by means of Mann's (1944) and Ebel's (1952a) methods. The chromatographic technic of Opieńska-Blauth and others (1952) was applied; solvents were prepared according to Ebel (1952b).

In enzymatic experiments homogenates of waxmoth larvae were prepared in the following way: 1 g. of fresh larvae was homogenized with 9 ml. of ice-cold buffer solution (glycine-carbonate and acetate buffers being used) and centrifuged at low speed in order to remove chitin parts. The homogenate was incubated in test tubes containing various substrates, and orthophosphate liberated in enzymatic reaction was determined. The following controls were used: 1) the polyphosphates or the whole extract and buffer, 2) the homogenate with buffer without substrates. The enzymatic reaction was stopped by putting the test tubes on ice and by deproteinizing with 10 per cent trichloracetic acid. The content of each test tube was cool filtered for phosphorus determination.

The following phosphates were used as substrates and standard substances for chromatographic tests: 1) sodium pyrophosphate $Na_4P_2O_7$, Merck p. a., 2) trimetaphosphate, tetrametaphosphate, tripolyphosphate, and Graham's salt (all as Na salts), prepared in the laboratory of Prof. T h i l o in Berlin (kindly supplied by Prof. J. H e l l e r); 3) sodium glycerophosphate Merck p. a., 4) metaphosphate obtained from yeast by the method of W i a m e (1949).

For the enzymatic experiments the pyrophosphatase of the waxmoth was purified by the method of M c E l r o y and others (1951) with following slight modifications: only one acetone precipitation was made and the absorption on silica gel was omitted. The pyrophosphatase prepared in this way is accompanied by some ATP-ase activity which can be eliminated by repeated freezing and thawing, as had been proved by K a p l a n and L i p m a n n (1948).

The pyrophosphatase kept in a frozen state even for a week retains at least a half of its original activity.

EXPERIMENTAL

I. Identification of phosphorus compounds excreted by the waxmoth larvae

Fractionating precipitation of phosphorus compounds from the water extract with $BaCl_2$ at various pH-values was applied. At pH 2 no precipitate appeared, at pH 4.5 to 5, however, about a half of the labile phosphorus compounds was precipitated as Ba salts. This would point to the presence of pyrophosphate and of metaphosphate of low molecular weight. When precipitated with $BaCl_2$, rather great differences between parallel tests were observed. This fact proves that some compounds were not quantitatively precipitated. In order to find out whether pyrophosphate was present, manganese acetate was added to the extract at pH 5, and this resulted in the precipitation of a half of the P_7-fraction. In order to separate metaphosphates from pyrophosphate the precipitation with 50 per cent alcohol, according to M a n n (1944), at pH 5 to 8 was applied. No separation occured, and the precipitate contained both pyro- and metaphosphates, as proved by Mn and Ba precipitation. Table I summarizes the results of experiments described above. As will be seen neither barium nor manganese salts precipitate all labile phosphorus compounds present in the extract when used separately. Thus the two salts were applied one after another. As a result as much as 90 per cent or more of the P_7-fraction was precipitated, while both barium and manganese salts, when used

separately, removed only about a half of the labile phosphorus from the solution. The quantity of phosphorus precipitated with Mn or Ba salts was very the same irrespectively of which of the two salts was used first. The sequence of Mn-Ba precipitation was followed as a more useful, because in this way more of the labile phosphorus compounds could be precipitated. Indeed, only a small part of the P_7-fraction remained in the solution (Table II). Both the Mn and Ba fractions were slightly contaminated with orthophosphate. The precipitates were dissolved in 1 N H_2SO_4, the resulting $BaSO_4$ was removed, and the solutions used in further analyses. Orthophosphate and labile phosphates were determined as described above.

Table I

Precipitation of labile phosphorus compounds (P_7) from the excreta of wax-moth larvae by means of $BaCl_2$ and $Mn(CH_3COO)_2$ at pH = 5.

Exper. No	Substance	Quantity of phosphorus precipitated in per cent of total labile P	
		$BaCl_2$	$Mn(CH_3COO)_2$
1	Water extract	50; 47; 45*	44; 44*
2	„ „	50; 60*	52; 52*
3	„ „	56	65
1	Alcohol precipitate	72; 75*	30; 30*
2	„ „	49	60
3	„ „	60	46; 47*
4	„ „	—	85
5	„ „	—	71; 72*

* Parallel determinations in the same extract.

In further experiments chromatography was applied in order to investigate the composition of Mn and Ba fractions. The whole extract as well as barium and manganese precipitates were examined. The following solvents were used: 1) the acid solvent to separate the long chained polyphosphates: 75 ml. of isopropanol, 25 ml. of water, 5 g. of trichloracetic acid, and 0.3 ml. of concentrated ammonia, and 2) the alkaline solvent to separate cyclic metaphosphates: 20 ml. of isopropanol, 20 ml. of isobutanol, 39 ml. of water, and 1 ml. of concentrated ammonia. The filter paper, Whatman No 1, before being used was washed with dilute acetic acid. Chromato-

Table II

Separation of labile phosphorus compounds from water extract of excreta by means of succesive precipitation with Ba and Mn salts at pH = 5.

Group of experiments	Experiment No	Mn-precipitation			Ba-precipitation			P_7 in filtrate		Sum of P_7 in both precipitates and filtrate	
		P-ortho in mg.	P_7		P-ortho in mg.	P_7		in mg.	in per cent of total P_7	in mg.	in per cent
			in mg.	in per cent of total P_7		in mg.	in per cent of total P_7				
in the whole extract										1.87	100
I	1	0.011	0.78	41.7	0.02	0.85	46.4	0.29	15.5	1.92	104
	2	0.014	0.95	50.7	0.17	0.81	43.3	0.08	4.3	1.84	98
	3	0.048	0.72	38.4	0.06	0.82	43.8	0.12	6.4	1.66	89
	4	0.045	0.76	40.7	0.04	0.90	48.2	0.08	4.3	1.75	93
in the whole extract										0.72	100
II	1	0.14	0.29	40.3	traces	0.35	48.7	0.09	12.5	0.73	101
	2	0.19	0.28	38.9	traces	0.34	47.2	0.09	12.5	0.71	99

I group of experiments: phosphorus compounds were precipitated from the extract with Mn-salt and next with Ba-salt;
II group of experiments: reverse precipitation.

grams were developed at 27°—30° for about 8 hours (ascending chromatography), and thereafter they were sprayed with 1 N HCl and exposed to 80° for 7—10 minutes in order to hydrolyze the labile phosphorus compounds. Spots were detected with molybdenum-benzidine reagent.

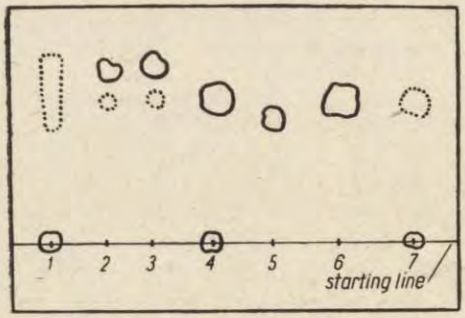

Fig. 1. Paper chromatogram of phosphorus compounds from the exreta of wax moth larvae and of synthetic phosphates: 1 — Graham's salt, 2 — tetrametaphosphate, 3 — trimetaphosphate, 4 — water extract of excreta, 5 — pyrophosphate, 6 — orthophosphate plus pyrophosphate, 7 — barium precipitate from excreta obtained after previous manganese precipitation. Alkaline solvent

The water extract when chromatographed in the alkaline solvent left two spots. One indicated the presence of orthophosphate and pyrophosphate (these two compounds do not separate from each other in this solvent), the other on the starting line indicated the presence of some phosphorus compounds that did note move in the alkaline solvent. Out of a number of synthetic meta- and polyphosphates examined only the Graham's salt left a spot on the starting line (look Fig. 1). The water extract as a whole left three distinct spots when chromatographed in the acid solvent. One was identified as orthophosphate, the second as pyrophosphate, and the third, very close to the starting line, had the same R_f value as trimetaphosphate (Fig. 2). The presence of trimetaphosphate in the water extract could, however, be excluded since no corresponding spot was visible on chromatograms developed in the alkaline solvent. There is a possibility that the trimetaphosphate appeared during the development in the acid solvent as a result of partial hydrolysis of some macromolecular polymer. When barium precipitate was chromatographed in the acid solvent three spots were visible, similar to those present when the water extract was used. Orthophosphate, pyrophosphate, and trimetaphosphate respectively were the source of these spots. But when barium precipitate was obtained, after preceding manganese precipitation, one spot was

visible either on the level of the trimetaphosphate when chromatographed in the acid solvent or on the starting line when the alkaline solvent was used (look Figures 1 and 2). The chromatogram of the manganese precipitate indicated the presence of orthophosphate and pyrophosphate only (Fig. 2).

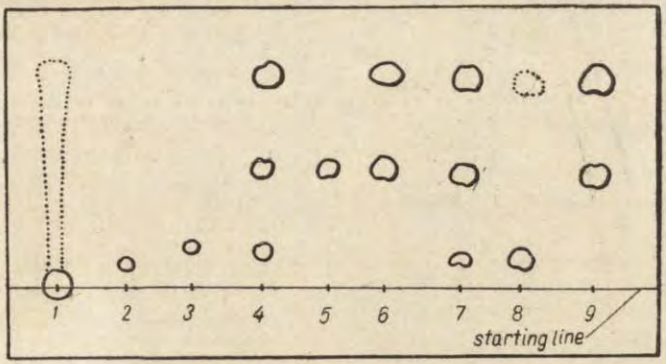

Fig. 2. Paper chromatogram of phosphorus compounds from the excreta of wax moth larvae and of synthetic phosphates: 1-Graham's salt, 2-tetrametaphosphate, 3-trimetaphosphate, 4-water extract of excreta, 5-pyrophosphate, 6-orthophosphate plus pyrophosphate, 7-barium precipitate from water extract, 8-barium precipitate from water extract obtained after previous manganese precipitation, 9-manganese precipitate. Acid solvent

In the course of further experiments dialysis was applied. The water extract was dialyzed through cellophane against distilled water at 0^0. It appeared that after 24 hours about 1/4 of the P_7-fraction remained in the bag. No precipitation with manganese acetate resulted, but $BaCl_2$ at pH 5 precipitated about 85 per cent of the phosphorus present. After 48 hours of dialysis only 13 per cent of the labile phosphorus remained, 70 per cent of it was precipitable as Ba salt at pH 5. When this phosphorus compound that did not dialyze was tested chromatographically there was only one spot on the starting line (the alkaline solvent was used) and similar to the barium precipitate there was a very strong metachromatic reaction with toluidine blue. No such reaction was observed, however, when the manganese precipitate was tested. All this indicated that some macromolecular phosphorus compound was present in the P_7-fraction. This compound either dialyzed very slowly through cellophane or it gets decomposed to some extent during dialysis.

When the barium precipitate was examined with regard to the presence of other constituents than phosphorus some pentose was detected. It could not be established whether this sugar was bound to the phosphorus compounds.

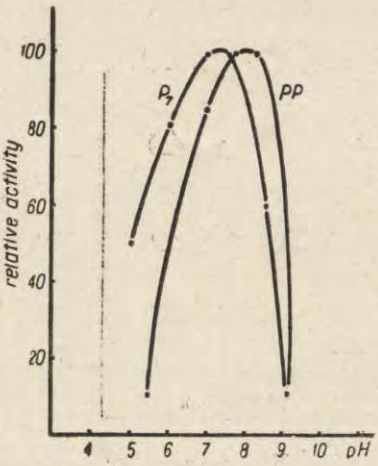

Fig. 3. The effect of pH on the enzymatic hydrolysis of pyrophosphate (PP) and of labile phosphorus fraction of excreta (P_7). Incubation mixtures contain, for pyrophosphate hydrolysis: 3 ml. of homogenate in buffer solution, 1 ml. of 0.02 M sodium pyrophosphate, and 0.5 ml. of 0.05 M $MgSO_4$ (incubation time 1 hour at 37°), for labile phosphorus (P_7) hydrolysis: 3 ml. of homogenate in buffer solution and 2 ml. of water extract of excreta containing about 0.5 mg. of labile phosphorus (incubation time 3 hours at 37°)

II. The enzymatic hydrolysis of pyro- and polyphosphates derived from excreta

The water extract or separate fractions of it were incubated together with the homogenates of the wax moth larvae. In the case of the water extract as a whole the effect was the sum of hydrolysis of pyro- and polyphosphates.

a) The effect of pH. The effect of pH on the enzymatic hydrolysis was examined in the pH range from 5 to 9. As will be seen from Figure 3 the optimum was about 7—7.5.

b) The effect of temperature. The whole water extract was incubated together with the homogenate at 24°, 37°, and 54° respectively. Incubation lasted for 3 hours and the increase in the amount of orthophosphate was measured in the following time intervals: 15 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours starting from the beginning of the incubation. As will be seen on Fig. 4, the temperature of 37° was the most favourable for the enzymatic hydrolysis: after 3 hours of incubation nearly all the labile phosphorus was converted to orthophosphate but as much as 80 per cent of it was converted already after 1 hour. At 24° the rate of hydrolysis was very low, since after 2 hours of incubation only 40 per cent of labile phosphorus compounds was hydrolyzed, while at 37° this percentage amounted to over 90. At 54° the initial

rate of hydrolysis was higher than at 37°, but thereafter a gradual thermic inactivation of the enzyme took place. After 1 hour when only 60 per cent of the labile phosphorus was hydrolyzed the reaction came to a stop.

c) The effect of Mg^{++}, Mn^{++}, Co^{++}, Zn^{++}, Ag^{+}, and Hg^{++}. The effect of various metal ions on the enzymatic hydrolysis of pyro- and polyphosphates was investigated. It must be taken into account that the water extract of excreta contains some quantities of various cations, especially Ca^{++} and Mg^{++} in percentages of the dry weight of excreta 0.4 and 0.3 respectively.

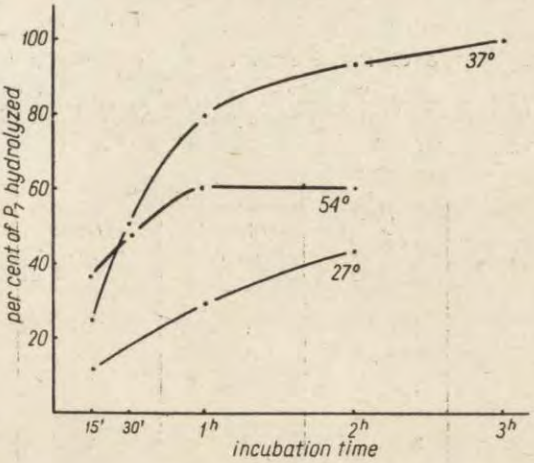

Fig. 4. The effect of temperature on the enzymatic hydrolysis of the P_7-fraction. Incubation mixtures contain: 3 ml. of homogenate in glicine-carbonate buffer of pH 7.5 and 2 ml. of water extract containing 0.5 mg. of labile phosphorus

It will be seen from the Table III that: 1) both Mg++ and Co++ activated the hydrolysis, this effect being more pronounced at pH 6.6 than at pH 5.5, 2) Mn^{++} ions though they activated the hydrolysis at pH 6.6 had an inhibitory effect at pH 5.5, 3) Zn^{++} ions exerted a strongly inhibitory influence at pH 6.6 and a slight one at pH 5.5, 4) both Hg^{++} and Ag^{+} had a strong inhibitory effect at 5.5.

As the influence of Mn^{++} ions on the enzymatic hydrolysis of the whole P_7-fraction was at various pH values different, separate barium and manganese precipitates were used in futher experiments. Table IV summarizes the results of these experiments as well as the effect of Mg^{++} and Mn^{++} on the hydrolysis of synthetic pyrophosphate. It will be seen that the phosphorus compound precipitated as manganese salt was hydrolyzed to orthophosphate like the synthetic pyrophosphate. The hydrolysis of both these substances was inhibited by Mn^{++} ions at pH 8. At pH 5.5 the pyrophosphate and the manganese precipitate were hydrolyzed to a small

degree. These experiments confirm the chromatographic data which showed that the manganese precipitate contained only pyrophosphate. The hydrolysis of the barium precipitate, however, was not inhibited by Mn^{++}, and the degree of hydrolysis at pH 5.5 was twice as high as in the case of the manganese precipitate.

Table III

Influence of metal ions on the enzymatic hydrolysis of labile phosphorus compounds (P_7) from excreta of waxmoth larvae

The incubation mixtures contained: 3.0 ml. of homogenate, 1 mg. of P_7, ion tested in final concentration $3 \cdot 10^{-3}$ M. Incubation was at 37° for 15 minutes

Salts added	pH = 5.5		pH = 6.6	
	P liberated in mg.	activity in per cent*	P liberated in mg.	activity in per cent*
No	0.12	100	0.20	100
$MgSO_4$	0.25	208	0.24	135
$MnCl_2$	0.08	67	0.25	145
$CoSO_4$	0.28	234	0.26	155
$ZnSO_4$	0.09	75	0.03	15
$AgNO_3$	0.05	42	.	.
$HgCl_2$	0.02	17	.	.

* The activity with no added salt was taken as 100.

d) Hydrolysis by means of frog organ homogenates. It was certainly worth while to find out whether the polyphosphate present in the excreta of the wax moth larvae was hydrolyzed by a special enzyme characteristic for those larvae or by a more widespread one *. In order to answer this question the water extract was incubated with homogenates of frog intestine and liver. For comparison, similar experiments were performed with pyrophosphate, glycerophosphate, various synthetic meta- and polyphosphates, and with the metaphosphate isolated from yeast by the method of W i a m e (1949). It appeared that only the water extract of excreta, synthetic pyrophosphate, and tripolyphosphate were easily hydrolyzed by all homogenates used. Glycerophosphate was hydrolyzed to a much lesser degree (Table V). Thus it has been established that the enzyme which hydrolyzes the

* In previous investigations N i e m i e r k o and N i e m i e r k o (1950a, b) had observed that the P_7-fraction was partly hydrolyzed when incubated with homogenates of rabbit muscles.

Table IV

The effect of Mg^{++} and Mn^{++} on the enzymatic hydrolysis of barium and manganese precipitates by homogenate of waxmoth larvae

Incubation mixture contained: 2 ml. of homogenate, 2) 0.5 ml. of 0.01 M $MgCl_2$ or 0.5 ml. of 0.01 M $MgCl_2$ plus 0.5 ml. of 0.01 M $MnCl_2$, 3) substrate containing about 1 mg. P_i, 4) glycine-carbonate buffer pH = 8 or acetate buffer pH = 5.5 to a final volume of 5.0 ml. Incubation was at 37° for 2 hours

pH	Substrate	Phoshorus liberated in per cent ions added	
		Mg^{++}	Mg^{++} and Mn^{++}
8	water extract of excreta	100	.
	pyrophosphate (synthetic)	100	50
	manganese precipitate	93	30
	" "	90	57
	barium precipitate	100	100
	" "	100	93
5.5	water extract of excreta	70	20
	pyrophosphate (synthetic)	10	7
	manganese precipitate	17	10
	barium precipitate	36	.

polyphosphate fraction of excreta is a widespread one and is present in insects and in vertebrate tissues as well. It must be emphasized here that the polyphosphate of excreta is not identical with tripolyphosphate, although both are hydrolyzed under the influence of the homogenates since other experiments previously described have proved it to be a rather macromolecular compound.

III. Properties of the waxmoth pyrophosphatase

The presence of the pyrophosphatase in the waxmoth tissues had been discovered by Niemierko and Wojtczak (1952). In the present investigations the properties of this enzyme are examined. It was worth while to find out whether the wax moth pyrophosphatase brings about the hydrolysis of polyphosphate fraction contained in the excreta or whether these hydrolysis is a result of the activity of another enzyme present in the homogenates.

In all experiments sodium pyrophosphate Merck p. a. was used as a substrate. For specifity investigations the enzyme was purified as described in the Methods. In all other experiments the whole homogenate was used.

Table V

Enzymatic hydrolysis of metaphosphates, polyphosphates, and glycerophosphate by homogenates of frog organs and of waxmoth larvae
Incubation mixtures contained: 3 ml. of homogenate (300 mg. tissue plus 2.7 ml. glycine-carbonate buffer pH = 8), 0.5 ml. of 0.01 M $MgCl_2$ and substrate containing about 1 mg. labile phosphorus.

Substrate	Phosphorus liberated in per cent		
	homogenates of		
	frog intestine	frog liver	wax moth larvae
water extract of excreta	100	100	100
pyrophoshate	100	100	100
tripolyphosphate	—	100	100
trimetaphosphate	—	3*	5
tetrametaphosphate	—	0	0
Graham's salt	—	0	0
metaphosphate of yeast	—	0	0
glycerophosphate	30	—	50

* Trimetaphosphate was slightly contaminated with tripolyphosphate as had been proved by chromatographic test.

a) pH optimum. The pyrophosphatase activity was measured in the pH range of 5.5 to 9.3. The pH optimum was found to be at 7.5 to 8.3 (Fig. 3). Similar pH values had been obtained for the pyrophosphatases of liver (Bamann and Gall 1937) and of fire-fly (Mc Elroy and others 1951). Whereas for the pyrophosphatase of yeast (Beiley and Weeb 1944) and of *Penicillium chrysogenum* (Krishnan 1952) the optimum pH was 7.0 and about 6 respectively.

b) Effect of temperature. The optimum temperature was found to be about 37°. At higher temperatures a gradual decline in the activity took place. A complete inactivation of the enzyme occured after 1 hour of heating at 60° and at pH 7.

c) Effect of Mg^{++}, Co^{++}, Mn^{++}, Ca^{++} and F^{-}. It was found that pyrophosphatase present in the homogenate of the wax moth larvae was not active in the absence of Mg^{++} ions. Optimum Mg^{++} concentration was 0.004 M. In much higher concentrations

Table VI

The effect of metal ions and fluoride on waxmoth pyrophosphatase. The mixtures were incubated at 37° for 15 minutes. pH and pyrophosphate concentration were 8 and 0.004 M respectively

Exper. No	Salts added	Final concentrations	P liberated in mg.	per cent activity*
1	$MgSO_4$	$5 \cdot 10^{-3}$ M	0.152	100
2	$MgSO_4$	$1 \cdot 10^{-3}$ M	0.099	66
3	$CaCl_2$ $MgSO_4$	$1 \cdot 10^{-3}$ M $5 \cdot 10^{-3}$ M	0.086	56
4	$CaCl_2$ $MgSO_4$	$5 \cdot 10^{-3}$ M $5 \cdot 10^{-3}$ M	0.008	5
5	$MnSO_4$ $MgSO_4$	$1 \cdot 10^{-3}$ M $5 \cdot 10^{-3}$ M	0.077	50
6	$MnSO_4$ $MgSO_4$	$1 \cdot 10^{-3}$ M $5 \cdot 10^{-3}$ M	0.039**	26
7	$CoSO_4$ $MgSO_4$	$1 \cdot 10^{-3}$ M $1 \cdot 10^{-3}$ M	0.150	100
8	NaF $MgSO_4$	$1 \cdot 10^{-3}$ M $5 \cdot 10^{-3}$ M	0.034	22

* Pyrophosphatase activity in the presence of Mg^{++} $5 \cdot 10^{-3}$ M was taken as 100.
** Pyrophosphate concentration was 0.002 M (in all other experiments it was 0.004 M).

of Mg salts, about 0.1 M, a marked inhibition of pyrophosphatase activity took place. Table VI summarizes the effects of various ions on the enzymatic hydrolysis of pyrophosphate. It is obvious that Mn^{++} inhibits the hydrolysis. This is in all probability due to the formation of the manganese pyrophosphate precipitate. The degree of the ensuing inhibition depends on the substrate concen-

tration, namely at a higher concentration of pyrophosphate the manganese ions are less inhibitory. The same has been observed with regard to pyrophosphatases of horse erythrocytes (S j ö b e r g 1954) and with regard to fire-fly pyrophosphatase (M c E l r o y and others 1951). In the present investigations an activatory effect of Co^{++} ions was observed, but, interestingly enough, this was the case only when Mg^{++} ions were present too, even if in a low concentration. The degree of inhibition caused by calcium ions depends on the Ca : Mg ratio. At Ca : Mg = 0.2 a 50 per cent inhibition took place, at Ca : Mg = 1 the inhibition was almost complete. The same had been noticed with regard to the fire-fly pyrophosphatase (M c E l r o y and others 1951). Fluoride had a strong inhibitory effect, as in the case of other pyrophosphatases.

d) T h e e f f e c t o f t h e s u b s t r a t e c o n c e n t r a t i o n. The homogenates of larvae were incubated with varying amounts of pyrophosphate and the orthophosphate liberated during the first 15 minutes measured. It was noticed (Fig. 5)

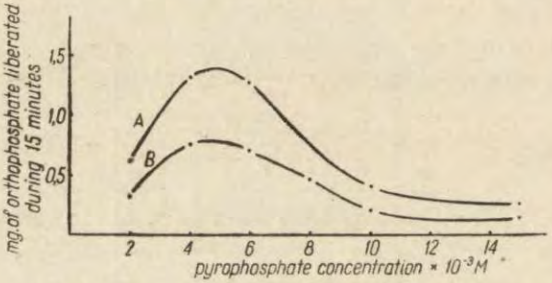

Fig. 5. The effect of substrate concentration on the rate of enzymatic hydrolysis of pyrophosphate. Curve A — 1 ml. of homogenate, curve B — 0.5 ml. of homogenate; Mg^{++} concentration 0.004 M, pH 8, temperature 37°, incubation time 15 minutes

that the optimum substrate concentration was 0.004 M. When the concentration increased to 0.008 M the activity of the pyrophosphatase dropped to a half, and at the concentration of 0.015 M was as low as several per cent of its optimum. The M i c h a e l i s constant obtained from the curve (Fig. 5) is $2.3 \cdot 10^{-3}$ M. The optimum substrate concentrations for other pyrophosphatases are as

follows: 0.0004 M for fire-fly pyrophosphatase (Mc Elroy and others 1951), 0.0001 M for yeast pyrophosphatase (Beiley and Weeb 1944), and 0.001 M for pyrophosphatase of horse erythrocytes (Sjöberg 1954). The Michaelis constant for all those enzymes is very low, namely from 10^{-4} to 10^{-3} M, what points to a great affinity between the enzyme and the substrate. Owing to that pyrophosphate can be effectively hydrolyzed even in a very low concentration. This is why in all those processes as nucleotide synthesis (Kornberg and others 1955), fatty acid activation (Kornberg and Pricer 1953), and light emission by the fire-fly (Mc Elroy and others 1953) in which pyrophosphate is generated the pyrophosphatase is supposed to play an important part as a regulatory agent.

e) Specifity studies. In investigations on the specifity of the waxmoth pyrophosphatase, purified enzyme preparation and following substrates were used: pyrophosphate, tripolyphosphate, trimetaphosphate, tetrametaphosphate, Graham's salt, yeast metaphosphate, wax moth polyphosphate (precipitated from the water extract as barium salt), ATP, and glycerophosphate. Of all these substances only pyrophosphate was hydrolyzed. Other compounds in the presence of Mg^{++} and Mn^{++} ions were not decomposed at pH 5 and pH 8. It is obvious that the pyrophosphatase of the wax moth is very specific, similar to the pyrophosphatases obtained from other sources. The only exception is the yeast pyrophosphatase which is active against hexametaphosphate to some extent (Mann 1944).

DISCUSSION

The experiments have shown that the labile phosphorus fraction discovered previously in the excreta of the waxmoth larvae (Niemierko and Niemierko 1950 a, b) was composed of approximate equal amounts of pyrophosphate and some non-identified polyphosphate. The presence of pyrophosphate was proved by the precipitation with manganese acetate, by the chromatographic analysis and by the enzymatic method as well. The nature of the polyphosphate precipitated from the water extract of excreta as barium salt, was investigated too. It was proved to be a macromolecular polymer, since it gave a positive metachromatic reaction with toluidine blue, did not dialyze or dialyzed very slowly and

did not move on chromatograms in the alkaline solvent. Thus the unknown compound was similar to one of the macromolecular synthetic polymers, the so called Graham's salt, although it differed from the latter because it could not be precipitated with $BaCl_2$ at pH 2 but only at pH 5. In addition, the polyphosphate of excreta was hydrolyzed enzymatically by homogenates of frog organs and of the wax moth larvae, while the Graham's salt was not.

It was not established which of the enzymes hydrolyzed the polyphosphate from excreta. One thing is sure, it was not the pyrophosphatase, since the purified pyrophosphatase from the wax moth larvae did not decompose the polyphosphate fraction, isolated from the excreta by means of barium precipitation. It is known that in yeast and moulds polyphosphatases are present which hydrolyze meta- and polyphosphates (M a t t e n h e i m e r 1951, M a l m g r e n 1952). No such polyphosphatase could be, however, detected in the wax moth larvae. There are two possibilities to be considered: either the existence in tissues of the wax moth larvae of a specific enzyme, able to hydrolyze the polyphosphate excreted by the larvae, or the presence of a non-specific phosphatase like alkaline or acid phosphatase, active against the polyphosphate of the excreta. This last possibility could be confirmed by the fact that the polyphosphate in question was hydrolyzed also by homogenates of some vertebrate tissues, able to decompose some micromolecular polyphosphates. M a t t e n h e i m e r (1951) was the only one who claimed to have proved that homogenates of the human and rat liver as well as of frog muscles could hydrolyze Graham's salt too. Thus it is obvious that the problem of polyphosphatases of animals has not yet been solved.

SUMMARY

1. The nature of the labile phosphorus fraction derived from the excreta of the waxmoth *(Galleria mellonella* L.) larvae was investigated. Pyrophosphate and some polyphosphate were found to be the main components of this fraction. Pyrophosphate was identified by means of the chromatographic analysis of the manganese precipitate from the water extract and by the way it was split by a very specific pyrophoshatase, obtained from the wax moth larvae. Pyrophosphate precipitated from the water extract with manganese acetate at pH 5 amounted to a half of the labile phosphorus compounds of excreta. Polyphosphate was isolated from

the excreta as barium salts. When examined chromatographically and by means of dialysis it appeared to be a rather macromolecular polymer, very labile in the acid medium. The metachromatic reaction with toluidine blue of this polyphosphate was very pronounced. It was easily hydrolyzed enzymatically to orthophosphate by the wax moth larvae homogenates and by the homogenates of frog organs.

2. Optimum conditions for the enzymatic hydrolysis of the whole labile phosphorus fraction of excreta brought about by the wax moth larvae homogenates were established. The temperature and pH optima are 37° and 7.0—7.5 respectively. To become fully active the hydrolyzing enzyme requires the presence of bivalent cations, Mg^{++} and Co^{++} being most effective. Mn^{++} activates the hydrolysis at pH 6.6, becomes inhibitory at pH 5.5. Zn^{++}, Ag^{+}, and Hg^{++} are strong inhibitors of the hydrolysis.

3. An enzyme which hydrolyzes inorganic pyrophosphate to orthophosphate was isolated from the waxmoth larvae and partially purified. The maximum rate of pyrophosphate hydrolysis was obtained at 37° and at pH range 7.5—8.3. Magnesium ions are essential for the enzyme activity, the optimum concentration of Mg^{++} being 0.004 M, Co^{++} ions are able to activate the rate of hydrolysis in the presence of some amount of Mg^{++} ions only. The enzyme was inhibited by Mn^{++}, Ca^{++} and F^{-}. The optimum substrate concentration proved to be 0.004 M. At higher substrate concentrations a decrease in the rate of hydrolysis was observed. Michaelis constant, as calculated from the substrate concentration curve, was $2.3 \cdot 10^{-3}$ M. A high specifity of the purified enzyme against pyrophosphate has been observed. No orthophosphate was liberated, when the enzyme was incubated with the following substances: tripolyphosphate, trimetaphosphate, tetrametaphosphate, Graham's salt, yeast polyphosphate, polyphosphate of the wax moth excreta, adenosine triphosphate, and glycerophosphate.

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LIPID AND PHOSPHORUS METABOLISM DURING GROWTH OF THE SILKWORM (*BOMBYX MORI* L.)

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Studies in the biochemistry of the postembryonic development of insects have been usually much less concerned with the period of growth than with their metamorphosis. The processes of growth, which lead frequently to a considerable accumulation of substance within a short time are not yet sufficiently known.

The larval growth of the insects, as is well known, is divided into several cycles, the periods of true growth, during which animals feed, alternating with periods of moulting. During the feeding periods intense anabolic processes take place. They lead to a considerable increase of the mass of the body, whereas morphological changes are insignificant.

The periods of activity are followed in some species by periods of comparative rest (as in the wax moth), whereas in others rest amounts to a full quiescence (as in the silkworm). During moulting biochemical processes take place, which lead to a partial transformation of body constituents. The external manifestation of these processes is the casting off of the skin and the formation of a new cuticle.

Of the older works on the biochemistry of the growth of the silkworm Kellner's (1897) deserves special mention. He studied not only the chemical composition of the larvae, but also the constituents of their food. Among other things he demonstrated that lipids accumulated in the body of the larvae during the fifth instar derive partially from the transformation of other compounds contained in the mulberry leaves. Later biochemical studies of the growth of the silkworm are mainly concerned with the last larval instar. Not only the storage of reserve materials, mainly lipids and proteins, but also processes linked with the production of silk occur in this period. The work of Białaszewicz (1936, 1937 a) on nutrition and assimilation of food constituents and on changes in the chemical composition of larvae during their growth (lipids, carbohydrates and proteins) confirmed the previous conclusions of Kellner about the origin of lipids in the body of the silkworm larvae. Niemierko (1947) observed that during the last larval instar of silkworm the increase of content of lipids is accompanied by changes of their composition, and the rate of increase of the unsaturated fatty acids is greater than that of the saturated acids.

A number of authors have studied the respiratory metabolism of insects during the period of growth. Worthy of mention are the works of Luciani and Lo Monaco (1897) on the silkworm and of Białaszewicz (1933, 1937b) and Balsam (1933) on some species of Lepidoptera. According to Białaszewicz the intensity of metabolism during moulting, as well as during metamorphosis, decreases considerably as compared with the intensity of metabolism during feeding. The whole period of growth is marked by cyclic waves of intense and low energy metabolism. Białaszewicz assumed that „it is possible that the character of the chemical metabolism during moulting may be similar to the phenomena of metamorphosis". He did not, however, conduct any biochemical experiments to confirm this hypothesis. As was pointed out by Niemierko (1947), in spite of a strong decrease in the intensity of metabolic energy exchange during ecdysis as compared with periods of feeding, numerous and varied biochemical transformations are going on in the larvae during the moultings.

Investigations of the biochemistry of growth of waxmoth larvae have been conducted by Teissier (1931), by Niemierko and Cepelewicz (1950, composition of lipids) and Nie-

m i e r k o (1952, lipids, phospholipids and nucleic acids). The last work demonstrated that during the growth of the larvae neutral fats are accumulated, the percentage of phospholipids in dry substance remains approximately constant, while the content of nucleic acids decreases. These investigations did not, however, take into account the changes occuring during the moulting, because it is difficult to catch this moment in the development of *Galleria mellonella* larvae. The larvae of the silkworm are much better suited for this kind of experiment, because their periods of activity and of rest are clearly distinguishable.

The purpose of the present study was to investigate the metabolism of lipid and phosphorus compounds during the whole period

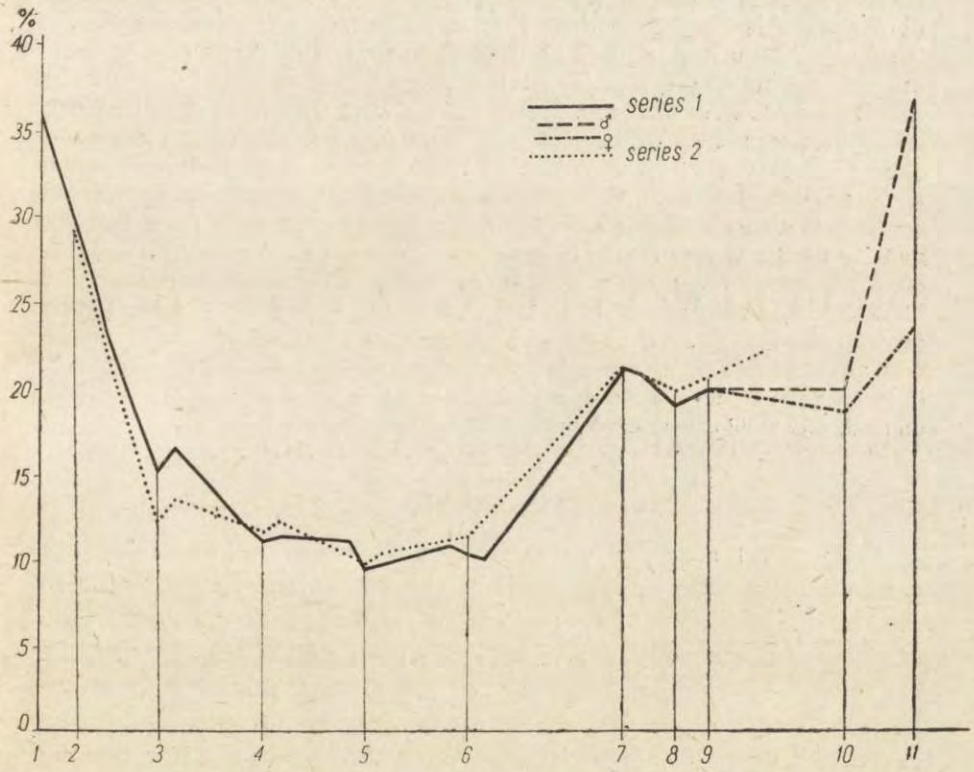

Fig. 1. Percentage of dry substance during the development of silkworm. 1 — diapause eggs, 2 — newly hatched larvae, 3 — first moulting, 4 — second moulting, 5 — third moulting, 6 — fourth moulting, 7 — cessation of feeding, 8 — larvae taken from the cocoon, 9 — one- day old pupae, 10 — ten- day old pupae, 11 — imagines, females and males

of growth of the silkworm. Special attention has been paid to periods of moulting. This work is completed by analyses of diapause eggs, as well as that of some selected moments of metamorphosis.

METHODS

The experiments were conducted on the yellow silkworm, Ascola race, under normal breeding conditions. The number of specimens used in each analysis depended on their size; from a single larva in the fifth larval instar up to several thousands newly hatched larvae or eggs. This corresponded to 2—4 g of fresh substance-weight. After the third ecdysis the experiments were made separately on males and females. Analyses were performed on diapause eggs, and on larvae immediately after hatching. During the whole period of growth the larvae were analysed one day before the expected moult, during the moulting, after each ecdysis before feeding, after cessation of feeding in fifth instar and lastly during spinning. During metamorphosis one-day-old and ten-day-old pupae, as well as adults before the excretion of sexual products were analysed. Measurements were performed simultaneously on 3 to 4 samples. In each sample the dry substance, lipids and some phosphorus compounds: phospholipids, nucleic acids and acid-soluble P-compounds were determined. The extraction of lipids and the fractionation of phosphorus compounds were performed according to Niemierko, Niemierko and Włodawer (1952); inorganic phosphorus and soluble P-compounds were determined by Lohmann's procedure. The lipid-P was determined after digesting with concentrated HNO_3; P in the RNA and DNA was estimated by method of Schmidt and Thannhauser (1945). The protein-P was not determined, as preliminary analyses have shown its content to be insignificant. P was determined by Fiske and Subba-Row (1925) method, but amidol was used instead of eukonogen.

RESULTS

Dry substance. Changes in the water and dry substance content during the development of the silkworm are presented in Fig. 1. The character of these changes confirms essentially the data of Kellner (1897) and Nakamura (1940). During the embryonic development one part of the organic matter is consumed and the dry substance, which amounts to about 36% of the diapause egg, drops to 28,5% in a newly hatched larva. After feeding has begun a distinct accumulation of water takes place together with a decrease of percentage of the solids. Towards the end of the second instar a balance between the accumulation of water and organic matter develops and it maintained up to the fifth instar. During this period the dry substance amounts to 10—11% of the body weight.

These results differ from those obtained by N a k a m u r a, who found that the maximum percentage of water is reached in the third instar and diminishes continually until the pupal instar. Our experiments showed, however, that only at the end of the fifth instar

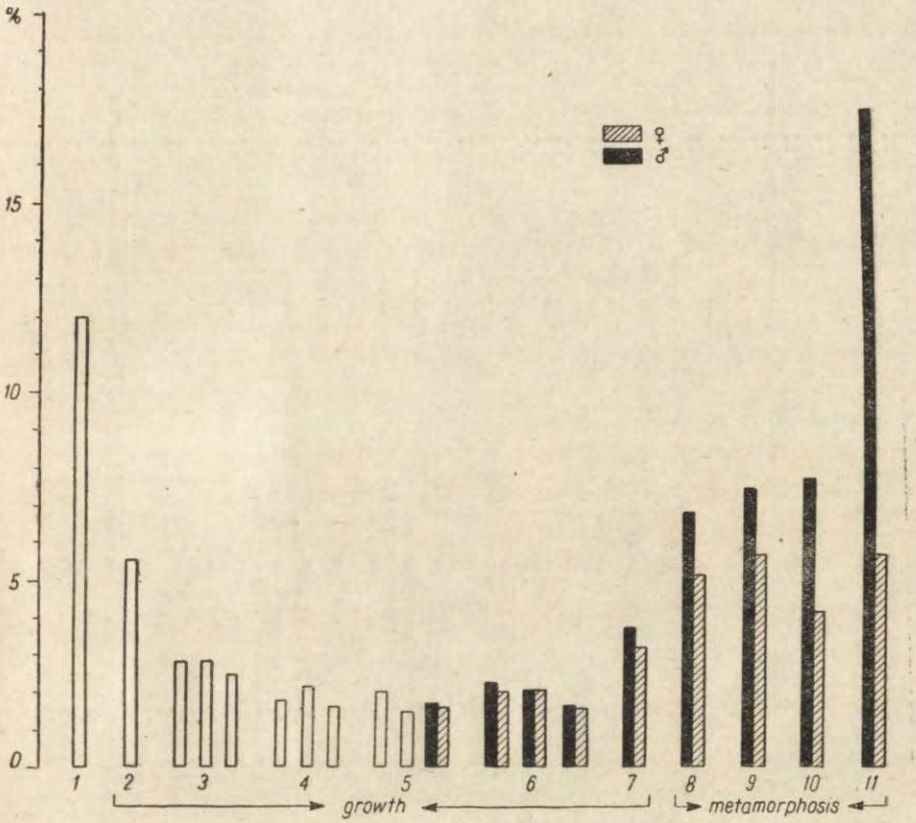

Fig. 2. Percentage of lipids in the fresh weight of silkworm during development. 1 — diapause eggs, 2 — newly hatched larvae, 3 first moulting, 4 — second moulting, 5 — third moulting, 6 — fourth moulting, 7 — cessation of feeding, 8 — larvae taken from the cocoon, 9 — one day old pupae, 10 — ten day old pupae, 11 —imagines

the increase of the solids is greater than that of the water. After cessation of feeding, the dry substance amounts to 21% of the body weight. This high content of the dry substance results from the accumulation of lipids and proteins on the one hand, and on the elimination of water before spinning on the other.

It has not been possible to establish any distinct changes in the content of water and dry substance during the moulting periods.

During metamorphosis the percentage of the dry substance of the analysed pupae did not change in comparison with larvae which commenced spinning. In adults, which have been examined after

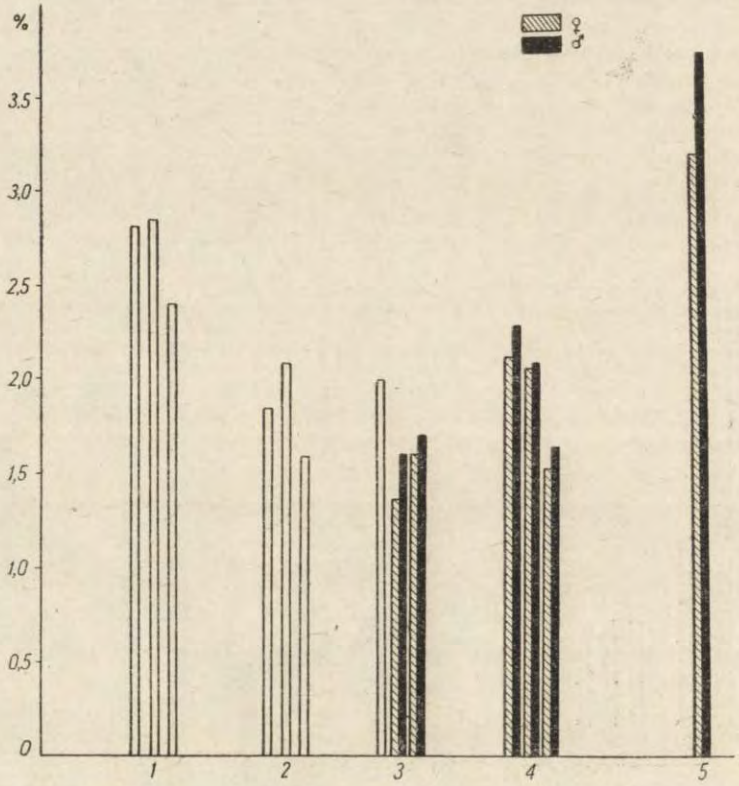

Fig. 2a — Percentage of lipids in the fresh weight of silkworm larvae during growth. 1 — first moulting, 2 — second moulting, 3 — third moulting, 4 — fourth moulting, 5 — cessation of feeding

excretion of meconium, the amount of dry substance was increased. Sexual differences in this respect became conspicuous, the females contained 24%, and the males — 36% of dry substance.

During spinning and metamorphosis part of the organic substances undergoes metabolic transformation: one-day-old pupae contain 40% and adults 25% of the dry substance found in larvae which ceased feeding.

L i p i d s. Changes in the content of lipids during development of the silkworm are presented in Fig. 2 and 2 a.

Lipids amount to 12% of the fresh mass of the diapause eggs, and 5.5% of newly hatched larvae. This means that a considerable amount of lipids is consumed during embryonic development. In the first and second instar the percentage of lipids decreases, (2.8%

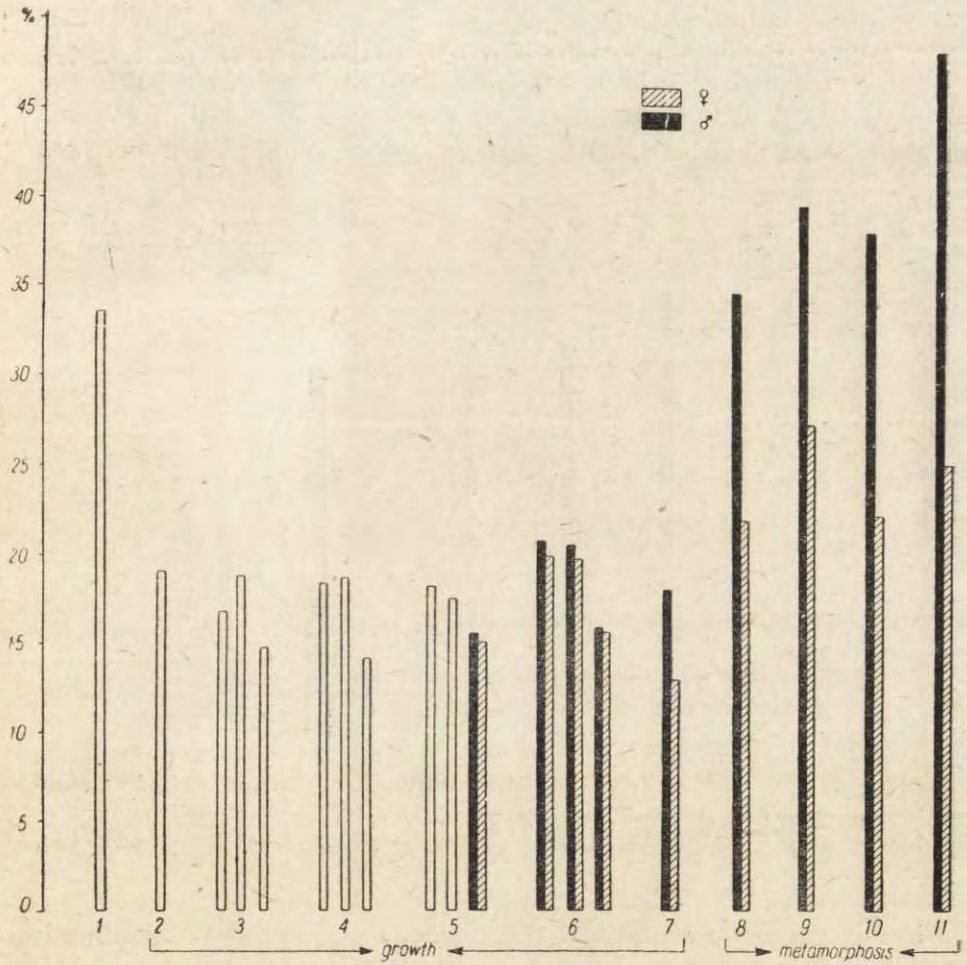

Fig .3. Percentage of lipids in the dry substance of silkworm during development. 1 — diapause eggs, 2 — newly hatched larvae, 3 — first moulting, 4 — second moulting, 5 — third moulting, 6 — fourth moulting, 7 — cessation of feeding, 8 — larvae taken from the cocoon, 9 — one- day old pupae, 10 — ten- day old pupae, — 11 imagines

before the first, 2,1% before the second moulting) in connection with the accumulation of water in the larval body. In the third and fourth instar the content of lipids remains almost constant (2.0%—2.2% of the fresh substance). Only in the last instar does the percentage of lipids in the fresh substance nearly double, reaching 3.8%. During every moulting period a decrease of 15—25% in the content of lipids takes place, as compared with the content before moulting. During spinning and pupation the content of lipids continues to increase, and the sex-conditioned differences to intensify. Female larvae taken from the cocoons contain 5.0% and males 6.8% of lipids. One-day old female pupae contain 5.8% and male — 4.7% of lipids. Differences between adults, when analysed

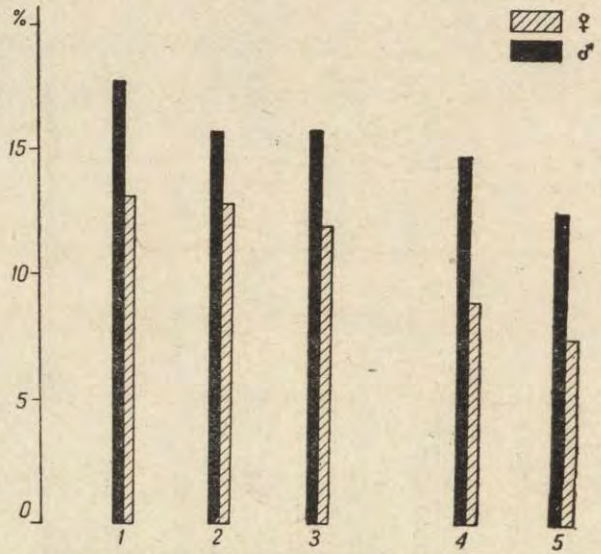

Fig. 4. Consumption of lipids by the silkworm after cessation of feeding (during spinning and metamorphosis). 1 — larvae after cessation of feeding, 2 — larvae taken from the cocoon, 3 — one day old pupae, 4 — ten day old pupae, 5 — imagines

before excretion of the sexual products are even more pronounced: the content of lipids in females amounts to 6%, in males — 17.5% of the fresh substance. Owing to the changes in the water content of the insect during the development, the changes of the lipids content in the dry substance and in the fresh weight proceed differently (Fig. 3). Lipids form about 34% of the dry substance of

the eggs, and 19% of the newly hatched larvae. During the four subsequent stages of growth the variations in the percentage of lipids are insignificant and lipids continue to form 18—20% of the dry substance.

The decrease of the percentage of lipids during each ecdysis amounts to 20—25% of the lipid content before the moulting period. In the fifth instar no changes in the percentage of lipids in males were observed, whereas females showed a small decrease. It appears, that the solid components of the body of males increase evenly in this period. Only during spinning and pupation does the chemical composition of the substance change. This change is asso-

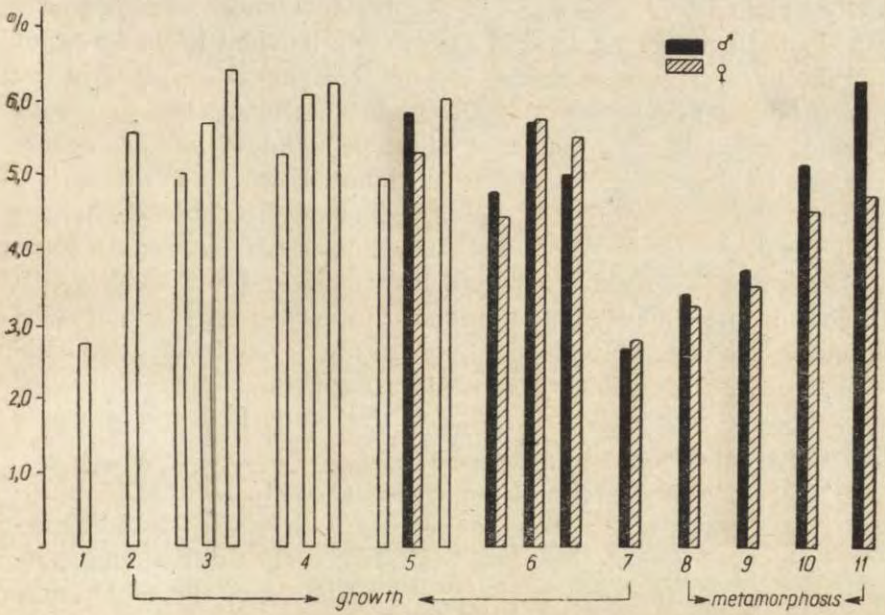

Fig. 5. Percentage of phospholipids in the dry substance of the silkworm during development. 1 — diapause eggs, 2 — newly hatched larvae, 3 — first moulting, 4 — second moulting, 5 — third moulting, 6 — fourth moulting, 7 — cessation of feeding, 8 — larvae taken from the cocoon, 9 — one day old pupae, 10 — ten day old pupae, 11 — imagines

ciated with the secretion of considerable amounts of silk. The percentage of lipids then rises sharply, amounting to 40% of the dry substance in one-day old male pupae and 27% in the female pupae. During metamorphosis the utilization of lipids in females

and males is different and male adults, just after emergence from the cocoons, contain 48%, whereas the females 25% of lipids in the dry substance.

The content of lipids in the adult insects compared with that in full grown larvae shows (Fig. 4), that the females have used from the commencement of spinning up to the time when they emerge from the cocoons 50% and the males 30% of the initial content of lipids.

P h o s p h o l i p i d s. Changes in the content of phospholipids during the development are shown in Fig. 5.

The content of phospholipids in eggs amounts to 3%, and in newly hatched larvae — 5.5% of the dry substance. Each egg contains in the average 7.0 μg. and each newly hatched larva 8.3 μg. of phospholipids. Thus a synthesis of phospholipids takes place in the course of the development of the embryo. During the four subsequent larval instars the level of phospholipids remains nearly constant at about 6% of the dry substance. During each moulting, although the total content of lipids decreases, the content of phospholipids continues to rise and the larva after ecdysis contain some 15—20% more phospholipids than before it. In the last instar, in which an intensified accumulation of organic components takes place, the percentage of phospholipids in the dry substance is smaller than in the previous stages. This proves, that the synthesis of other body components, including neutral fats, is more intensive than the synthesis of phospholipids.

During spinning and metamorphosis certain amounts of phospholipids are also utilized simultaneously with other lipids. A considerable utilization of phospholipids was observed during the spinning of cocoons (male larvae before pupation contain only some 50%, females — 60% of the total of phospholipids contained in larvae after cessation of feeding).

The period of metamorphosis was studied only incidentally, but it appears that a synthesis of phospholipids takes place in the second part of the metamorphosis (ten-day old pupae contain some 25% more phospholipids than those one-day old). During the whole period of metamorphosis the insects utilize about 35% of the phospholipids contained in the larvae after cessation of feeding.

Phospholipids form about 8% of all lipids in diapause eggs and 30% in newly hatched larvae. During feeding in subsequent stages

of growth phospholipids amount to 25—30% of all lipids and during moulting to some 40%. In the fifth instar because of the accumulation of neutral fats, the proportion of phospholipids in lipids decreases, so that when larvae cease feeding phospholipids constitute only 15% of all lipids. In larvae taken from the cocoon and one-day old pupae phospholipids amount to 10% of all lipids in males and to 13% in females. Approximate data concerning the period of metamorphosis suggests that the relative consumption of lipids during this period is greater than that of phospholipids. In 10-days old pupae and female adults phospholipids amount to 20% of all lipids, in male adults — to 12.5%. Thus the content of phospholipids in the total lipid is greater towards the end of metamorphosis than at its beginning.

Table I

The nucleic acid content during growth of the silkworm larvae (mean values expressed as mg nucleic acid phosphorus per 100 g dry substance)

Stage of growth	Series 1	Series 2
before I ecdysis	458	—
I „	438	405
after „ „	387	399
before II ecdysis	342	—
II „	328	366
after „ „	411	366
before III ecdysis	345	—
III „	371	374
after „ „	414	352
before IV ecdysis	303	—
IV „	298	246
after „ „	309	283

N u c l e i c a c i d s. Table I presents the content of nucleic acids in consecutive stages of growth. The results indicate that within each ecdysis there are no significant differences in the content of nucleic acids. Fig. 6 shows how the content of the total nucleic acids and that of DNA and RNA in the dry substance changes during the whole period of growth and during embryonic development.

The diagram shows, that during the embryonic development the content of nucleic acids increases sharply. Phosphorus of the nucleic acids amounts to 60 mg% of the dry substance in eggs and to 463 mg% in newly hatched larvae. Nucleic acid phosphorus (NAP) content in one egg is 0.18 μg, in one newly hatched larva — 0.63 μg. Thus during the embryonic development of the silkworm a synthesis of nucleic acids takes place, which is much more intensive than the synthesis of phospholipids described above.

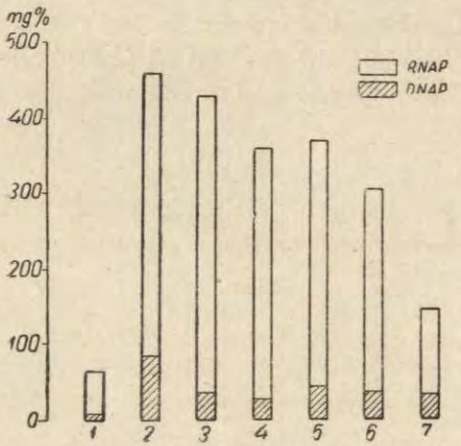

Fig. 6. Nucleic acids content in dry substance of silkworm larvae during growth. 1 — diapause eggs, 2 — newly hatched larvae, 3 — first moulting, 4 — second moulting, 5 — third moulting, 6 — fourth moulting, 7 — cessation of feeding

During the whole period of growth of the larvae a gradual decrease of the percentage of nucleic acids occurs. During the second and third ecdysis the level of nucleic acids in the dry substance remains constant; it decreases somewhat during the fourth; but with the end of the fifth instar in larvae which ceased feeding, the percentage of NAP is much lower and amounts to only one half of the fourth ecdysis value.

The metamorphosis was studied, as already mentioned, only in 1- and 10-days old pupae in one series and in 2- and 6-day old pupae in another.

Table II shows the amount of NAP during spinning and metamorphosis calculated in relation to the weight of the larvae before spinning. The results indicate, that the spinning of cocoons is associated with a strong decrease of the content of nucleic acids, which is even greater in males than in females. The decrease of the content of nucleic acids in the insects body appears also during the period when the adults emerge from their cocoons. Considerable utilization of nucleic acids during spinning has also been noticed in the wax moth (N i e m i e r k o and W o j t c z a k 1954).

Table II

The nucleic acid content during metamorphosis of silkworm. Mean values (expressed as mg of nucleic acid phosphorus per 100 g of „initial"* larval weight)

Series No	1		2	
Stage of development	♀	♂	♀	♂
Larvae after cessation of feeding	29,6	27,7	27,9	26,4
Larvae taken from cocoon	15,5	15,1	11,7	11,3
1-day old pupae	8,6	8,1	—	—
2 „ „ „	—	—	10,0	9,6
6 „ „ „	—	—	5,5	6,5
10 „ „ „	7,8	9,3	—	—
Imago	4,8	2,9	—	—

* „Initial" larval weight= the weight of larvae just after cessation of feeding.

Table III

The total acid soluble phosphorus and inorganic phosphorus content during development of silkworm (expressed as mg per 100 g of dry substance)

Stage of development	P total acid soluble mg%		P inorganic mg%		P inorganic % of total acid soluble	
diapause eggs	511		214		42	
newly hatched larvae	340		194		49	
before I ecdysis	758		196		26	
I ecdysis	797		185		23	
after „ „	796		324		41	
before II ecdysis	837		320		39	
II ecdysis	825		266		32	
after „ „	795		348		44	
before III ecdysis	755		256		34	
	♂	♀	♂	♀	♂	♀
III ecdysis	715	711	263	256	37	36
after „ „	745	789	357	336	48	43
before IV ecdysis	742	801	238	248	32	31
IV ecdysis	775	807	192	228	25	28
after „ „	915	860	306	286	33	33
larvae after cessation of feeding	216	225	58	71	27	32
larvae taken from cocoon	593	631	325	331	55	53
10 day old pupae	652	679	300	271	46	40
imago	246	301	111	130	45	43

Acid-soluble phosphorus compounds. The percentage of acid soluble P compounds, as shown in Table III, after decreasing during the embryonic development, increases considerably during the first instar, without any distinct changes during the remaining periods. A considerable decrease of the percentage of these constituents does not take place until the larvae cease feeding.

No differences have been found in the content of total soluble phosphorus compounds during ecdysis, when compared with the period of feeding; but the amount of inorganic P grows after each ecdysis. This occurs most probably at the expense of difficultly hydrolysable and nonhydrolysable P compounds. The easily hydrolysable compounds (phosphoarginine and ATP) vary only slightly in this period. Moreover, as the P of these compounds forms only a small part of the total soluble P content (about 10—12%) its

Table IV

The total phosphorus content during growth of silkworm (calculated from means of different P-fractions: acid soluble compounds, nucleic acids and phospholipids, mg per 100 g of dry weight)

Instar	I	II	III	IV
before ecdysis	1414	1410	1298	1265
ecdysis	1463	1434	1302	1316
after ecdysis	1426	1456	1413	1406

changes could not account for the increase of the inorganic phosphorus. The level of the inorganic P after ecdysis attains 40 and more per cent of the total soluble phosphorus (Table III). A high P inorganic percentage was also observed in newly hatched larvae and in larvae removed from cocoons. During metamorphosis the value of the soluble P is relatively greater than during the period of growth.

The total phosphorus has been calculated from the amounts found in each fraction (Table IV). The above data indicate that the percentage of the total phosphorus in the dry substance does not vary markedly during the growth of silkworm larvae.

Some biochemical processes appear to be common to embryonic development, to moulting periods and to certain stages of metamorphosis. They are synthesis of phospholipids, consumption of

lipids, and as known from other work from this laboratory (Z a ł u s k a, personal communication) hydrolysis of glycogen and formation of chitin.

DISCUSSION

The experiments reported here concern mainly the period of growth of the silkworm larvae. Although changes occuring during the embryonic development will be described later, a few incidental results obtained on diapause eggs as compared to those from newly hatched larvae bring out far reaching biochemical transformations, as for instance, the utilization of lipids, and the synthesis of phospholipids and nucleic acids. The consumption of lipids amounts to 60% of their total content in diapause eggs. Many authors who studied the embryonic period, have found that fats, and to a lesser extent carbohydrates, form the main source of energy of the developing embryo of the silkworm (T i c h o m i r o w 1885, F a r k a s 1904). Similar phenomena have been described also in several other insects (R u d o l f s 1926, in *Malacosoma americana,* S l i f e r 1930, 1932, and also N e e d h a m 1950, in *Melanoplus differentialis).* Not all lipid fractions are, however, utilized during the embryonic development. The comparison of the amount of phospholipids of the egg and of the newly hatched larva indicates that the embryo synthesizes the phospholipids (7.0 µg. in the egg and 8.3 µg. in the larva). According to the old data of T i c h o m i r o w (1885) the content of lecithin during the development of the silkworm egg increases by 70%.

The synthesis of nucleic acids in the embryo is much more intense than the synthesis of phospholipids. A newly hatched larva contains three and a half as many nucleic acids as the diapause egg. A similar phenomenon has been observed by L u and B o d i n e (1953) during the development of the *Melanoplus* eggs. It should be mentioned that the rate of increase of the DNA content is greater than that of RNA; the amount of DNA increase 8-fold, of RNA — fourfold.

The participation of each phosphoric fraction expressed in percentage of total P during life-history of the silkworm is shown in Fig. 7. In diapause eggs P of acid soluble compounds amounts to 75%, P of lipids — to 16%, P of nucleic acids — to 10% of the total phosphorus.

In young larvae the soluble phosphorus compounds fraction is much smaller and P of this fraction amounts only to about 30% of total phosphorus, whereas P of the nucleic acids forms more than 40%, and P of the phospholipids — 26% of the total P. The increased amounts of phospholipids and of nucleic acids are most probably synthetized at the expense of phosphorus from acid- soluble P-compounds as the content of the latter decreases sharply.

The inorganic P in per cent of the total P is smaller in young larvae than in the eggs. The increase of the acid insoluble phospho-

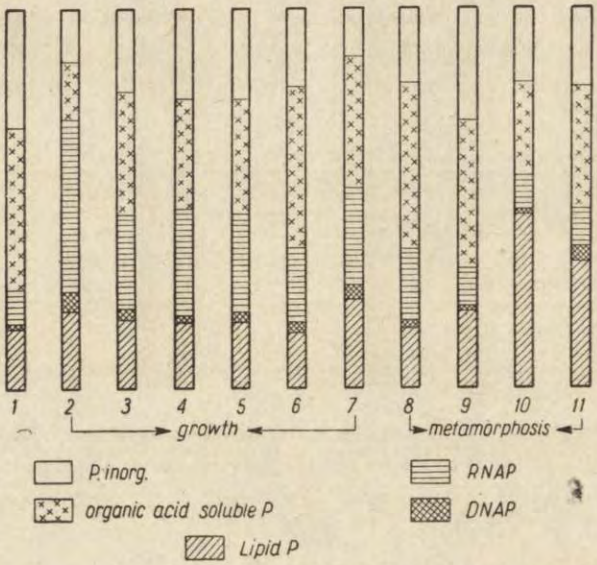

Fig. 7. Phosphorus fraction in per cent of total phosphorus during the development of silkworm. 1 — diapause eggs, 2 — newly hatched larvae, 3 — first moulting, 4 — second moulting, 5 — third moulting, 6 — fourth moulting, 7 — cessation of feeding, 8 — larvae taken from the cocoon, 9 — ten-day old pupae, 10 — male imagines, 11 — female imagines

rus compounds fraction at the expense of the acid soluble P compounds has also been found by K a r p i a k (1949) in the eggs of *Celerio euphorbiae* during their development.

Changes in the quantitative relations between various fractions of phosphorus compounds have also been observed in the first instar of the silkworm larvae. During the first moulting relative content of the lipid and nucleic acids phosphorus decreases, whereas that

of the acid-soluble compounds increases. More than half of the total P is contained in acid soluble compounds, about 19% in lipid and 28% in nucleic acids. During the second and the third moulting the quantitative relations between individual P fractions do not change. Only during the fourth moulting does a certain decrease of the NAP fraction become noticeable. As indicated in the experimental part, the percentage of nucleic acids in the dry substance decreases during the growth of the larvae. The same phenomenon has been observed also during the growth of *Galleria mellonella* larvae (N i e m i e r k o, 1952).

In larvae, after cessation of feeding, the percentage of NAP and lipid phosphorus increases, while the share of acid soluble phosphorus compounds decreases.

In larvae which spun cocoons, the relative quantity of the nucleic acids and lipid phosphorus fraction is smaller than in those which have stopped feeding, whereas quantity of the soluble P is greater. This leads to the assumption that during cocoon spinning, phospholipids and nucleic acids are utilised to a greater extent than the soluble phosphorus compounds. The content of P in silk is insignificant (L e w i ń s k a, unpublished data) and thus can not account for the decrease of the content of the nucleic acids and phospholipids in the larval body after spinning. Hydrolysis of phospholipids and nucleic acids is associated with the liberation of inorganic phosphates. The amount of the latter in larvae removed from the cocoon is relatively greater than in larvae before spinning.

The NAP in 1-day old pupae amounts to 10% of the total P, the lipid P — 20% and the P of acid soluble compounds — 70%. In adults distinct sex linked differences appear: in males 45% of the total P is the lipid phosphorus, in females this value amounts to over thirty, whereas the NAP fraction is greater in females.

Changes in content of the dry matter and of lipids during the whole period of growth of the silkworm, described here, coincide in general with those of K e l l n e r (1897) and B i a ł a s z e w i c z (1936, 1937). These authors, however, did not investigate the differences which occur between the moulting and the feeding periods. The present investigations concentrate especially on the biochemical changes during moulting periods. In spite of the interruption of feeding and of immobility this is a period of deep and varied biochemical transformations. The decrease of the content of

lipids which follows each ecdysis proves that during moulting 20—25% of fats is metabolized. Utilization of lipids during moulting was assumed by Białaszewicz (1937b) on the basis of changes of RQ. Immediately after ecdysis the RQ was 0.75, whereas during feeding this quotient increases. It seems that the disappearance of lipids during moulting is brought about not only by starvation during quiescence, but also by some simultaneous transformations occuring in this period.

The cyclic increase of the phospholipid content, simultaneously with the decrease of neutral fats seems to indicate that during moulting, as well as during the embryonic development, processes occur which lead to the synthesis of phospholipids. During moulting phospholipids constitute about 40% of the total lipids, during feeding, however, 30%. The increase of the relative content of the phospholipids in lipids during moulting of the silkworm has also been demonstrated by Niemierko and Kurowski (1954).

It is not easy to explain the meaning of the synthesis of phospholipids during moulting. It seems that no attempts have been made so far to elucidate the role of phospholipids in insects. In the vertebrates, on the other hand, it has frequently been found that the activity of a tissue or organ and the content of phospholipids are interdependent. We may quote Bloor (1934, 1940) for instance, who found that the degree of activity of various muscles and their content of phospholipids interlinked. Moreover, the content of phospholipids in organs which show cyclical changes of physiological activity has been frequently found to increase in periods of higher activity. Boyd (1935, 1938) observed this phenomenon in the ovaries of frogs and rabbits; Bloor et al. (1930) — in the corpus luteum of the sow; Weinhouse (1942) in the corpus luteum of women. Bloor (1930) expressed the view, that „the phospholipid content of a tissue in an expression or a measure of the extent and variety of the physiological activities of that tissues".

In our experiments the increase in phospholipids content was observed during moultings. It is possible that this increase appearing during decisive moments of transition from one instar to another is a reflexion of important biochemical processes.

Fundamental biochemical processes during moulting are evident from the results of the investigations of Niemierko, Kąkol, Załuska (1954), showing a decrease of the glycogen content to

1/5 of the value during the feeding period and a simultaneous increase of the chitin content (Z a ł u s k a). The studies of S m o l i n (1952) on *Antherea pernyi* demonstrated occurence of glycolysis during moulting.

Thus, the periods of moulting are characterized by a variety of important anabolic and catabolic processes: a part of lipids is oxidized, phospholipids are synthetized, a greater part of the glycogen hydrolyses, glycolysis takes place, inorganic phosphates are liberated from the phosphorylated esters, chitin is formed. The question arises how to explain and link together the all these facts, which show the intensity of biochemical processes, with the simultaneous decrease o f gas exchange observed by many authors (B i a ł a s z e w i c z, 1933 and others). It seems that this decrease in oxygen consumption is associated in the first place with absence of movements. During feeding the larvae move about and ingest great quantities of food which causes a great expense of energy, whereas during the period of moulting larvae remain motionless. In bugs which feed intermittently E d w a r d s (1953) observed a different course of oxygen consumption than that of silkworm. The intensity of respiratory exchange during the whole period of growth of *Oncopeltus fasciatus* undergoes, like that of the silkworm, a continuous decrease and periodical oscillations. But contrary to the silkworm, the maxima of oxygen consumption of the *Oncopeltus fasciatus* coincide with the periods of moulting. The intensity of respiratory exchange is thus greater during moulting than in the periods between moultings, that means, in the periods of true growth.

As E d w a r d s does not describe the details of his experiments, it is difficult to judge what is the real cause of increased oxygen consumption during the moulting of *Oncopeltus fasciatus*. It might be due to movements executed during casting off of the skin (as noticed H e l l e r, 1928, in larvae of *Celerio euphorbiae)* but it may be surmised also that it is a reflexion of profound biochemical transformations intimately connected with this stage of development. Another fact seems to substantiate the last supposition. Thus W o j t c z a k (1956) who studied the activity of respiratory enzymes on the pulp of larvae from various stages found an increased activity in the pulp from moulting larvae as compared with that from feeding larvae. Under such experimental conditions the overwhelming influence of the active movements on the total

respiratory exchanges does not interfere with the detection of the intimate biochemical transformations going on during moulting period.

The behaviour of respiratory enzymes in the pulp shows that different phases of the growth cycle have a repercussion even on a sub-cellular level. It seems justifiable therefore to assume that they influence also fundamental biochemical processes in living larvae.

SUMMARY

1. The water content, metabolism of lipids, nucleic acids and acid soluble phosphorus compounds during growth of silkworm larvae are described. Special attention has been paid to moulting periods.

2. Moulting periods are characterized by various and important biochemical processes. It was established, that during ecdysis about 20—25% of lipids are consumed, phospholipids are synthetized and after ecdysis inorganic phosphates are liberated from phosphorylated esters.

3. It seems that synthesis of phospholipids, consumption of lipids and liberation of inorganic phosphates from phosphorylated esters are common biochemical features of embryonic development, of moulting periods and of certain stages of metamorphosis.

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THE ROLE OF SULFHYDRYL GROUPS IN MUSCULAR CONTRACTION

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Recent experiments carried out on isolated muscle preparations (myofibrils, threads, protein preparations) point to the role of sulfhydryl groups in muscle physiology. It was shown that the ATP-ase properties of myosin become evident only if its sulfhydryl groups are intact (Singer and Barron 1944). Blocking of the — SH groups of actomyosin inhibits its ATP-ase activity and contraction properties (Goddeaux 1946, Korey 1950, Bailey, Perry 1947, Kuschinsky, Turba 1951, Portzehl 1952), prevents the interaction of myosin and actin (Bailey, Perry 1947) and, in the case of actin inhibits the transformation of G actin into F-actin (Feuer et al. 1948, Turba, Kuschinsky 1952).

In our previous experiments on isolated muscle (Brahms 1955) it was shown that muscular contraction and neuromuscular transmission are differently influenced by the degree of inhibition of — SH groups.

The importance of systematic studies in the role of — SH groups in muscular activity follows from recent findings which show the existence of high energy sulfur compounds in addition to the known

energy — rich phosphorus compounds. The purpose of the experiments reported here was to determine how contractile activities of the muscle influence its content of — SH groups.

Experiments were carried out on symmetrical pairs of the gastrocnemius or rectus abdominis muscles of the frog — *Rana esculenta*. One of the symmetrical mucles which was stimulated while the other acted as the control. At a given moment the muscles were suddenly immersed in liquid oxygen and pulverized. The powder thus obtained was subjected to analysis.

The total content of sulfhydryl groups in the muscles was determined according to F l e s c h and K u n (1950) using 1 — (4-chlormercuriphenylazo) naphtol — 2. Non — protein-SH groups were determined in filtrates following precipitation by 3% metaphosphoric acid (G r u n e r t and P h i l l i p s 1951).

In control experiments the liquid oxygen fixation procedure was compared with a mild cooling procedure (cooling period 90 min.; change of temperature from room temp. to 0°C and — 10°C), and prooved to be without significant influence on the the content of total — SH groups (Table 1), and on non-protein sulfhydryl groups.

Table I

The content of — SH groups in the gastrocnemius muscle. Mild cooling procedure compared with liquid oxygen fixation method (results expressed in μM/g wet wt.)

Mild cooling	Liquid oxygen	Difference in %
3.01	2.90	— 1.8
3.88	3.94	+ 2
3.32	3.26	— 1.7
3.34	3.34	0

The experiments were carried out with electrically stimulated muscles (tetanus) and with acetylcholine contracted muscles.

The action of the acetylcholine — Ringer solution (1 : 10000) on the rectus abdominis muscle causes a slow apparent tonical contracture. At the moment of maximal contraction (which usually occured 6 sec. after the beginning of the contraction) the muscles were fixed in liquid oxygen. Results (Table 2) show that the total content of — SH groups in frozen muscle at the moment of maximal contraction is greater than the total content of these groups in resting muscle. It should be noted that in experiments with autumn

Table II

The content of -SH groups in the rectus abdominis muscle in acetycholine contracture fixed at the moment of maximum contracture (in μM/g. wet wt.)

No	Date	Control	Contraction	Difference in %
1	11.VI	2.80	3.08	+ 8.87%
2	13.VI	2.12	2.28	+ 7.15%
3	8.VI	2.79	2.95	+ 5.3 %
4	28.XI	1.96	2.36	+17.2 %
5	30.XI	1.83	2.30	+20.5 %
6	2.XI	1.89	2.49	+24.1 %

Table III

The content of -SH groups in the gastrocnemius muscle frozen at the moment of maximum contraction (Ringer — acetylcholine perfusion, in μM/g. wet. wt.)

No	Date	Control	Contraction	Difference in %
1	21.V	2.42	2.79	+12.8
2	28.V	2.39	2.71	+12.0
3	30.V	2.41	2.61	+ 7.5
4	31.V	2.90	3.10	+ 6.3
5	17.VI	2.35	2.51	+ 6.5

Table IV

The content of — SH groups in the gastrocnemius muscle in tetanus at the moment of maximum contraction (in μM/g. wet wt.)

No	Date	Control	Tetanus	Difference in %
1	3.VII.	2.03	3.04	+33.3
2	12.VII.	2.27	3.03	+26.1
3	26.VII.	2.76	3.77	+27.6
4	28.VII.	3.08	3.30	+ 7.6
5	30.VII.	3.05	3.32	+ 8.2
6	10. IX.	2.02	2.96	+32.0

frogs (experiments No 4, 5, 6), the increase of — SH groups is greater than in analogus experiments with spring frogs.

Similar experiments were carried out with the gastrocnemius muscle perfused with acetylcholine solution according to Pisemski's method (P i s e m s k i j 1913). The results confirm previous findings with rectus abdominis (Table 3).

Table V

The content of — SH groups in the gastrocnemius muscle in tetanus contraction after reaching maximum contraction (in μM/g wet wt.)

No	Control	Tetanus	Difference in %
1	3.47	2.88	—17.2
2	3.41	2.75	—19.4
3	2.73	2.33	—13.3
4	4.07	3.67	— 9.0

Table VI

The content of — SH groups in the rectus abdominis muscles in acetylcholine contracture fixed at the moment of the maximum contracture after treatement with 2,4 DNP (in μM/g wet wt.)

No	DNP concentration (M)	Time of treatment (min)	Control	Contraction	Difference in %
1	5.10^{-4}	40	3.72	3.26	—12.4
2	10^{-3}	20	4.16	3.76	— 9.5
3	10^{-4}	30	2.97	2.69	— 9.7
4	10^{-4}	30	2.71	2.26	—19.4
5	10^{-4}	15	3.50	2.54	—27.6
6	10^{-4}	15	3.26	2.66	—18.8

Electrically stimulated gastrocnemius muscle (tetanus) fixed at maximal contraction shows a significant increase (reaching 1/3 of initial value) of — SH groups as compared with the resting muscle. Measurements were made about 0.5 sec. after the beginning of tetanus (Table 4).

The measurements made under the above conditions (cf. Table 4), but after the moment of maximal contracture (the stimula-

tion period in this experiment was longer about 1 sec.), show the decrease of total — SH groups in the muscles (Table 5).

The decrease of — SH groups with acetylcholine contracted rectus abdominis has been demonstrated with 10^{-4} — 10^{-3} M 2,4-dinitrophenol (DNP) treated muscles, at the moment of maximum contracture (Table 6). In these experiments both symmetrical muscles were treated with 2, 4-dinitrophenol.

In order to explain what kind of — SH groups participate in the described changes, investigations were conducted on the content of non — protein-SH groups. Determinations of the content of non — protein-SH groups in the rectus abdominis muscle were made during acetylcholine contracture by fixing muscle with liquid oxygen at various time intervals beginning with the moment of contraction. Results of the experiment (Table 7) show that at the moment maximum amplitude is reached, (usually 6 seconds after the commencement of contraction) no changes are observed in the content of non — protein-SH groups in a contracting muscle, as compared with a resting muscle.

Table VII

The content of non-protein — SH groups in the rectus abdominis muscle during acetylcholine contracture (in μM/g. wet wt.)

Time of contraction (secs.)	Control	Contraction	Difference in %
3	0.31	0.32	—
5	0.51	0.50	—
6	0.31	0.31	—
18	0.57	0.57	—
20	0.40	0.41	—
30	0.29	0.28	—

These experiments show that in the described changes in the total content of — SH groups during the process of muscle contraction, a fundamental role is played only by protein — SH groups.

The results prove that, during contraction of a sceletal muscle, a higher content of protein — SH groups is observed at the moment of maximal contraction, as compared to a muscle at rest.

This increase in the content of — SH groups takes place both in the case of slow, apparent tonical, acetylcholine contracture of the rectus abdominis and gastrocnemius muscle, as also in the case of tetanus contraction of the gastrocnemius muscle. The primary increase followed by an decrease in the content of — SH groups during somewhat longer lasting contraction seem to point to the fact that — SH groups take specific part in processes connected with muscle contraction.

The experiments in which the muscle was treated with 2, 4-dinitrophenol give tentative indications as to the character of the compounds with which protein — SH groups of the muscle interact. Existing data point to the fact that, 2, 4-dinitrophenol uncouples oxidative phosphorylation, activates ATP-ase, and causes splitting of ATP and creatine phosphate in the muscle (R o n z o n i, E h r e n f e s t 1936). It can therefore be assumed that changes in the content of protein — SH groups in the muscle treated with DNP take place under the influence of ATP and creatine phosphate splitting. The possibility of interaction between — SH groups of the muscle and ATP is proved by model experiments in which it was shown that in blocking or oxidizing these groups, loss of ATP-ase and contraction properties takes place (S i n g e r, B a r r o n 1944, B a i l e y, P e r r y 1947, G o d d e a u x 1946, K o r e y 1950, K u s c h i n s k y, T u r b a 1951, P o r t z e h l 1952). The mechanism of interaction between ATP and contracting muscle proteins remains heretofore unexplained. According to W e b e r and P o r t z e h l (1954) the action of ATP is twofold: in the first place it acts as a plasticizer, in the second as a contraction agent. In the action of ATP as a contracting agent, it is simultaneously split; in this case preservation of — SH protein groups is a necessary condition. The question arises as to whether explanation of the character of ATP interaction with myosin should not be sought for in phosphorylation of — SH myosin groups? In view of the fact that B u c h t a l and al. (1949) have demonstrated the existence of phosphorylated myosin, the observed increase of the content of — SH groups in muscle frozen at the moment of maximal contraction may be dependant on the splitting of the thiophosphoric bond in myosin. On the other hand splitting of the supposed thiophosphoric bond in myosin could explain the observation of F l e c k e n s t e i n and al. (1954) that liberation of

inorganic phosphorus from an unknown compound takes place during muscle contraction, while no changes in ATP content were observed.

SUMMARY

A study has been made of the sulfhydryl groups content of muscles in different stages of activity.

During acetylcholine contracture of the rectus abdominis and gastrocnemius muscle a higher content of sulfhydryl groups was observed at the moment of maximum contracture as compared with muscle at rest.

During tetanus of the gastrocnemius muscle, the content of -SH groups is greater at the moment of maximum contraction than the content of sulfhydryl groups in muscle at rest.

After the attainment of maximum contraction and somewhat longer lasting tetanus, the content of sulfhydryl groups decreases.

A decrease in the content of sulfhydryl groups at the moment of maximum contracture takes place in muscle treated with 2, 4 dinitrophenol.

The content of non-protein sulfhydryl groups is unchanged during initial periods of acetylcholine contracture.

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