KOMITET BIOCHEMICZNY I BIOFIZYCZNY
POLSKIEJ AKADEMII NAUK

COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS
POLISH ACADEMY OF SCIENCES

# ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. 16
No. 4

WARSZAWA 1969
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ADDRESS

Krakowskie Przedmieście 26/28, Warszawa 64.
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 1818+132. Ark. wyd. 8,25, ark. druk. 5.25+1,0 wkładek + +0,375 wklejek kred. Papier druk. sat. kl. III, 80 g. B1
Oddano do składania 1.VIII.1969. Podpisano do druku 19.XI.1969.
Druk ukończono w listopadzie 1969.

Zam. 663/69 P-72 Cena zł 25.—

Warszawska Drukarnia Naukowa — Warszawa, Śniadeckich 8

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

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# THE NEPHRIDIAL EXCRETION OF GUANINE, XANTHINE AND URIC ACID IN SLUGS (LIMACIDAE) AND SNAILS (HELICIDAE)

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1. Two species of Limacidae (*Limax maximus* and *Deroceras agreste*) and four species of Helicidae (*Cepaea nemoralis*, *Helicigona arbustorum*, *Cepaea vindobonensis* and *Helix pomatia*) were investigated during the feeding period. 2. The amounts of nephridial excreta eliminated by the animals varied from day to day. 3. The slugs, like snails, were found to be purinotelic. 4. The nephridial excreta of all land Gastropoda studied consist mainly of purines (72 - 91%) with varying ratios of uric acid to xanthine and to guanine. 5. The mean amount of purine-N eliminated per day, calculated per 100 g. of soft body weight, was for *H. pomatia* about 5.5 mg., with a maximum of about 9 mg. being excreted during the egg-laying period. The values for Limacidae at the egg-laying period were of the same order.

There have been practically no data concerning the form of nitrogen excretion in slugs, therefore a study has been undertaken to identify and determine the nitrogen compounds in nephridial excreta of two species of Limacidae: *Limax maximus* and *Deroceras agreste* during the feeding period. Since the latter slug feeds on burdock (*Arctium lappa*) leaves, its nitrogen excretion was compared with that of two snails, *Helix pomatia* and *Cepaea nemoralis*, which feed on the same plant. The other slug studied, *L. maximus*, lives under widely different ecological conditions: it lives in cellars, where the temperature does not fall below 0°, and feeds on potatoes, sometimes even in winter.

In view of the rather irregular elimination of the nephridial excreta by land Gastropoda, the average amounts per day of excreted purines and purine-N were determined.

## *Animals*

The land Gastropoda to be studied were kept in the laboratory at room temperature in large vessels provided with Petri dishes containing water; the vessels were kept away from direct sunlight.

*Helicidae.* Twenty one snails *Helix pomatia* (width and height of the shell 40 - 55 mm.) were collected near Zielona Góra (district of Wrocław, Silesia) in summer and were hibernated for 6 months in a cold room at 4°. In April they were trans-

ferred to the laboratory and fed with burdock leaves until incipient next hibernation.

Twenty snails *Cepaea nemoralis* L. (width of the shell 21 - 27 mm., height 17 - 20 mm.) were collected in a garden in Warsaw and kept for a few months; they were fed with burdock and garden lettuce leaves.

Forty snails *Cepaea vindobonensis* C. Pfr. (width of the shell 20 - 26 mm., height 17 - 24 mm.) were collected in the neighbourhood of Warsaw and kept during June. They were fed with garden lettuce and cauliflower leaves.

Forty snails *Helicigona arbustorum* L. (width of the shell 18 - 25 mm., height 15 - 27 mm.) were collected in the neighbourhood of Warsaw. They were kept during June and fed with garden lettuce and cauliflower leaves.

*Limacidae*. Ten slugs *Limax maximus* L. (length of the body 120 - 200 mm., width 20 mm.) were collected in a cellar and kept for a few months. They were fed with potatoes, and a burdock leaf was provided as shelter.

Seventeen slugs *Deroceras agreste* L. (earlier name: *Limax agrestis* L.) (length of the body 50 - 60 mm.) were collected in a garden in Warsaw and kept for a few weeks; they were fed with burdock leaves.

### *Elimination of nephridial excreta*

Both the snails and slugs studied deposit their nephridial excreta in the form of yellowish "nubs" which can be easily separated from faeces. The frequency of elimination of nephridial excreta by land Gastropoda has not so far been determined. Needham (1935) mentioned that *Helix pomatia* deposit the excreta about once a fortnight.

The nephridial excreta deposited on leaves and on walls of the vessel were collected, depending on their amount, either every day or at intervals of a few days. The collected excreta were air-dried and weighed. The amounts of excreta of *H. pomatia*, *L. maximus* and *D. agreste* collected from the whole groups of the animals are presented in Fig. 1. It is apparent that on some days large amounts of the excreta were deposited, whereas at other times these amounts were small for several consecutive days. These differences were more pronounced in *H. pomatia* and *L. maximus* than in *D. agreste*. When the total amount of the excreta was large, the size of the particular nubs was also greater, which seems to indicate that the voiding was not dependent on the amount of excreta accumulated in nephridium. There was no correlation between the total weight of the excreta and changes in temperature, atmospheric pressure or humidity of the air, but the greatest amounts of excreta were deposited after rainfalls although the animals, being indoors, were not exposed to the rain.

Dry weight of the individual nubs ranged in *Helix pomatia* from a few milligrams to as much as 55 mg. Twice the excreta of *H. pomatia* were collected in the moment of their voiding; the fresh weight was 63 and 23 mg., and after drying it was 43 and 14 mg., respectively; thus the content of water amounted to 32 - 39%.

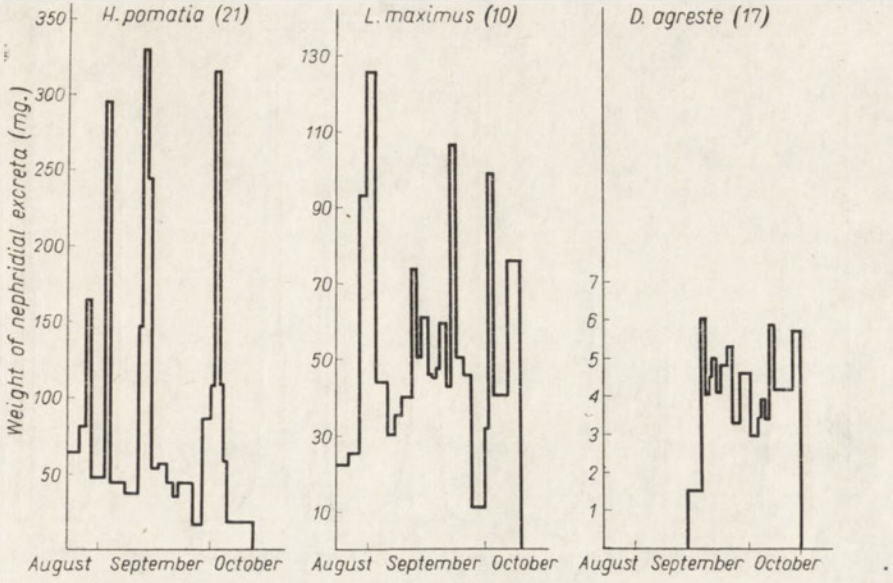


Fig. 1. The amount of nephridial excreta eliminated by *Helix pomatia*, *Limax maximus* and *Deroceras agreste*. The number of animals in each group is given in parentheses. The excreta were collected and air-dried.

*Purine composition of the nephridial excreta*

The air-dried excreta of *L. maximus*, *D. agreste* and *C. nemoralis*, 1 - 3 mg., were ground to a fine powder and dissolved in a minimum volume of water. The solution was not alkalized because this interfered with the subsequent chromatographic resolution. The aqueous solution was applied to a Dowex 1X8 (formate form) column and eluted first with water and then with a linear gradient of formic acid up to 0.4 M. Fractions of 4 ml. were collected and the extinction of each fraction examined at 260 mμ on a Unicam spectrophotometer. For each of the three species three peaks were obtained (Fig. 2). The fractions corresponding to each peak were pooled, evaporated until dry on a boiling-water bath, dissolved in 0.1 N-HCl and the absorption spectra examined. The spectra of peaks *I* were characteristic of guanine, peaks *II* of xanthine and peaks *III* of uric acid. The amounts of the respective purines were calculated from the molar extinction coefficients at suitable wavelengths (Dorough & Seaton, 1954).

The excreta from *C. vindobonensis* and *H. arbustorum* were analysed as described previously for *H. pomatia* (Jeżewska, Gorzkowski & Heller, 1963a). The air-dry excreta were dissolved in water with the addition of lithium carbonate, purines were separated by paper chromatography and purine nitrogen was estimated by the Kjeldahl method.

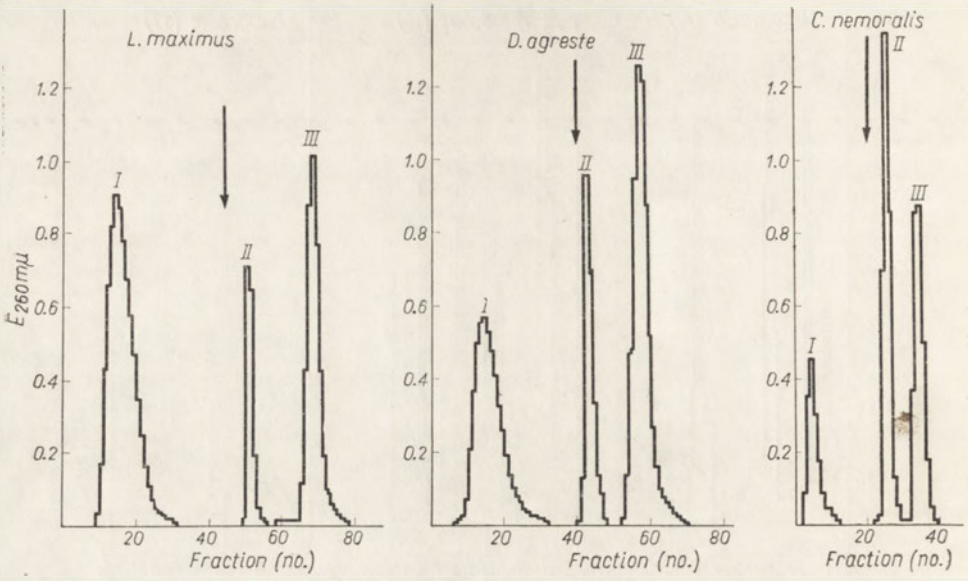


Fig. 2. Elution diagram of purines from nephridial excreta of *Limax maximus*, *Deroceras agreste* and *Cepaea nemoralis*, on Dowex 1X8 (25×1 cm.) column, formate form (200 - 400 mesh). The elution was first with water, then at the point indicated by arrow, 0.4 N-formic acid was applied. Fractions of 4 ml. were collected. *I*, Guanine; *II*, xanthine; *III*, uric acid.

A preliminary communication was presented at the Ist Polish Congress of Biochemistry (Jeżewska, Gorzkowski & Porembska, 1963).

The results of purine estimation in the excreta of the studied Gastropoda together with those for *H. pomatia* in spring and autumn, obtained previously (Jeżewska, Gorzkowski & Heller, 1963a,b), are presented in Table 1. All the land Gastropoda studied, both Limacidae and Helicidae, were found to be purinotelic, similarly as *Helix pomatia* (Jeżewska *et al.*, 1963a,b) and *Otala lactea* (Lee & Campbell, 1965). The dry nephridial excreta contained 72 - 91% of purines, lower values being found with slugs. Traces of ammonia were found in all excreta solutions on addition of the Nessler reagent.

The composition of purines in excreta differed from species to species. Uric acid accounted for about a half of total purines in Limacidae, whereas in Helicidae it varied from 34% to as much as 84%. Except for *H. pomatia* in autumn it was the greatest single purine component. The content of guanine was higher in Limacidae and in L. *maximus* it equalled that of uric acid. In Helicidae the content of guanine was low, and in *H. arbustorum* and *C. vindobonensis* that base has not been found at all. Xanthine, the more soluble purine, represented a minor component in the excreta of slugs but a large one in those of snails, in *H. pomatia* its content in autumn being as high as 55% of total purines. It should be noted that individual variations might occur in the composition of purines in nephridial excreta. As

Table 1

*Purines in nephridial excreta of some Helicidae and Limacidae species*

| | μmoles/100 mg. of dry excreta | | | | % of total purines | | | Total purines (mg./100 mg. of dry excreta) |
|---|---|---|---|---|---|---|---|---|
| | uric acid | xanthine | guanine | sum | uric acid | xanthine | guanine | |
| Limacidae | | | | | | | | |
| *Limax maximus* | | | | | | | | |
| September-October | 206 | 35 | 220 | 461 | 45 | 7 | 47 | 72 |
| *Deroceras agreste* | | | | | | | | |
| September-October | 277 | 70 | 124 | 471 | 58 | 15 | 26 | 77 |
| Helicidae | | | | | | | | |
| *Cepaea nemoralis* | | | | | | | | |
| July | 209 | 184 | 111 | 504 | 44 | 35 | 21 | 80 |
| *Helicigona arbustorum* | | | | | | | | |
| June | 369 | 137 | 0 | 506 | 73 | 27 | 0 | 84 |
| *Cepaea vindobonensis* | | | | | | | | |
| June | 466 | 86 | 0 | 552 | 84 | 15 | 0 | 91 |
| *Helix pomatia* | | | | | | | | |
| September-October* | 177 | 285 | 56 | 517 | 34 | 55 | 11 | 81 |
| April-July* | 261 | 176 | 86 | 523 | 49 | 34 | 16 | 88 |

* Data from Jeżewska *et al.* (1963b).

demonstrated in a previous work (Jeżewska *et al.*, 1963b) in *H. pomatia* the ratio of uric acid to xanthine may vary during active life, and moreover during hibernation guanine was absent from nephridia of some specimens.

### *Excretion of purine*-N

Little is known about the amount of nitrogen excreted by land Gastropoda. Hesse (1910) established that *H. pomatia* excretes 9.1 mg. of nitrogen per day per 100 g. soft body weight. Recently Speeg & Campbell (1968) in one-day experiments determined the excretory capacity for nitrogen in active *Otala lactea* by measuring the rate of [l-$^{14}$C]glycine incorporation into purines accumulated in nephridia. These data, however, give no information concerning the possible changes in excretory capacity during the active period of snail's life. According to Hunter (1964) the snails do during the active period alternately feed and spend some time withdrawn into the shells and isolated from the environment by a transparent pellicle, but not all the specimens do so simultaneously and for the same time. These periods are dependent neither on the accessibility of water nor of food.

Taking into account these variations and the fact that the snails accumulate the nephridial excreta and eliminate them at irregular intervals, it was thought

advantageous to collect the excreta from a group of snails over a prolonged period and to calculate the average amount of purine nitrogen excreted from haemolymph to nephridia per day.

The mean purine nitrogen elimination in *Helix pomatia* and in two species of slugs is presented in Tables 2 and 3. Speeg & Campbell (1968) expressed their results

Table 2

*Nephridial excretion of purine*-N *in* Helix pomatia

Nephridial excreta of 21 snails were collected.

| Period of excreta collection | Total dry weight of excreta collected (mg.) | Mean weight of one individual without shell* (g.) | Purine-N | |
|---|---|---|---|---|
| | | | mg./individual/day | mg./100 g. of body weight without shell*/day |
| 25.IV - 30.IV | 536 | 18.5 | 1.3 \| 0.5** | 7.0 \| 2.7** |
| 1.V - 31.V | 2857 | 18.5 | 1.3 \| 0.5** | 7.0 \| 2.7** |
| 1.VI - 30.VI | 2175 | 20.0 | 1.0 | 5.0 |
| 1.VII - 17.VII | 2164 | 20.0 | 1.8 | 9.0 |
| 25.VIII - 31.VIII | 616 | 22.0 | 1.3 | 5.9 |
| 1.IX - 30.IX | 2184 | 19.3 | 1.0 | 5.2 |
| 1.X - 11.X | 776 | 19.3 | 1.0 | 5.2 |
| Mean value for eighteen weeks | | | 1.2 1.0** | 6.7 5.5** |

* The shell accounts for about 1/4 to 1/5 of total weight of the animal.

** After subtracting purine nitrogen accumulated during the hibernation and eliminated after this period (about 31 mg. N per one individual).

Table 3

*Nephridial excretion of purine*-N *in slugs*

| Period of excreta collection | Total dry weight of excreta collected (mg.) | Mean weight of one individual (g.) | Purine-N | |
|---|---|---|---|---|
| | | | mg./individual/day | mg./100 g. of body weight/day |
| *Limax maximus* L. (10 slugs) | | | | |
| 24.VIII - 31.VIII | 330 | 10.6 | 1.2 | 11.0 |
| 1.IX - 30.IX | 1599 | 10.6 | 1.5 | 13.7 |
| 1.X - 11.X | 545 | 12.1 | 1.4 | 11.6 |
| Mean value for seven weeks | | | 1.4 | 12.3 |
| *Deroceras agreste* L. (17 slugs) | | | | |
| 11.IX - 11.X | 116 | 0.83 | 0.066 | 8.4 |

concerning the excretory potential for purine nitrogen in *Otala lactea* per gram total body weight. As the shell accounts for 50 - 60% of the total weight of *O. lactea*, this reduced the differences in the state of hydration of the animals. However, the relative shell weight varies widely from species to species, being about 30% in *Pila virens* (Meenakshi, 1958), and 20 - 25% in *H. pomatia*, whereas the slugs have but vestigial shells. Therefore for comparing the values of nitrogen elimination, the results were expressed in milligrams of purine nitrogen per 100 g. of soft tissue weight. To reduce the error due to differences in hydration, the animals were given free access to water. The data on nitrogen excretion (Tables 2 and 3) were also calculated per one individual, as this seems to characterize better the changes in excretion occurring during the active period.

The snails after awakening excrete the purines accumulated during hibernation. It has been found by Jeżewska *et al.* (1963b) that in *H. pomatia* the amount of accumulated purine nitrogen is about 31 mg. per individual. If this value is subtracted from the amount of nitrogen excreted by the snails from 25th of April till 31st of May (Table 2), the elimination at this time of the non-accumulated nitrogen would amount only to 0.5 mg. per day. The elimination of nitrogen increased with time, and in the first half of July, at the egg-laying period, it was the highest; then it decreased till the beginning of hibernation.

*L. maximus* and *D. agreste* were investigated in autumn at the time they lay eggs; the mean nitrogen excretion per 100 g. body weight in these slugs was of the same order as in *H. pomatia* in July.

The accumulation of purine nitrogen in nephridia of *H. pomatia* during 5 1/2 months of hibernation was 31 mg. N per individual, i.e. about 0.2 mg. N per individual per day and 1.2 mg. N per 100 g. soft tissue weight per day (Jeżewska *et al.*, 1963b), which is about five times less than the mean amount of nitrogen eliminated during the feeding period, 1.0 mg. and 5.5 mg. N, respectively. The obtained values are in good agreement with those reported by Speeg & Campbell (1968) for *Otala lactea* (Helicidae): 1.94 mg. N per 100 g. soft tissue weight during hibernation and 7.82 mg. N during the feeding period.

It is difficult to interpret the differences found in the rate of nitrogen excretion in *Helix pomatia*, because there are no data concerning the nitrogen balance in land Gastropoda. It is probable that following hibernation *H. pomatia* replaces the proteins lost in winter, and before hibernation stores them. So the small excretion in those periods of time would be related to the positive nitrogen balance of the animal. On the other hand, the observed variations in nitrogen excretion could reflect real changes in the rate of protein metabolism.

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My thanks are due to Mgr. Grzegorz Soszka from the Department of Hydrobiology of the Warsaw University for the taxonomic classification of slugs and snails used, and to the Institute of Hydrology and Meteorology in Warsaw for the data concerning temperature, atmospheric pressure, humidity and rainfalls in the period from April to October, 1968.

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## WYDALANIE GUANINY, KSANTYNY I KWASU MOCZOWEGO Z NEFRIDIUM U ŚLIMAKÓW LĄDOWYCH Z RODZINY LIMACIDAE I HELICIDAE

### Streszczenie

1. Wydalanie azotowe badano u dwóch gatunków z rodziny Limacidae (*Limax maximus* i *Deroceras agreste*) oraz czterech gatunków z rodziny Helicidae (*Cepaea nemoralis*, *Helicigona arbustorum*, *Cepaea vindobonensis* i *Helix pomatia*) podczas okresu żerowania.

2. Dobowe ilości wydalin z nefridium są zmienne.

3. Zarówno Limacidae, jak i Helicidae są purynoteliczne.

4. U badanych lądowych Gastropoda wydaliny z nefridium składają się głównie z puryn (72 - 91%). Stosunek kwas moczowy:ksantyna:guanina jest różny u różnych gatunków.

5. U *H. pomatia* ilość azotu puryn wydalanych w ciągu doby, w przeliczeniu na 100 g. masy ciała bez skorupy, wynosi średnio około 5,5 mg., a w okresie składania jaj zwiększa się do około 9 mg. U Limacidae wydalanie azotowe w okresie składania jaj jest tego samego rzędu.

Received 11 February, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

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# ACTIVITY OF THE PHENYLALANINE HYDROXYLATING SYSTEM IN LIVER OF NEWBORN, SUCKLING AND ADULT RATS

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1. In liver extracts, the activity of phenylalanine hydroxylation was the lowest in newborn animals, being 25% of the activity found in adult rats; this difference was also observed in experiments with cofactor added. 2. $K_m$ values for phenylalanine, and inhibition by excess of substrate were the same in all age groups, whereas maximum velocities, $V_{max}$, were different. 3. Preincubation of liver extracts from young animals resulted in inactivation of both the endogenous and the added cofactor; in liver of sucklings the inactivation was complete. With extracts from adult animals, the effect of preincubation was much smaller.

The enzymic system hydroxylating phenylalanine in mammalian liver is composed of two enzymes: (1), phenylalanine 4-hydroxylase (EC 1.14.3.1) which catalyses the reaction:

$$\text{phenylalanine} + \text{tetrahydropteridine} + O_2 \rightarrow \text{tyrosine} + \text{dihydropteridine} + H_2O$$

and (2), a dihydropteridine reductase which catalyses the generation of tetrahydropteridine by the reduced NAD(P):

$$\text{dihydropteridine} + \text{NAD(P)}H_2 \rightarrow \text{tetrahydropteridine} + \text{NAD(P)}.$$

Kaufman (1963) has demonstrated that although various pteridine derivatives, including folic acid, may act as a cofactor in phenylalanine hydroxylation, the natural pteridine present in rat liver is the 2-amino-4-hydroxy-6(1,2-dihydroxypropyl)-5,6 or 7,8-dihydropteridine. The biopteridine is synthesized by the rat (Pabst & Rembold, 1966) but it is not known whether it is synthesized in man.

Menkes & Avery (1963) in 25% of the 71 premature human infants studied, observed transient hyperphenylalaninaemia which appeared on the third day of life and reached a maximum (5 - 20 mg.%) during the second week. Simultaneously, the phenylalanine tolerance test became abnormal. The authors interpreted these observations as indicating that on the first and second day of life the infants are able to metabolize phenylalanine in a normal manner but subsequently lose this ability. Also in full-term infants in the first months of life, a transient hyperphenylalaninaemia (20 - 60 mg.%) not accompanied by phenylketonuria, was observed (LaDu, Howell, Michael & Sober, 1963; Kleinman, Twiss & Day, 1966; and other

authors). It has not been elucidated whether this hyperphenylalaninaemia is due to the impairment of 4-hydroxylase, like in the genetic disease phenylketonuria, or to a decrease of the amount of biopteridine or of dihydropteridine reductase.

In experiments *in vitro* Kenney, Reem & Kretchmer (1958) and Kenney & Kretchmer (1959) found negligible phenylalanine hydroxylation in liver of foetal and newborn rats, which they ascribed to the absence of 4-hydroxylase. On the other hand, Freedland, Krakowski & Waisman (1962) observed in newborn rat liver 25% of the activity found in adult rat liver. Brenneman & Kaufman (1965), in studies on rabbit, guinea pig, chicken and various strains of rats, found lower phenylalanine hydroxylation in livers of newborn animals than in adults; on addition of the cofactor, the activity increased to adult level. Brenneman & Kaufman considered the low activity of the system in newborn animals to be due to a deficiency of the cofactor and, probably, dihydropteridine reductase.

To reinvestigate the problem of changes occurring in the phenylalanine hydroxylating system during development, experiments were performed on liver extracts from newborn, suckling and adult rats. In the present paper the obtained kinetic data and the effect of biopteridine are reported.

## MATERIALS AND METHODS

*Reagents.* L-Phenylalanine and $NADH_2$ were products of Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A.); L-tyrosine was from British Drug Houses (Poole, Dorset, England); NAD from Boehringer und Soehne GmbH (Mannheim, German Federal Republic); $\alpha$-nitroso-$\beta$-naphthol from Merck (Darmstadt, G.F.R.); other reagents were analytical grade, supplied by Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland). L-[U-$^{14}$C]Tyrosine was a product of the Radiochemical Centre (Amersham, Bucks., England).

*Animals.* Newborn (less than 18 hr. old, mostly 2 - 4 hr. old and unfed) and suckling (5 - 8 days old) white rats of both sexes, and adult male rats (2 - 3 months old) were used for experiments. Adult animals were fed *ad libitum* with a mixed diet.

*Liver extract.* The animals were stunned, killed by decapitation and bled; then the liver was isolated and chilled on ice. All further steps of the procedure were carried out at 0 - 2°. The liver from adult animals was passed through a fine sieve and homogenized in a glass Potter-type homogenizer with 2 volumes of 1.1% KCl. Livers from newborn and suckling rats from one or more litters were pooled and homogenized. The homogenate was centrifuged for 60 min. at 13 000 *g*, then a small layer of fat was removed, and the supernatant, further referred to as the extract, was immediately used.

*Phenylalanine hydroxylation assay.* The standard incubation mixture was prepared after Udenfriend & Cooper (1952); it contained in 1 ml.: 3 µmoles of L-phenylalanine, 0.44 µmole of NAD, 4.0 µmoles of nicotinamide, 0.4 ml. of the liver extract equivalent to 0.132 g. of fresh liver tissue, and 6 µmoles of Na,K-phosphate buffer,

pH 7.0. The incubation was carried out at 37° in air with shaking. The reaction was stopped by adding 0.5 ml. of 5% trichloroacetic acid, and in the supernatant the tyrosine was estimated according to Ceriotti & Spandrio (1957). The control sample was deproteinized before incubation. The enzyme activity was expressed as the amount of tyrosine formed per 1 g. of liver.

*Experiments with tyrosine as substrate.* In the standard incubation mixture phenylalanine was replaced by 0.55 μmole of L-tyrosine alone or with 0.015 μmole of L-[U-$^{14}$C]tyrosine (180 mμc). The radioactivity was measured using the scintillation fluid of Bray (1960) in a type LL 1 liquid scintillation counter (Biuro Urządzeń Techniki Jądrowej, Warszawa, Poland).

*Protein determination* was carried out by the tannin method of Mejbaum-Katzenellenbogen (1955).

*Preparation of the natural cofactor of phenylalanine hydroxylase.* The cofactor was obtained according to Brenneman & Kaufman (1965) by boiling for 2 min. the homogenate from adult rat liver (1 g. of tissue and 2 ml. of glass-distilled water). The homogenate was then rapidly cooled and centrifuged at 13 000 *g* for 60 min., and the supernatant used as the source of biopteridine.

## RESULTS

The amount of protein extracted from the liver with 1.1% KCl varied with the age of the animals but the differences were not statistically significant (Table 1). From 1 g. of tissue, 75±10.4, 90±8.9 and 105±8.5 mg. of protein was extracted from newborn, suckling and adult rats, respectively.

Under standard conditions, with 3 mM-phenylalanine, the rate of tyrosine formation for all age groups was linear with the amount of extract to at least 0.5 ml. per sample. For experiments, 0.4 ml. of the extract was used, which corresponded to 0.132 g. of liver tissue.

Table 1

*Age of rats, body weight, liver weight, and amount of protein extracted from the liver*

Mean values, ± S.D., are given.

| Age of rats | No. of determinations | Body wt. (g.) | Liver wt. (g.) | Protein extracted from 1 g. of liver (mg.) | Protein in extract (mg./ml.) |
|---|---|---|---|---|---|
| Newborn (1 - 18 hr. old) | 11 | 5.1± 0.75 | 0.24±0.055 | 75±10.4 | 25±3.7 |
| Suckling (5 - 8 days old) | 18 | 11 ± 2.7 | 0.3 ±0.06 | 90± 9.8 | 30±3.7 |
| Adult (2 - 3 months old) | 28 | 210 ±23 | 8.6 ±1.5 | 105± 8.5 | 35±2.8 |

The formation of tyrosine as a function of time presented in Fig. 1 shows that in liver extract of adult rats the amount of tyrosine formed increased during 30 min. of incubation, and in extracts of young animal livers only during 10 - 20 min. The highest activity was observed in adult rats (about 6.6 μmoles of tyrosine formed per 1 g. of liver), and much smaller activity, 1.6 and 1.8 μmoles, respectively, in liver extracts of newborn and suckling animals.

The time-course of the reaction was not changed when phenylalanine was applied at concentrations of 0.7 or 6.0 mM instead of 3 mM. The application of NADH instead of NAD or an increase in NAD concentration had also no effect.

The time-course of the reaction suggested that liver extracts from adult animals catabolized the tyrosine arising from phenylalanine. This was confirmed by the experiments in which tyrosine instead of phenylalanine was added to the incubation mixture. In samples containing the extract from adult animals, the tyrosine decreased during incubation, whereas in samples containing the extract from sucklings the

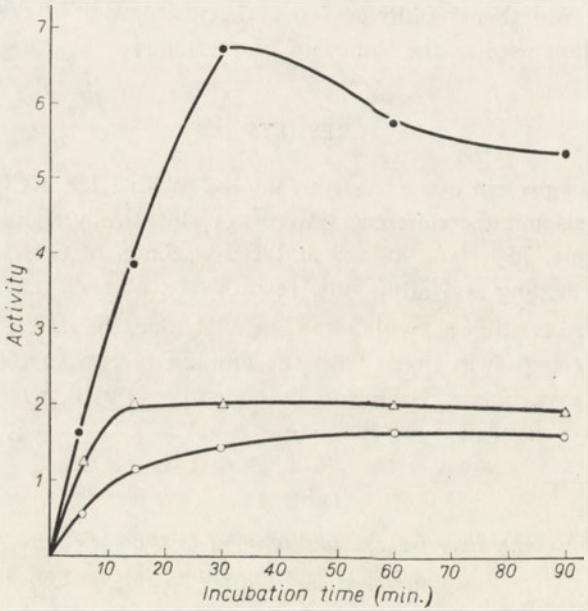


Fig. 1. Time-course of phenylalanine hydroxylation by liver extracts from (○), newborn, (△), suckling, and (●), adult rats. Standard incubation mixture as described in Methods. Activity is expressed in μmoles of tyrosine formed/g. of liver.

amount of tyrosine was unchanged (Fig. 2). In view of the poor solubility of tyrosine there was a possibility of tyrosine being adsorbed on the protein in adult animals, but not in young ones. This has been ruled out in experiments where [U-$^{14}$C]tyrosine was used and the radioactivity was determined both in the sedimented protein and in supernatant (Fig. 2). No radioactivity was found in the protein sediment in the samples with extracts from either young or adult rats. With extracts from young

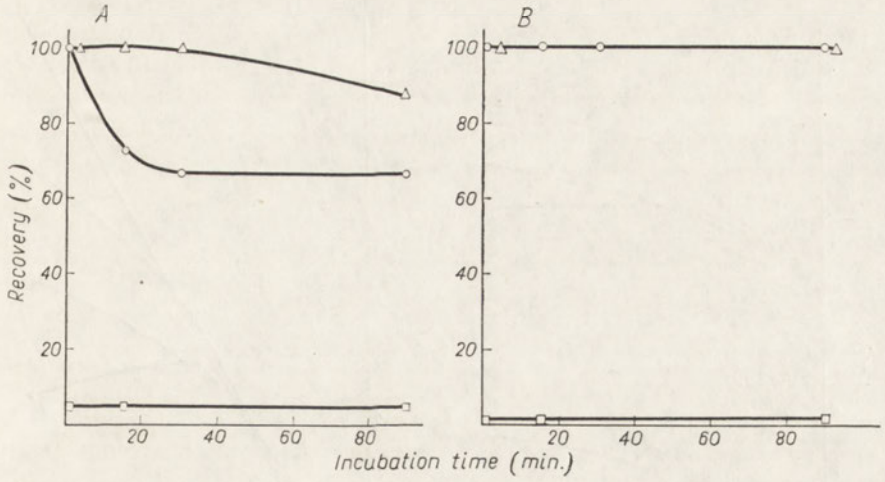


Fig. 2. Changes in tyrosine and radioactivity during incubation with liver extracts from (*A*), adult and (*B*), suckling rats. (○), Tyrosine content, and (△), radioactivity in deproteinized supernatant; (□), radioactivity in protein sedimented by trichloroacetic acid. The composition of the reaction mixture was the same as for the phenylalanine hydroxylation assay, except that tyrosine (0.55 μmole) and [U-$^{14}$C]tyrosine (0.015 μmole, 180 mμc) were added as substrate instead of phenylalanine.

animals, the total radioactivity added was recovered in the supernatant whereas with those of adult rats the radioactivity in the supernatant decreased with the time of incubation, indicating that the decarboxylation of tyrosine was involved.

It should be noted that when NAD was omitted from the incubation mixture, in the experiments with extracts from adult rats tyrosine disappeared almost completely within 90 min., and the radioactivity decreased to a greater extent than in the presence of NAD. The omission of NAD had no effect in the experiments with extracts from young animals, and no changes in tyrosine content and radioactivity in its absence were observed.

Due to some decomposition of tyrosine by the liver extracts from adult rats, the obtained values for phenylalanine hydroxylation were somewhat lowered. This, however, does not affect the interpretation of the results because the differences observed in enzymic activity between extracts from young and adult rats, were very large.

*The effect of the natural cofactor and benzaldehyde.* To determine which reaction of the phenylalanine hydroxylating system is the rate-limiting one, the effect of biopteridine and benzaldehyde were investigated. As biopteridine, the cofactor prepared according to Brenneman & Kaufman (1965) from adult liver homogenate deproteinized by heating, was applied. Benzaldehyde was used to regenerate $NADH_2$, as Mitoma (1956) has demonstrated that it acts as a NAD-reducing agent. In preliminary experiments it was found that under the standard conditions NAD was not decomposed, and that the addition of benzaldehyde did not influence

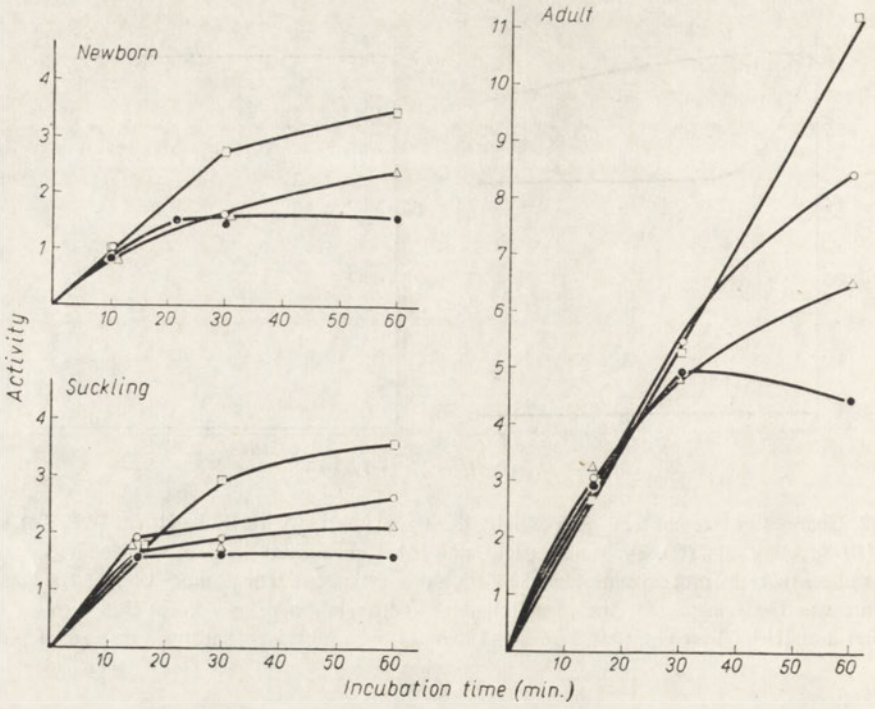


Fig. 3. The effect of the natural cofactor and benzaldehyde on phenylalanine hydroxylation by liver extracts from newborn, suckling and adult rats. Standard reaction mixtures as described in Methods, (●) without additions, and with the addition of: (△), 0.2 ml. of the cofactor preparation; (○), 3 μmoles of benzaldehyde; (□), cofactor and benzaldehyde. Activity is expressed in μmoles of tyrosine formed/g. of liver.

the decomposition of tyrosine by liver extracts from adult rats, nor did it interfere with the determination of tyrosine by the method of Ceriotti & Spandrio (1957).

The cofactor and benzaldehyde added at the beginning of incubation had no effect on the initial rate of the reaction; their influence became apparent only when the reaction ceased to proceed (Fig. 3). In all age groups of the animals, the cofactor added together with benzaldehyde had the greatest effect, causing a twofold increase in activity at 60 min. of incubation. Benzaldehyde alone gave a smaller effect, and the cofactor alone was still less active. The lack of effect of the cofactor and benzaldehyde on the initial rate of the reaction suggested that the extracts contained originally sufficient amounts both of biopteridine and of a system involved in its reduction to tetrahydropteridine. This points to 4-hydroxylase being the rate-limiting enzyme. At longer incubation time the reaction was arrested due, probably, to lack of tetrahydropteridine generation and partial inactivation or destruction of biopteridine.

An attempt was made to investigate the inactivation of biopteridine and the exhaustion of the NAD-reducing system by studying the effect of preincubation,

and of the addition of the cofactor and benzaldehyde before or after the preincubation. The liver extracts were preincubated with phosphate buffer, pH 7, and nicotinamide for 30 min. at 37°, then the reaction was initiated by the addition of phenylalanine and NAD, and after 15 min. the activity was determined (Table 2). In liver extracts from newborn rats, the preincubation caused a loss of 50% of the activity but the addition of the cofactor alone or with benzaldehyde restored 80% of the activity. In extracts from sucklings, the preincubation resulted in complete loss of the activity, which was restored to about 60% by the cofactor or cofactor together with benzaldehyde, but not by benzaldehyde alone. With extracts from adult rats, the preincubation lowered the activity by 50%; benzaldehyde restored 80% of the activity, whereas on addition of the cofactor alone or with benzaldehyde the activity was even higher than in control sample.

When the preincubation was carried out with the addition of the cofactor and/or benzaldehyde, the results obtained with liver extracts from newborn and suckling rats were the same as in the case of preincubation without these additions. With extracts from adult animals, the preincubation with the cofactor had little effect, with benzaldehyde it gave the same result as the addition of benzaldehyde after preincubation, and with both benzaldehyde and the cofactor the activity was almost completely restored.

These results indicate that the biopteridine present in liver extracts as well as the biopteridine added were inactivated during 30 min. preincubation; in extracts from sucklings this inactivation was complete, whereas in extracts from newborn and adult rats it was but partial.

Table 2

*The effect of preincubation, addition of natural cofactor and benzaldehyde on the hydroxylation of phenylalanine by liver extracts*

The liver extracts were preincubated for 30 min. at 37° with phosphate buffer, pH 7.0, and nicotinamide, then the reaction was started by the addition of phenylalanine and NAD, and after 15 min. the activity was determined. The cofactor (0.2 ml./sample) and benzaldehyde (3 μmoles) were added after or before preincubation, as indicated in the Table. The activity is expressed as μmoles of tyrosine formed/15 min./1 g. of wet liver wt. Results of a typical experiment are presented.

| Age of rats | Control | After preincubation | Addition | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | after preincubation | | | before preincubation | | |
| | | | Cofactor | Benzaldehyde | Cofactor and benzaldehyde | Cofactor | Benzaldehyde | Cofactor and benzaldehyde |
| Newborn | 1.1 | 0.5 | 0.8 | — | 0.7 | 0.55 | — | 0.5 |
| Suckling | 1.3 | 0.0 | 0.8 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 |
| Adult | 2.8 | 1.4 | 3.2 | 2.3 | 3.0 | 1.7 | 2.3 | 2.6 |

*Kinetic studies.* In all age groups of the animals, at the initial rate of the reaction, phenylalanine 4-hydroxylase was the rate-limiting enzyme, and the observed differences in activity could be due to either the different affinity of the enzyme for the substrate, or different amounts of the enzyme being present in liver.

The effect of phenylalanine concentration on enzyme activity was studied at the initial reaction rate (5 and 10 min. of incubation), that is at the time when the amount of tetrahydropteridine was sufficient. The results are shown in Fig. 4. The $K_m$ values determined by extrapolation of the Lineweaver-Burk plots, similarly as those obtained by the method of Hofstee (Dixon & Webb, 1966), were practically

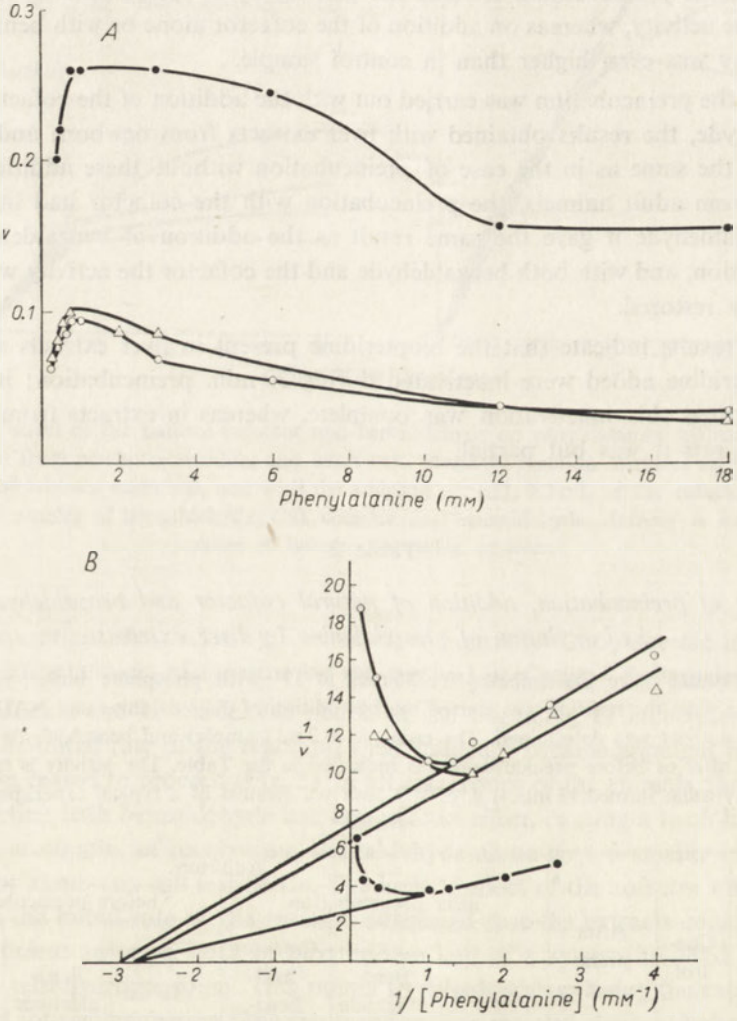


Fig. 4. *A*, Effect of substrate concentration on the initial rate of phenylalanine hydroxylation by liver extracts from (●), adult rats; (△), sucklings; and (○), newborn rats. Standard reaction mixtures were as described in Methods, with phenylalanine concentration ranging from 0.25 to 18 mM. Velocity, *v*, is expressed as μmoles of tyrosine formed/min./g. wet liver wt. *B*, Lineweaver-Burk double-reciprocal plots of the same data.

identical for all age groups of the animals. The average values obtained from 2 - 5 determinations were 0.28 mM for newborn, 0.32 for suckling and 0.33 for adult animals (Table 3). $K_m$ of the same order were reported by Mitoma (1956) and McCormic, Young & Woods (1965). The values of $V_{max}$ differed markedly and were, respectively, 0.11, 0.17 and 0.45 μmole of tyrosine formed/min./g. wet liver weight.

Table 3

*Michaelis constant for phenylalanine, maximum velocity, and substrate inhibition constant for the phenylalanine hydroxylating system in liver extracts from rats of different age*

The values are averages from the indicated number of determinations; in parentheses the limit values are given.

| Age of rats | No. of determinations | $K_m$ (mM) | $V_{max}$ (μmole/min./g. wet liver wt.) | $K_i$ (mM) |
|---|---|---|---|---|
| Newborn | 3 | 0.28 (0.22 - 0.33) | 0.11 (0.08 - 0.14) | 7.5 |
| Suckling | 2 | 0.32 (0.28 - 0.36) | 0.17 (0.16 - 0.18) | 8.2 |
| Adult | 5 | 0.33 (0.25 - 0.36) | 0.45 (0.4 - 0.6) | 11 |

The hydroxylation of phenylalanine is known to be inhibited by an excess of substrate (Udenfriend & Cooper, 1952; Mitoma, 1956; Bełżecka, Jakubiec & Pużyńska, 1967), which is also apparent from the results obtained with liver extracts from animals of all age groups studied (Fig. 4). To check whether substrate concentrations at which $K_m$ and $V_{max}$ values were determined, were within the range at which the Michaelis law is obeyed, the method of Dixon & Webb (1966) was applied. Double reciprocal plots of relative velocity $\left(\Phi = \frac{v}{V_{max}}\right)$ *versus* relative substrate concentration $\left(\sigma = \frac{s}{K_m}\right)$ gave a slope which intersected the axis of $1/\Phi$ at 1 and the axis of $1/\sigma$ at -1, indicating that the determinations at substrate concentration of 0.25 - 1.0 mM were correct.

The substrate inhibition constant, $K_i$, was calculated from the plot of reciprocal velocity *versus* phenylalanine concentration (Dixon & Webb, 1966). At initial reaction rate, $K_i$ values for different age groups (Fig. 5) were practically the same (8 - 11 mM) indicating that the susceptibility to an excess of substrate did not change during development.

The obtained mean values of $K_m$, $V_{max}$ and $K_i$ for the three age groups of the animals are presented in Table 3. It should be noted that these values were not altered by the addition of the cofactor and/or benzaldehyde to the reaction mixture.

## DISCUSSION

In the presented experiments, the activity of the phenylalanine hydroxylating system in liver extracts from newborn rats was much lower (about 1/4) than that found in adult rat liver. These results are in agreement with those of Freedland

*et al.* (1962) and Brenneman & Kaufman (1965). However, at variance with the observations of the latter authors, the addition of cofactor to the liver extract from newborn rats did not eliminate this age-dependent difference in activity.

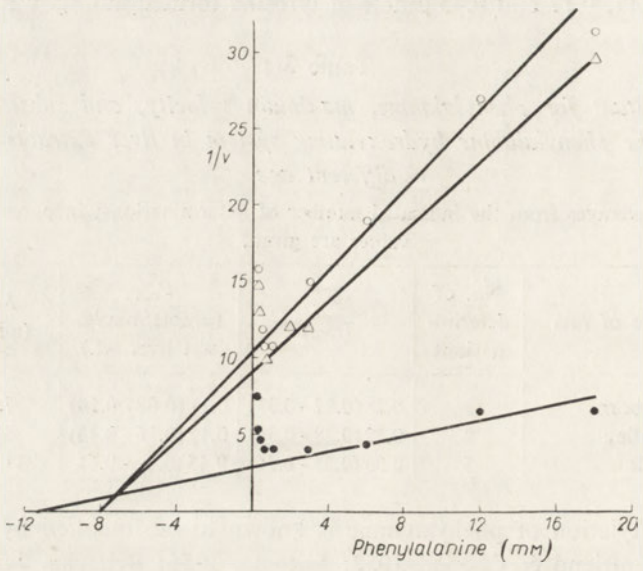


Fig. 5. Plots of reciprocal velocity *versus* phenylalanine concentration (data from Fig. 4A). Liver extract from (○), newborn, (△), suckling, and (●), adult rats. Velocity, *v*, is expressed as μmoles of tyrosine formed/min./g. wet liver wt.

The Michaelis constants for phenylalanine and susceptibility to excess of substrate were the same for all age groups studied. On the other hand, maximum velocities, $V_{max}$, were found to differ; in adult liver, $V_{max}$ was fourfold higher than for newborn rats, and 2.5-fold than that found in sucklings. These results seem to indicate that in newborn animals the amount of phenylalanine 4-hydroxylase is lower than in adults.

Differences were also found in the extent of inactivation of the endogenous and added cofactor during preincubation; the inactivation was much greater in liver extracts from young rats than from adults, and was especially pronounced in extracts from sucklings.

In newborn human infants the phenylalanine hydroxylating system is known to be operating, as shown by the normal blood phenylalanine concentration and normal phenylalanine tolerance test. Also in immature human foetus the formation of tyrosine from radioactive phenylalanine has been demonstrated (Ryan & Orr, 1966).

The transient hyperphenylalaninaemia, which appears relatively often in infants on the 2 - 3 day of life, could be due to rapid inactivation of biopteridine, similar to that observed in the present experiments in the liver of young rats. On the basis

of our results it is possible to suppose that also in infants the whole phenylalanine hydroxylating system is labile due both to the lower amount of phenylalanine 4-hydroxylase and the easy inactivation of biopteridine, which under adverse conditions may lead to transient hyperphenylalaninaemia.

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## HYDROKSYLACJA FENYLOALANINY W WĄTROBIE NOWORODKÓW, OSESKÓW I DOROSŁYCH SZCZURÓW

### Streszczenie

1. Badano hydroksylację fenyloalaniny w wyciągach z wątroby szczurów w różnym wieku. Aktywność w przeliczeniu na 1 g wątroby była najniższa u noworodków; stanowiła ona ok. 1/4 aktywności stwierdzonej u szczurów dorosłych. Różnice te występowały również po dodaniu kofaktora.

2. Stałe Michaelisa dla fenyloalaniny i stałe hamowania substratowego były takie same dla wszystkich grup wieku, natomiast wartości $V_{max}$ znacznie się różniły.

3. W wyciągach z wątroby młodych zwierząt, szczególnie osesków, podczas preinkubacji następowała inaktywacja kofaktora zarówno endogennego, jak i dodanego. U dorosłych efekt preinkubacji był dużo słabszy.

Received 9 March, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

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# POLYACRYLAMIDE-GEL ELECTROPHORESIS OF APPLE-SEED ENZYMES

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1. From acetone-dried powder of stratified apple seeds, proteins were extracted with tris-HCl buffer, pH 7.2, and separated by polyacrylamide-gel electrophoresis.
2. On the electrophoretograms, 30 bands were detected, corresponding to 12 enzymes, mainly oxidoreductases and hydrolases.

A period of secondary ripening is necessary before germination of apple seeds can occur. Ninety-day storage of the seeds at 4 - 5° at appropriate humidity, called stratification, ensures appropriate conditions for the secondary ripening. Duczmal (1963) demonstrated that some enzyme activities underwent changes during stratification of apple seeds, but the nature of the biochemical changes occurring at this time has not been extensively studied. For such studies, a simple technique enabling detection of numerous enzymes is necessary.

The present paper describes the application of polyacrylamide-gel electrophoresis for separation of proteins and detection of some oxidoreductases controlling the energy generating systems, and of hydrolases participating in degradation of reserve materials, in stratified apple seeds.

## MATERIAL AND METHODS

*Material.* For experiments, apple seeds cv. Antonovka, obtained from the Experimental Station of the Institute of Pomology in Sinołęka collected in 1967 and 1968, were used. The seeds were stratified under conditions described by Lewak & Smoleńska (1968). One hundred of the stratified seeds were washed free of sand, dried on filter paper, weighed, and acetone powder was prepared. The seeds were homogenized in 80 ml. of acetone (chilled to -20°) for 5 min. at 12 000 rev./min. in type 309 homogenizer (Unipan, Warszawa, Poland). The homogenate was filtered and the procedure was repeated twice using the same amount of cold acetone. The precipitate was dried for about 1 hr. at room temperature, placed in a vacuum desiccator and stored over KOH at 4° for 1 week up to 3 months.

*Chemicals.* The following reagents were used: Acrylamide, *N,N'*-methylene-bis-acrylamide, naphthalene Black 12 B, phenazine methosulphate (British Drug Houses, Poole, Dorset, England), *N,N,N',N'*-tetramethylenediamine (Koch - Light, Colnbrook, Bucks, England), *p*-nitrotetrazolium Blue (Mann Research Lab., New York, N.Y., U.S.A.), Fast Blue B and Fast Blue RR (Gurr, London, England), NADP (Sigma, St. Louis, Mo., U.S.A.), DL-isocitric acid trisodium salt (Fluka, Buchs, Switzerland), NAD and crystalline horse liver dehydrogenase (Reanal, Budapest, Hungary). α-Naphthyl acetate was prepared as described by Chattaway (1931) and α-naphthyl phosphate as described by Kunz (1894).

*Preparation of protein extracts.* Neutral extract: the acetone powder (about 2 g.) prepared from 100 seeds was extracted for 1 hr. with stirring at 4°, by 15 ml. of a solution containing 0.05 M-tris-HCl buffer, pH 7.2, 0.5 M-sucrose, 6 mM-cysteine hydrochloride and 6 mM-ascorbic acid (Macko, Honold & Stahman, 1967). The resulting suspension was centrifuged to remove the undissolved matter and then centrifuged at 100 000 *g* for 1 hr. at 4°. The clear supernatant was divided into 1 - 2 ml. samples and kept in deep-freeze for up to 2 weeks without marked changes in enzyme activity.

Acidic extract: the acetone powder obtained from 100 seeds was extracted as above with 15 ml. of 0.05 M-acetic acid. No cysteine or ascorbic acid was added.

The residue remaining after the acidic extraction was washed with water and then extracted with 15 ml. of 0.05 M-tris-glycine buffer, pH 8.4, as described above. The resultant extract is further referred to as the alkaline extract.

*Protein content* was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

*Disc electrophoresis* was performed by the method of Ornstein (1964) and Davis (1964) with running pH of 8.4, with tris-glycine buffer, using riboflavin instead of ammonium persulphate as polymerizing agent.

The glass tubes were about 64 mm. in length and had a 7.0 mm. outside, and 5.0 mm. inside diameter. The column of polyacrylamide gel was composed of two layers: a bottom layer of small-pore 7.5% acrylamide gel in which the electrophoretic separation takes place, and a large-pore 2.5% acrylamide gel (spacer gel) in which electrophoretic concentration takes place. The protein extract was placed directly on the spacer gel by underlayering the reservoir buffer. The concentrated extract would often deform the surface of the spacer gel; this was prevented by preparing the spacer gel with 40% sucrose solution instead of water. A few drops of bromophenol blue were added to the upper buffer solution to mark the ion front. The gels for electrophoresis and the buffer solutions were cooled to 0°.

Electrophoresis was carried out in an apparatus similar to that described by Ornstein (1964). The current was adjusted to 2 mA per tube for about 15 min. until the dye front migrated through the spacer gel. Then the current was adjusted to about 4 mA per tube and electrophoresis was continued for 1 - 1.5 hr. until the blue dye front migrated to 35 - 45 mm. into the separation gel. The electrophoresis was performed at room temperature; to avoid an increase in temperature above 10 - 15°, frozen buffer solution was added in the form of ice cubes.

The gels removed from the tubes were stained with 1% naphthalene Black in 7% acetic acid, usually overnight. The excess dye was removed by electrophoresis.

*Enzyme activities* were detected following electrophoresis by immersing the gels in appropriate solutions at 37°, as described below.

## RESULTS

Relatively few publications on polyacrylamide-gel electrophoresis of plant proteins recommend separation of crude extracts from plant tissues (Steward & Barber, 1964; Steward, Lyndon & Barber, 1965; Honold, Farkas & Stahman, 1966; Johnson, Barnhart & Hall, 1967; Mc Cown, Beck & Hall, 1968). According to other authors, extracts from acetone powders should be used (Clementis, 1966; Bhatia, Buiatti & Smith, 1967; Macko *et al.*, 1967). This procedure allows to remove lipids and other acetone-soluble components from the plant material. Since apple seeds contain considerable amounts of lipids (up to 15% of dry weight), in the present work extracts from acetone-dried seeds were used.

The neutral extract contained 2 - 5 mg. protein per 1 ml., the acidic one 4 - 6 mg., and the alkaline one as much as 14 - 16 mg. The considerable amount of protein present in the alkaline extract resulted probably from solubilization of reserve proteins.

For electrophoretic separation, the extracts were diluted with 0.5 M-sucrose solution so as to obtain 100 µg. protein in a volume of 0.05 - 0.1 ml. The obtained electrophoretograms are shown in Fig. 1. The neutral extract was separated into ten distinct bands, the acidic extract into seven bands, and the alkaline extract into eight bands partially masked by a slowly migrating fraction.

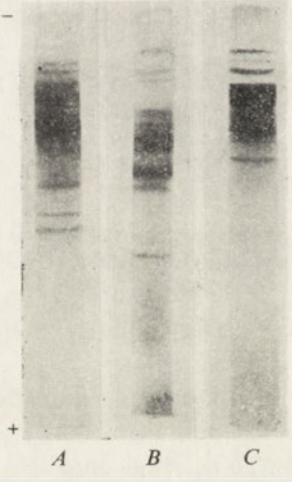


Fig. 1. Polyacrylamide gel electrophoresis of (*A*), neutral, (*B*), acidic and (*C*), alkaline extracts of acetone-dried powders from apple seeds. On the gel, 100 µg. of protein was applied and the separation was carried out at pH 8.4, naphthalene Black being used for staining.

Irrespective of the manner of extract preparation, all electrophoretograms which were stained with naphthalene Black had a blue background. Steward & Barber (1964) reported similar difficulties during electrophoretic separation of lipid-free plant extracts, and assumed that this background results from the presence in plant material of non-protein interfering substances.

*Assays of enzymic activities.* After electrophoretic separation of the neutral extract, the assays for enzymic activities were performed (Fig. 2). As the methods used for detecting the enzymes were more sensitive than the staining of protein, it was not always possible to ascribe enzymic activities to the individual protein fractions. However, for comparison, mobilities of the enzymic bands relative to the mobility of bromophenol Blue ($R_x$) are presented.

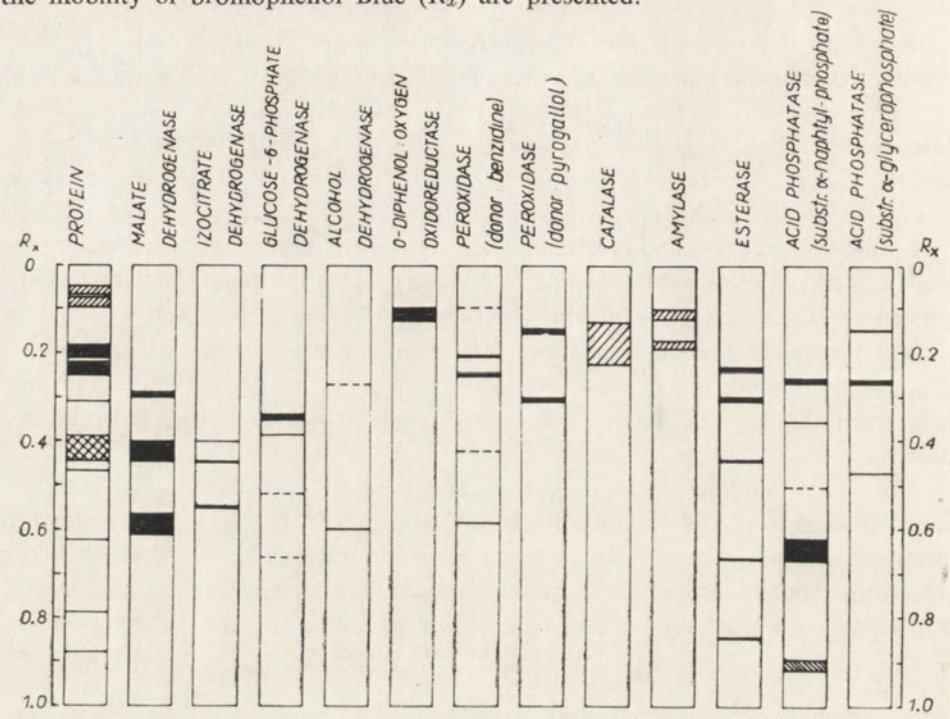


Fig. 2. Electrophoretic patterns of protein and enzyme activities of the neutral extract of acetone-dried powder from apple seeds. $R_x$ represents the mobility relative to the mobility of bromophenol Blue. Band intensities are graphically represented as follows: black area, very strong colour; black line, medium intensity; dashed line or area, very weak colour; hatched area, wide diffuse band.

The dehydrogenases were checked by the method of Honold *et al.* (1966) using appropriate substrates, NAD or NADP, and tetrazolium salt for colour development. Malate dehydrogenase activity was found, after 15 min. incubation, in three bands, the faster moving bands being more intensely stained. Isocitrate dehydrogenase appeared after 20 - 30 min. as three very weak bands. All three isoenzymes were NADP-dependent and no colour developed when NAD was used as coenzyme. Glucose-6-phosphate dehydrogenase was found in the presence of NADP in four bands. To detect alcohol dehydrogenase activity, NAD was added as coenzyme and it was necessary to apply on the gel a double amount of protein (200 μg.) as compared with the amounts required for demonstration of the presence of other enzymes. Alcohol dehydrogenase activity was found in two bands with considerably different $R_x$ values. The mobility of the faster one corresponded to that of crystalline horse liver alcohol dehydrogenase.

No lactate, glutamate or α-ketoglutarate dehydrogenases were detected in the presence of NAD.

*o*-Diphenol - oxygen oxidoreductase was detected by a method similar to that described by Nye, Kern & Aldrich (1968). Electrophoretograms were incubated for 10 - 15 min. in 0.05 M-acetate buffer, pH 5.1, with 0.01 M-catechol as substrate; then the gels were immersed into a solution of 1% $FeCl_3$ in ethanol containing 1% HCl, until colour developed. Usually a single band was obtained, but sometimes two diffuse bands of very similar mobility were formed.

Peroxidase activity was checked according to Scandalios (1965). The electrophoretograms were soaked for 1 min. in a mixture of equal amounts of 1% hydrogen peroxide solution and of saturated aqueous benzidine solution. Of the five bands showing peroxidase activity, those with $R_x$ values of 0.21 and 0.25 were more intensely stained, and sometimes they overlapped to form one wide band. When pyrogallol instead of benzidine was used as hydrogen donor, the electrophoretograms were incubated for 1 min. in 1% $H_2O_2$ and then in 5% pyrogallol (Nicolas, Maravdo, Garber & Voth (1967). In this case, a different pattern was observed. Two intensely stained, slightly diffuse bands of $R_x$ 0.15 and 0.30 were formed.

For detection of catalase, the method of negative staining according to Macko *et al.* (1967) was used. The gels were prepared with 0.3% soluble starch. The electrophoretograms were incubated in 1% $H_2O_2$ for 1 min. and transferred to a solution of 0.5% KI containing a few drops of acetic acid. A wide, diffuse, non-stained area was observed, suggesting the presence in the extract of more than one enzyme possessing catalase activity.

Also for amylases the negative staining according to Macko *et al.* (1967) was applied. The gels contained 0.3% starch and the electrophoretograms were incubated in a solution containing 0.004% of iodine and 1.5% of KI in 0.2 M-acetate buffer, pH 5.0. Two distinct bands were formed, both located near the start.

Aryl esterases were assayed according to Scandalios (1965). The reaction mixture consisted of 5 ml. of 0.1 M-phosphate buffer, pH 6.0, 0.05 ml. of α-naphthyl acetate (10 mg. dissolved in 1 ml. of acetone) and 2 mg. of Fast Blue RR salt. The electrophoretograms were incubated until dark colour developed. Five bands appeared, of which two, with $R_x$ values of 0.22 and 0.29, formed much more rapidly than the others and were more intensely stained.

Acid phosphatase was estimated according to Rudolph & Stahman (1966) using the diazonium dye method. The electrophoretograms were preincubated in acetate buffer, pH 5.1, then incubated with α-naphthyl phosphate as substrate and Fast Blue B as dye coupler. Five distinct bands were observed, and sometimes a sixth band could be seen between those with $R_x$ values of 0.25 and 0.35. The band at $R_x$ 0.62 was very intensely stained and a little diffuse. The diffusion of this band was somewhat reduced when Fast Blue RR salt was used, but then the slower migrating bands were less distinct and required longer incubation. Application of other diazonium salts such as Fast Red RC or Fast Red 3GL led to formation of weaker diffuse bands. In these cases longer incubation exceeding 20 min. was

required, whereas 15 - 20 min. was sufficient when Fast Blue B or Fast Blue RR were used.

Attempts were also made to characterize the acid phosphatase specificity. For this purpose, α-glycerophosphate was used as substrate, and the inorganic phosphate liberated was visualized by the lead salt method. Three bands were observed; two of them corresponded to the bands detected with α-naphthyl phosphate, whereas the band at $R_x$ 0.15 appeared to be specific for the natural substrate.

## DISCUSSION

The extraction of acetone-dried powder from stratified apple seeds with tris-HCl buffer, pH 7.2, containing cysteine and ascorbic acid, permits to obtain a high number of protein fractions on polyacrylamide-gel electrophoresis and eliminates the masking effect of reserve proteins. The neutral extract was found to be suitable for demonstration of enzymic activities. When the alkaline extract was assayed, the activity of peroxidases could not be detected, and with the acidic extract it was not possible to demonstrate the presence of aryl esterase and of the dehydrogenases.

In most cases, the enzymic activities studied appeared in several bands; if this happened when a natural substrate was applied, the occurrence of isoenzymes should be considered. On the other hand, with unnatural substrates the presence of several active bands could be indicative of the occurrence of non-specific enzymes.

The results obtained indicate that the application of neutral extracts from acetone-dried apple seeds may permit to study changes in activities of dehydrogenases and phosphatases in the course of development of apple seeds, and to estimate the role of these enzymes in the processes of secondary ripening and germination.

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## ROZDZIAŁ ELEKTROFORETYCZNY NA ŻELU POLIAKRYLAMIDOWYM ENZYMÓW NASION JABŁONI

Streszczenie

1. Białka ekstrahowane buforem tris-HCl, pH 7.2, z proszków acetonowych ze stratyfikowanych nasion jabłoni rozdzielano elektroforetycznie na żelu poliakrylamidowym.

2. Na elektroforogramach stwierdzono ok. 30 pasm wykazujących aktywność dwunastu enzymów, należących do oksydoreduktaz i hydrolaz.

Received 22 May, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

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# THE MUREIN FROM *SALMONELLA TYPHI* CELL WALL

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Isolation of *Salmonella typhi* murein is described. The murein is composed of muramic acid, glucosamine, alanine, glutamic acid and diaminopimelic acid in molar ratios 0.9:1.1:1.6:1.0:0.8. The murein of *Escherichia coli* B prepared by the same method gave the following ratios: 0.8:1.0:1.9:1.0:0.9.

In spite of the ample literature dealing with the cell walls of Gram-negative bacteria, few data have been published (Colobert & Creach, 1960; Weidel, Frank & Leutgeb, 1963) concerning the murein of *Salmonella* cell wall. Our interest in the structure of the murein layer of *Salmonella typhi* arose from studies on the mechanism of penetration of Vi-bacteriophages, continued for several years. The present paper describes the isolation and analysis of murein. The procedure is based on the use of sodium dodecyl sulphate (Weidel, Frank & Leutgeb, 1963; Pelzer, 1962; Mardarowicz, 1966) and enzymic digestion (Leutgeb, Maas & Weidel, 1963) including pronase digestion (Mardarowicz, 1966).

## MATERIAL AND METHODS

*Salmonella typhi*, strain no. 21802 (Vi-phage type A) was obtained from the National Reference Laboratory for Enteric Phage Typing, Gdańsk, Poland. *Escherichia coli* B was kindly given by Dr. U. Schwarz from the Max Planck Institute of Biology in Tübingen, West Germany.

The bacteria were grown on the modified Stockes & Bayne culture medium (mSB) (Taylor & Taylor, 1963), composed of solution *A*: 20 g. of glucose in 400 ml. of water, and solution *B*: 2 g. of $(NH_4)_2SO_4$, 1 g. of trisodium citrate·$2H_2O$, 1 g. of $MgSO_4 \cdot 7H_2O$, 25 g. of $Na_2HPO_4 \cdot 12H_2O$, 4 g. of $KH_2PO_4$, 20 g. of lactalbumin hydrolysate in 1600 ml. of water. Solutions *A* and *B* were autoclaved separately and mixed after cooling. The pH of the medium was adjusted to 7.4 with sodium hydroxide.

*Reagents*: lactalbumin hydrolysate, enzymic, Difco (Detroit, U.S.A.); pancreatine, K and K Laboratories (New York, U.S.A.); sodium dodecyl sulphate (SDS)

and pronase, B.D.H. (Poole, England); tubings for dialysis, Kalle (Wiesbaden, West Germany); silica gel containing 13% of $CaSO_4$ (silica gel G) for thin-layer chromatography, Merck (Darmstadt, West Germany); diaminopimelic acid (DAP), Light (Colnbrook, England). Muramic acid was synthesized according to Kent & Strange (1962) by Eng. L. Dubowik. The remaining reagents, of analytical grade, were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Sugars were estimated by the anthrone method as used by Goebel & Barry (1958), diaminopimelic acid (DAP[1]) according to Work (1957) and aromatic amino acids by the method of Lowry, Rosebrough, Farr & Randall (1951).

## RESULTS AND DISCUSSION

The bacteria were grown with aeration in 1 l. flasks in a water bath at 39°. Each of the four flasks containing 0.5 l. of mSB was inoculated with bacteria from one (*E. coli* B) or two (*S. typhi*) overnight agar slants. The bacterial growth was followed by turbidity determination. When the extinction at 585 mμ, 1 cm., reached 1.5 (after about 5 hr. of incubation) the cells were chilled in ice and harvested by centrifugation (5000 *g*, 10 min., 0°). The sediment was suspended in 100 ml. of water at 0°. About 1.5 g. of acetone-dried bacterial mass was obtained from 2 l. of the culture.

The bacterial suspension, 100 ml. (from 2 l. of culture), was immediately poured into 100 ml. of boiling 4% solution of SDS and kept at 100° for 45 min., then incubated overnight at 37°. On the next day the suspension was centrifuged (11 000 *g*, 40 min.) at 30° to prevent the precipitation of SDS. The cell walls were washed three times with warm water by centrifugation. The yield of the cell wall preparation from 2 l. of the medium amounted on average to 177 mg., corresponding to 12% of the dry weight of bacteria. Figure 1A represents the *S. typhi* cell walls at this stage of preparation; the granular nature of the external layer of the thick multilayer cell walls deserves notice. In Table 1 the results of the analyses of the cell wall are compared with those for the preparation of murein.

The cell walls served as the starting material for the isolation of murein; they were washed once with 0.1 M-phosphate buffer, pH 7.8, and suspended in 30 ml. of this buffer containing 12 mg. of pancreatine and saturated with chloroform. This suspension was digested at 37° in a dialysis bag immersed in 400 ml. of the same buffer. After 24 hr. the external buffer was changed; to the dialysis bag 12 mg. of pancreatine in 30 ml. of buffer was added and the digestion was continued for another 24 hr. After centrifugation (17 000 *g*, 40 min., 10°) the sediment was washed once with phosphate buffer and twice with 50 ml. of water. The sediment was suspended in 10 ml. of water and mixed with 10 ml. of 0.2 M-phosphate buffer, pH 7.4, containing 0.5 mg. of pronase. After overnight digestion at 58° and centrifuga-

[1] Abbreviations used: SDS, sodium dodecyl sulphate; DAP, diaminopimelic acid; MA, muramic acid; $GlcNH_2$, glucosamine; Ala, alanine; Glu, glutamic acid.

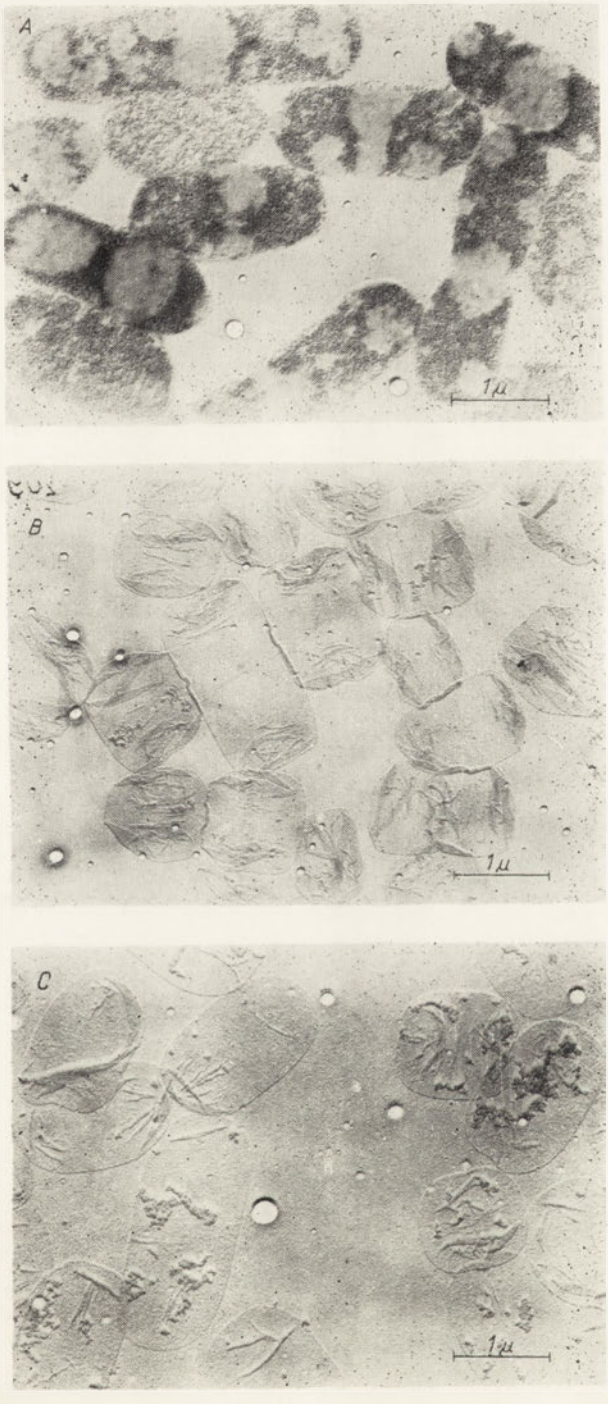


Fig. 1. Electron micrographs of (*A*), cell walls of *S. typhi*; (*B*), murein of *S. typhi*, and (*C*), murein of *E. coli*. The preparations were shadowed with chromium.

 

tion (20 000 *g*, 60 min., 10°) the sediment was washed three times with 50 ml. of water. The pellet was composed of two distinct layers; the bottom layer was white and the top one — transparent and colourless. The bottom layer was discarded at each centrifugation. The transparent sediment was resuspended in 6 ml. of water and added dropwise to 18 ml. of boiling 4% SDS. The suspension was kept for 1 hr. in a boiling water bath and incubated overnight at 37°. After centrifugation, the murein was washed twice with 50 ml. of warm water (11 000 *g*, 60 min., 30°) and four times with water saturated with chloroform (20 000 *g*, 60 min., 10°). The insignificant amounts of incidental contaminations were removed from the final suspension by centrifugation (1000 *g*, 10 min.). The murein was kept in the refrigerator as a suspension in water saturated with chloroform; the samples taken for analyses were freeze-dried. The yield of the murein preparation from a 2 l. batch amounted on average to 12 mg. corresponding to 0.8% of dry bacteria, by weight.

Pure murein has a strong tendency to aggregate. During the preparation of specimens for electron microscopy, aggregation was prevented by shaking the murein suspension with an equal volume of 0.1% solution of SDS at 37° for 2 hr. Then the agar-diffusion technique based on the method of Kellenberger & Arber (1957) was applied. Diffusion occurred for 20 min. at 37° in the humid chamber. The specimens were fixed with formaldehyde vapours and, after being mounted on the electron-microscope grids, shadowed with chromium at an angle of 8 - 10°. The specimens were examined and the photographs were taken in the Zeiss D2 electron microscope at the resolving power of 20 Å. Figure 1B represents the preparation of the *S. typhi* murein.

The murein of *E. coli* B was prepared by the same method. The composition and structure of the murein of these bacteria were the object of several studies (Weidel, Frank & Martin, 1960; Primosigh, Pelzer, Maas & Weidel, 1961; Pelzer, 1962; Leutgeb, Maas & Weidel, 1963) and reviews (Perkins, 1963; Leutgeb, Pelzer & Schwarz 1963; Weidel & Pelzer, 1964), therefore we adopted this preparation as a standard. The electron micrograph of the preparation of *E. coli* murein is presented in Fig. 1C. The purity of the murein preparations was checked by estimating the contents of DAP, the specific component of the murein, and the contents of sugars (reacting with anthrone) and aromatic amino acids, which are the measure of contaminations. If one assumes a muropeptide composed of *N*-acetylmuramic acid, *N*-acetylglucosamine, alanine, glutamic acid and DAP in molar ratios of: 1:1:2:1:1 to be the structural unit of the murein (Weidel & Pelzer, 1964; Martin, 1966), the calculated percentage of DAP amounts to 18.6 by weight. The results of analyses presented in Table 1 show that a quite satisfactory purity of the preparations of murein was attained, with concomitant preservation of its structure (Fig. 1B,C). Taking into account the simplified structure of murein mentioned above, the purity of the preparations examined amounted to at least 90%.

The qualitative analysis of *S. typhi* and *E. coli* mureins was performed by thin-layer chromatography and paper electrophoresis. The murein was hydrolysed in 4 N-HCl for 17 hr. at 103°. Hydrochloric acid was removed *in vacuo* over sodium

Table 1

*The analyses of cell walls and murein of* S. typhi

Cell walls and murein were prepared as described in the text. Samples for analysis were freeze-dried and analysed as described in Methods. The values are percentages of dry weight of the preparation.

| Material | Diaminopimelic acid (%) | Sugars (%) | Aromatic amino acids (%) |
|---|---|---|---|
| *S. typhi* cell walls | 1.1 | 27.0 | 4.4 |
| *S. typhi* murein | 16.7 | 2.3 | 0.9 |
| *E. coli* murein | 17 | 3 | 1.6 |

hydroxide. Thin-layer chromatography was carried out on plates coated with silica gel G. Figure 2 represents the chromatograms of the hydrolysates of *S. typhi* murein, *E. coli* murein and of the mixture of reference substances. The spots from bottom to the top correspond to DAP, glutamic acid, alanine, muramic acid and glucosamine. The identification of the components of murein was confirmed by electrophoresis on Whatman no. 1 paper in a buffer composed of 0.6 N-formic acid and 2 N-acetic acid (1:1), pH 1.9. Electrophoresis was performed under a constant voltage of 9 v/cm. for 5 hr. The spots were detected with 0.2% isatin solution in acetone.

Quantitative analysis of the murein components of *S. typhi* and *E. coli* was carried out in an automatic amino acid analyser (Beckman-Spinco model 120 B, U.S.A.) due to the kindness of Doc. Dr. Z. Szafran. The mole ratios of the components of both mureins are confronted in Table 2. Besides the characteristic murein components, small quantities of other amino acids (not exceeding 0.1 mole per 1 mole of glutamic acid) were found; glycine and leucine, and traces of aspartic acid, threonine, serine and isoleucine were identified.

On the basis of qualitative and quantitative analyses it may be assumed that the above-described method of isolation of *S. typhi* murein yields a product of high purity, at the same time preserving the integrity of its structure. The elongated shape of murein bags shows that the action of autolytic enzymes present in bacterial

Table 2

*Molar ratios of murein constituents of* S. typhi *and* E. coli *B*

Samples for analysis were freeze-dried and hydrolysed in 4 N-HCl, 12 hr., at 106°. HCl was removed in vacuum, over sodium hydroxide. Determinations were performed in the amino acid analyser Beckman-Spinco, model 120 B (U.S.A.).

| Material | MA | $GlcNH_2$ | Ala | Glu | DAP |
|---|---|---|---|---|---|
| *S. typhi* murein | 0.9 | 1.1 | 1.6 | 1.0 | 0.8 |
| *E. coli* B murein | 0.8 | 1.0 | 1.9 | 1.0 | 0.9 |

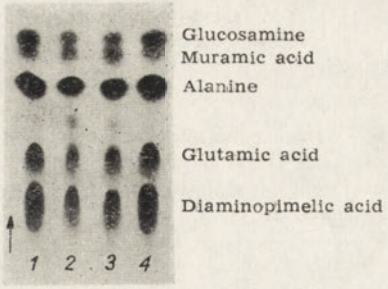


Fig. 2. Thin-layer chromatograms on silica gel G of: *1* and *4*, standard solutions; *2*, murein hydrolysate of *S. typhi*; *3*, murein hydrolysate of *E. coli* B. The plates (9×12 cm.) coated with silica gel G were dried for 2 hr. at 130° prior to use. The hydrolysates were applied in quantities equivalent to 40 μg. of murein, and developed twice in the same direction in a solvent containing *n*-butanol - pyridine - acetic acid - water (6:4:0.3:3, by vol.). The spots were detected with 0.3% ninhydrin solution in acetone.

envelopes has been inhibited (Weidel, 1951; Martin, 1966). The comparison of the mureins of *S. typhi* and *E. coli* B indicates that they have an identical qualitative and very similar quantitative composition.

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We wish to express our thanks to Dr. I. A. Georgadze and Dr. T. Tschanishvili who provided facilities for the preparation of large part of the material and offered warm hospitality during the stay of one of the authors (A.T.) in the Institute in Tbilisi. We are grateful to Doc. Dr. Z. Szafran for performing quantitative analyses and to Eng. L. Dubowik for the synthesis of muramic acid. The skilful technical assistance of Mrs. Józefa Zaba, Miss Anna Butler and Miss Zofia Walentynowicz is greatly appreciated.

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## MUREINA Z BŁON KOMÓRKOWYCH *SALMONELLA TYPHI*

Streszczenie

Wyizolowano mureinę z błon komórkowych *Salmonella typhi*. Składnikami mureiny *S. typhi* są: kwas muraminowy, glikozamina, alanina, kwas glutaminowy i kwas dwuaminopimelinowy w stosunkach molowych 0,9:1,1:1,6:1,0:0,8. W mureinie *Escherichia coli* B, przygotowanej w ten sam sposób dla porównania, stosunek molowy składników wynosił odpowiednio 0,8:1,0:1,9:1,0:0,9.

Received 28 May, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

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# CHEMILUMINESCENCE IN REACTIONS OF CYTOCHROME *c* AND HAEMATIN WITH HYDROGEN PEROXIDE

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1. The weak chemiluminescence accompanying the reaction of cytochrome *c* and haematin with hydrogen peroxide in carbonate buffer, pH 8.8, at 30° was examined. 2. The kinetics of the luminescence, changes in absorption spectra of the oxidized substrate, and the effect of free-radical reactions inhibitors on light emission were measured. 3. The obtained data indicate that the chemiluminescence is probably accompanied by oxidative scission of porphyrin ring, and in the case of cytochrome *c* also by oxidation of cyclic amino acid residues of the protein.

Hydrogen peroxide is formed in the living cells under the action of ionizing radiation, by auto-oxidation of some polyphenols and thiol groups of proteins, or direct oxidation of FMN-$H_2$ by molecular oxygen. $H_2O_2$ can be decomposed to water and hydrogen or it can oxidize natural substrates; these reactions may be accompanied by generation of excited molecules emitting weakly in the visible region of the spectrum (Dure & Cormier, 1964; Vladimirov, 1966). So far chemiluminescence has been reported to appear in the reaction of $H_2O_2$ with compounds present in liver extracts (Tarusov, Palivoda & Juravlev, 1961), with DNA, cysteine, glycine, proteins (Vladimirov, 1966), riboflavin (Strehler & Shoup, 1954), plant tannins and other polyphenols (Sławińska & Sławiński, 1964, 1967). Very little is known about the chemiluminescence accompanying the reaction of $H_2O_2$ with catalase or peroxidase, and its very existence has been discussed (Stauff & Wolf, 1964; Likhtenstein & Purmal, 1966). Hitherto, the chemiluminescence appearing in the reaction of $H_2O_2$ with haematin exhibiting peroxidase activity, and with the cytochromes has not been studied. In the present work an attempt was made to establish whether the reactions of cytochrome *c* and haematin with $H_2O_2$ are accompanied by chemiluminescence and, if so, which components are involved in this phenomenon.

## MATERIALS AND METHODS

*Special reagents*: Cytochrome *c*, mol. wt. 13 530 in 0.9% NaCl, was a product of Biosedra (France); crystalline haemin (chloride of haematin) was prepared from

bovine blood by the method of Nencki & Siber (1884) and recrystallized twice from glacial acetic acid; for experiments a suspension in water was used. Alkaline haematin was obtained by dissolving haemin in a small volume of 0.1 N-NaOH and adding Na-carbonate buffer to the required dilution. Haemoglobin, electrophoretically pure (Institute of Haematology, Warsaw, Poland) was a kind gift of Prof. Dr. K. Murawski. Bovine α-globulin, Cohn fraction IV, and bovine albumin, Cohn fraction V, were products of Koch-Light Lab. (Colnbrook, Bucks., England). Catalase and peroxidase were from Reanal (Budapest, Hungary). Other reagents were of analytical purity, produced by Fabryka Odczynników Chemicznych (Gliwice, Poland). Water twice distilled with $KMnO_4$ from glass was used throughout. The concentration of $H_2O_2$ was determined by titration with $KMnO_4$.

*Light-measurement apparatus.* The intensity of chemiluminescence was measured by the photoelectric method using RCA 6655A photomultiplier (Radio Corporation of America, Harrison, N.J., U.S.A.) of spectral sensitivity in the range 300 - 650 mμ and maximum at 440 mμ. The signal from the photomultiplier was amplified by a D.C. amplifier WF-70 (Politechnika Śląska, Gliwice, Poland) and recorded with a Radiometer Rec 1a Recorder (Copenhagen, Denmark). The apparatus was adapted to record the luminescence continuously from the onset of the reaction. The time constant of the measuring system was 0.5 to 1 sec. The solution was vigorously stirred in the reaction cuvette mounted directly on the entrance window of the photomultiplier, as it was described previously (Sławińska & Sławiński, 1965, 1968).

Spectrophotometric measurements were made using a Spectromom-202 spectrophotometer (Budapest, Hungary).

*Experimental procedure.* The reaction was started by rapid injection of 1 ml. of the substrate solution, through a glass syringe, to a solution of 10 mM-$H_2O_2$ in 20 mM-$NaHCO_3$ - $Na_2CO_3$ buffer, pH 8.8. As substrates were used: solution of cytochrome *c* or haematin at 1 μM final concentration, or aqueous suspension of haemin. The incubation was carried out at 30°, and all determinations were made in triplicate.

The technique of the determinations has been described in detail in earlier communications (Sławińska & Sławiński, 1965, 1968).

## RESULTS

*Characteristics of the light-producing reaction.* The addition of cytochrome *c* or haematin to the solution of $H_2O_2$ in carbonate buffer gave a flash of light lasting about 10 sec. and followed by very weak but long-lived emission (Fig. 1). The intensity of the flash was dependent on the rate of substrate injection, i.e. upon the concentration gradients arising at the moment of mixing; however, slow addition of the substrate also resulted in luminescence. When in the absence of $H_2O_2$ a stream of $O_2$ was passed through the solution of cytochrome *c* or haematin in carbonate buffer, no emission was measured by our detection system.

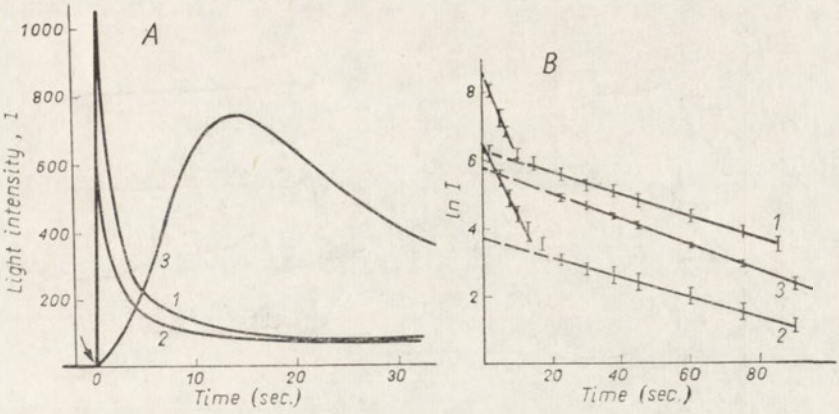


Fig. 1. *A*, Chemiluminescence resulting from injection of: *1*, cytochrome *c* (final concn. 1 μM); *2*, haematin (1 μM); *3*, haemin suspension (0.1 mM), to a solution containing 10 mM-$H_2O_2$ in 20 mM-$NaHCO_3$ - $Na_2CO_3$ buffer, pH 8.8. *B*, Semilogarithmic plots of the same data.

The supplementary addition of $H_2O_2$ to the whole system after the reaction had been completed, gave no effect; the addition, however, of cytochrome *c* or haematin evoked another flash of about the same intensity. Similar results were obtained with haemoglobin, catalase and peroxidase.

The intensity of chemiluminescence of all the compounds studied increased strongly with increasing pH value. The maximum intensity was obtained at pH 12, that is at a value close to the dissociation constant ($K_{diss}$) for $H_2O_2$, $2.5\times10^{-12}$.

The spectrum of the luminescence could not be determined because of its very small intensity and short duration. Only two filters could be used: a bluish-green with maximum transmittance at 490 mμ and an orange one with the maximum at 610 mμ. At 490 mμ the light intensity ($I$) was 87.2% of $I_{max}$ and at 610 mμ, 3.8%. These data indicate only that the light emitted had a blue component; it was not possible to determine whether the red component was also present because of low sensitivity of the photomultiplier in the red region of the spectrum.

*Effect of particular components of haemin enzymes on the light-producing reaction.* The addition of an aqueous suspension of haemin resulted in a luminescence of smaller intensity but of longer duration as compared with the addition of haematin at the same pH value. Substrate added to the buffer alone gave no emission; however, the mixture of $H_2O_2$ with buffer without substrate produced an emission the intensity of which was 2.8% of that observed in the presence of cytochrome *c*.

Vladimirov (1966) demonstrated that the oxidation of some amino acids, especially the aromatic ones, and of proteins is accompanied by weak chemiluminescence. To determine whether the observed chemiluminescence of cytochrome *c* was due to the amino acid components of the protein, phenylalanine, tryptophan, tyrosine, histidine and bovine globulin were used as substrates (Fig. 2). Both globulin and the

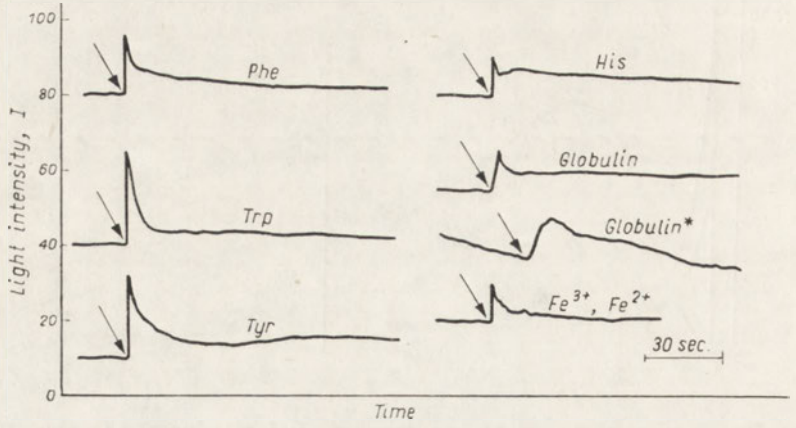


Fig. 2. Chemiluminescence resulting from the injection of amino acids or protein into the solution containing 10 mM-$H_2O_2$ in 20 mM-carbonate buffer, pH 8.8. The indicated amino acids (final concn. 0.1 mM) and bovine albumin (0.02%) were injected in a volume of 1 ml. at the points shown by arrows. The curve "globulin*" represents the final part of curve *3* (for haemin suspension) from Fig. 1 after 2.5 min.

amino acids gave a chemiluminescence the intensity of which was 0.2 - 7% of that for cytochrome *c* (cf. Fig. 1) although the concentration of the latter was lower by two orders of magnitude. The addition of globulin or albumin to the light-producing reaction mixture containing haematin, also led to an increase in luminescence by only 5%.

The reaction of $FeSO_4$, $Fe_2(SO_4)_3$, or $K_3Fe(CN)_6$ with $H_2O_2$ resulted in an emission with an intensity by two orders of magnitude lower than that appearing on addition of haematin.

*Effect of inhibitors.* Inhibitors of free-radical reactions and thermal inactivation of cytochrome *c* and haemoglobin were applied to obtain more information concerning the mechanism of chemiluminescence. Thermal inactivation (1 hr., 100°) had little effect on $I_{max}$ and on the sum of light emission, $\Sigma I$. Ascorbic acid and $\alpha$-naphthol at 1 mM concentration and hydroquinone (10 mM) inhibited the luminescence of the systems studied. Ascorbic acid and hydroquinone, being strong reducing agents, reduced cytochrome *c* to a form which gave no luminescence. As regards haematin, ascorbic acid at 0.1 mM concentration gave an induction period of about 120 sec., altering slightly the luminescence intensity as compared to that without the inhibitor. The value of the induction period was proportional to the concentration of the inhibitor. The appearance of the induction period, typical of the occurrence of radical chain reactions, is associated with scavenging of free OH and OOH radicals propagating decomposition of $H_2O_2$, and with the reduction of haematin $Fe^{3+}$ to the catalytically ineffective $Fe^{2+}$ compound. The addition of 1 mM-KCN in the case of haemoglobin gave an increase in $I_{max}$ and $\Sigma I$ which was due to the strong chemiluminescence of the KCN-$H_2O_2$ system alone;

the analogous effect was observed by Likhtenstein & Purmal (1966) in the reaction of catalase with hydrogen peroxide.

*Spectrophotometric observations and kinetics of the reaction.* Absorption spectra of haematin and cytochrome *c* were taken before and after oxidation (Fig. 3). The absorption at 400, 515 and 540 - 600 mμ characteristic of porphyrin disappeared on oxidation, indicating the opening of the porphyrin ring. The increased absorption in the range of 240 - 320 mμ for cytochrome *c* suggested oxidation of a component of protein, or formation of some porphyrin-degradation products which show absorption in this region.

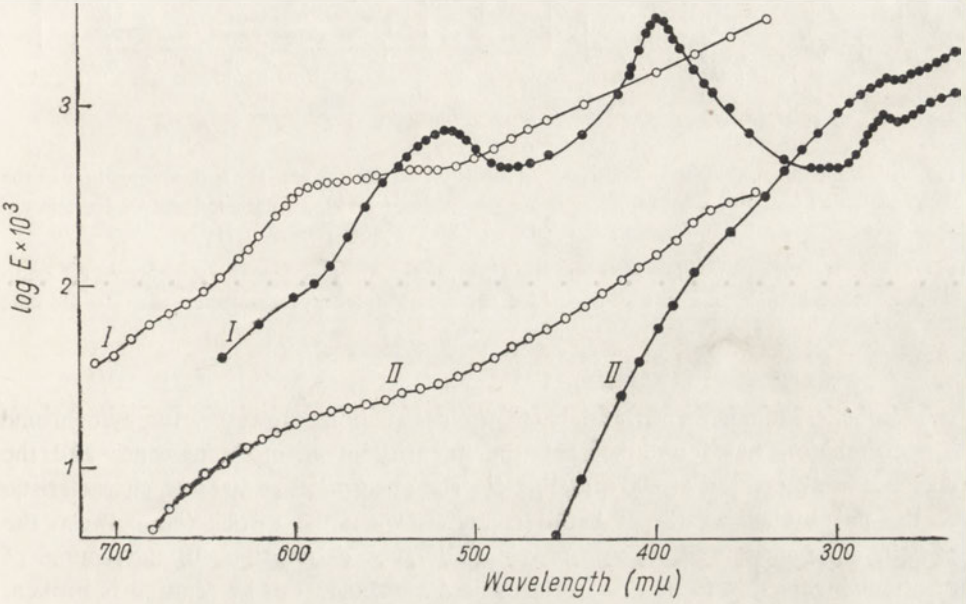


Fig. 3. Absorption spectra of (○), haematin and (●), cytochrome *c* before (curves *I*) and after (curves *II*) oxidation. The reaction mixture contained 200 mM-$H_2O_2$ in 20 mM-sodium carbonate buffer, pH 8.8, and 0.1 mM substrate. The incubation time was 60 min. for cytochrome *c* and 20 hr. for haematin. Temp. 20°.

The kinetics of the reaction was followed by luminescence measurements, $H_2O_2$ decomposition and changes in absorption at 580 mμ for haematin and at 515, 400 and 277 mμ for cytochrome *c*. For haematin (Fig. 4) no correlation was observed between the time-course of $H_2O_2$ decomposition and luminescence. The absorption at 580 mμ after 2 - 3 min. of incubation increased temporarily indicating formation of a transient species absorbing in this range of spectrum. The shape of the time-absorption curve may thus result from two parallel processes: decrease in absorption at 580 mμ due to opening of the porphyrin ring, and accumulation of an intermediate, probably biliverdin, which shows absorption in this region. That is why it is impossible to conclude whether there was really no correlation

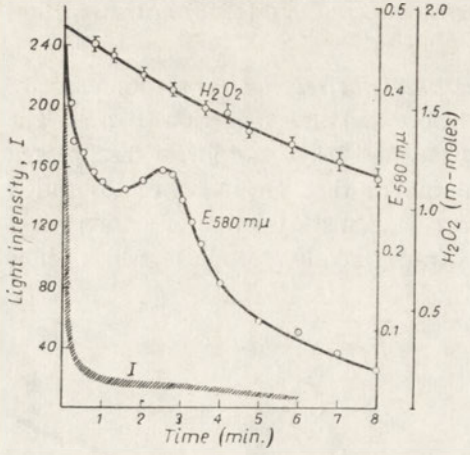


Fig. 4

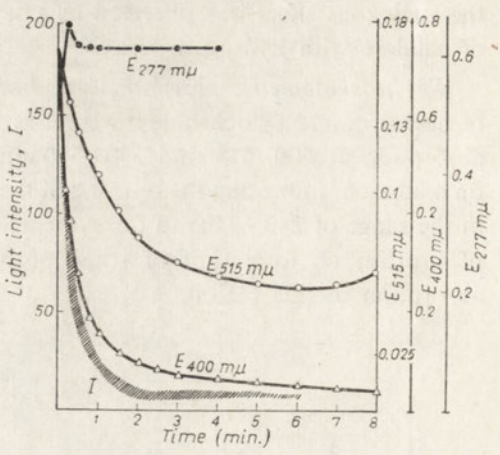


Fig. 5

Fig. 4. Time-course of chemiluminescence, absorption at 580 mμ and $H_2O_2$ decomposition in the reaction with haematin. Conditions of the experiment as in Fig. 3. Shaded curve, light intensity; (○), absorption at 580 mμ; (●), concentration of $H_2O_2$.

Fig. 5. Time-course of chemiluminescence and absorption in the reaction with cytochrome *c*. Conditions of the experiment as in Fig. 3. Shaded curve, light intensity; absorption at: (○), 515 mμ; (△), 400 mμ; (●), 277 mμ.

between chemiluminescence and haematin destruction. However, for cytochrome *c* a correlation was found between the intensity of chemiluminescence and the decrease in absorption at 400 mμ (Fig. 5). The absorption at 400 mμ, characteristic of the porphyrin ring (Soret band) decreases when the association between the protein component and iron-porphyrin undergoes destruction. In the course of reactions involved in formation of the excited molecules, this association is broken. At 277 and 515 mμ no correlation was observed between the changes in absorption and in chemiluminescence. Unfortunately, the changes in absorption were too fast to be followed with a common spectrophotometer, and no determinations could be made during the first 15 sec. of the reaction.

The time-course of chemiluminescence presented in Fig. 1a shows that the drop in intensity was at first fast and then became much slower. This was due to the existence of more than one step of the light-producing reaction. The kinetic curves could be resolved into two components by plotting ln *I versus* time (Fig. 1b).

$$I \approx \eta_1 \exp(-k_1 t) + \eta_2 \exp(-k_2 t)$$

where $k_1$, $k_2$, $\eta_1$, $\eta_2$ are respectively rate constants and quantum yields of the light-producing reaction. The values of $k_1$ obtained from six experiments were very close to each other, the average being $1.8 \pm 0.3$ (S.D.) $\times 10^{-1}$ sec.$^{-1}$. The values of $k_2$ obtained by extrapolation to zero time were $3.1 \times 10^{-2}$, $3.4 \times 10^{-2}$, $2.9 \times 10^{-2}$ and $2.7 \times 10^{-2}$ sec.$^{-1}$ for haematin, haemin suspension, cytochrome *c* and haemoglobin, respectively. The confidence interval for $P = 0.95$ was about $0.8 \times 10^{-2}$.

## DISCUSSION

The light-producing reaction between hydrogen peroxide and iron-porphyrin is a stoicheiometric reaction and not a catalytic one. The obtained results indicate that $H_2O_2$ is a reagent indispensable for the formation of the excited molecules, and that the disappearance of luminescence in the course of the reaction is caused by exhaustion of cytochrome *c* or haematin. Thus, the iron-porphyrin compounds studied did not merely catalyse the decomposition of $H_2O_2$ but entered into the reaction.

The inhibitors of free-radical reactions quench very strongly the chemiluminescence of the investigated compounds. This indicates that the free-radical reactions participate in generation of the excited molecules.

The results reported indicate that the observed chemiluminescence may be associated with three groups of reactions, namely:

1. The decomposition of $H_2O_2$ in the presence of cytochrome *c* or haematin in alkaline solutions which results in formation of excited molecular oxygen (Stauff & Schmidkunz, 1962):

$$H_2O_2 \rightarrow \text{radicals and/or ions: OH, OOH, } O_2$$
$$OH + OOH \rightarrow H_2O + O_2^* \text{ or } (O_2)_2^*$$
$$O_2\ ^1\Sigma_g^+ \text{ and/or } ^1\Delta_g \rightarrow O_2\ ^3\Sigma_g^- + h\nu$$

2. The oxidation of iron-porphyrin:
   a) charge-transfer reaction by which the porphyrin could be generated in an excited state:

$$HOOH + \text{porphyrin-Fe}^{2+} \rightarrow (\text{porphyrin-Fe}^{3+} - OH^-) + OH$$
$$(\text{porphyrin-Fe}^{3+} - OH^-) \rightarrow (\text{porphyrin-Fe}^{2+})^* + OH$$
$$(\text{porphyrin-Fe}^{2+})^* \rightarrow \text{porphyrin-Fe}^{2+} + h\nu$$

   b) oxidative degradation of the iron-porphyrin initiated by an oxidative scission at methene bridge.

3. Oxidation of protein component in the case of cytochrome *c*, haemoglobin or peroxidase by the decomposition products of $H_2O_2$.

The reactions 1 and 2a,b may provide the main component of luminescence of the flash. From the kinetic data (Fig. 1a,b) it is evident that the first light-producing reaction with $k_1 = 1.8 \times 10^{-1}$ sec.$^{-1}$ is associated with one rapid process, probably the formation of the excited $O_2$. In a number of reactions involving decomposition of $H_2O_2$, analogous flashes having very similar $k_1$ values were observed by different authors. The rapid injection of ferrous or ferric salts, haematin or cytochrome *c* into a $H_2O_2$ solution results in formation of bubbles of gaseous oxygen. This process is associated with luminescence from the excited molecules $O_2^*$ or their van der Waal's complexes $(O_2)_2^*$ (Stauff, Schmidkunz & Hartmann, 1963).

The flash accompanies also the opening of the porphyrin ring (Fig. 4, 5). From the two possible reactions, the reaction 2b seems to be more probable but to resolve this problem detailed spectral investigations are neces ary.

Special attention should be paid to the role of porphyrin ring which in the absence of protein component, e.g. as haematin, gives with $H_2O_2$ a comparatively strong light emission. Chemiluminescence accompanies also the photo-oxidation of chlorophyll (Litvin & Krasnovsky, 1967) and the decomposition of peroxide in the presence of derivatives of Zn-, Cu- and Sn-porphyrin (Linschitz & Abrahamson, 1953; Linschitz, 1961). Therefore it is very probable that the main reaction in the observed chemiluminescence is the oxidative degradation of the iron-porphyrin ring initiated by an attack of the decomposition products of $H_2O_2$ on the methene bridge. In this reaction, unstable hydroperoxides may be formed which decompose to carbonyl derivatives in excited state. These reactions are known to liberate about 70 kcal/mole (Bowen, 1964) which corresponds to 410 mμ of the short-wavelength limit of the emission spectrum. Since with the increase of pH the intensity of chemiluminescence increases and decay of chemiluminescence intensity obeys the first-order reaction with $k_2$ slightly lower for iron-porphyrin proteins, the formation of intermediate hydroperoxides of the iron-porphyrins seems very probable.

The low long-lived emission of cytochrome *c* could be connected with oxidation of the protein or its cyclic amino acid residues, especially tryptophan and tyrosine. Likhtenstein & Purmal (1966) demonstrated the pH dependence of $I_{max}$ of chemiluminescence and of the enzymic activity of catalase, and suggested that the light-emitting reaction is associated with the oxidation of imidazole of histidine residues and $NH_2$-groups.

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The authors wish to express their gratitude to Prof. Dr. J. Dobrowolski of the Department of Biochemistry, College of Agriculture, Szczecin, for his interest in this work and gifts of α-globulin and albumin.

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## CHEMILUMINESCENCJA W REAKCJACH CYTOCHROMU *c* I HEMATYNY Z NADTLENKIEM WODORU

Streszczenie

1. Badano słabą chemiluminescencję towarzyszącą reakcji utleniania cytochromu *c* i hematyny z nadtlenkiem wodoru w buforze węglanowym o pH 8.8 w temperaturze 30°.

2. Zmierzono kinetykę luminescencji, zmiany widm absorpcji utlenianych substratów i działanie inhibitorów reakcji rodnikowych na emisję światła.

3. Otrzymane wyniki wskazują, że chemiluminescencja jest prawdopodobnie związana z utleniającym rozerwaniem pierścienia porfirynowego, a w przypadku cytochromu *c* również z utlenianiem cyklicznych aminokwasów części białkowej.

Received 28 May, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

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# THE BIOSYNTHESIS OF CYTIDINE DIPHOSPHATE ESTERS IN RAT LIVER AND *NEUROSPORA CRASSA*

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1. The biosynthesis of phosphatidylethanolamine and phosphatidylcholine in wild type of *Neurospora crassa*, similarly as in rat liver, involves the formation of the respective cytidine diphosphate esters. Monomethylaminoethanol phosphate and dimethylaminoethanol phosphate can also be incorporated into phospholipids *via* the cytidine mechanism. 2. The cytidylyltransferase responsible for the formation of cytidine diphosphate dimethylaminoethanol is probably identical with the enzyme synthetizing cytidine diphosphate choline. The transferases responsible for the formation of cytidine diphosphate monomethylaminoethanol and cytidine diphosphate ethanolamine are probably separate enzymes.

The cytidine mechanism of the formation of phospholipids described by Kennedy (1961) involved three intermediates: cytidine diphosphate diglyceride, cytidine diphosphate choline (CMP-PC[1]) and cytidine diphosphate ethanolamine (CMP-PE). The last two are the precursors, respectively, of phosphatidylcholine and phosphatidylethanolamine. The possibility of formation of *N*-monomethyl- and *N*-dimethyl analogues of phosphatidylethanolamine *via* this mechanism has also been proved by Ansell & Chojnacki (1962). These two phospholipids were known to occur in trace amounts in animal tissues where their role is believed to be the intermediate steps in the formation of phosphatidylcholine from phosphatidylethanolamine and *S*-adenosylmethionine (Bremer, Figard & Greenberg, 1960). The MMAE- and DMAE-phospholipids occur in large amounts in *Neurospora crassa* (Hall & Nyc, 1959), however it has not been established whether they are only the intermediate products of the methylation of phosphatidylethanolamine or arise *via* the respective cytidine diphosphate mono- and dimethylaminoethanols (CMP-PMMAE and CMP-PDMAE). In the present paper the participation of the cytidine pathway in the formation of phospholipids containing ethanolamine, MMAE,

[1] Abbreviations used: PC, choline phosphate; PDMAE, dimethylaminoethanol phosphate; PE, ethanolamine phosphate; PMMAE, monomethylaminoethanol phosphate; CMP-PC, CMP-PDMAE, CMP-PE and CMP-PMMAE are the CMP derivatives of the respective aminoalcohol phosphates.

DMAE and choline has been demonstrated in wild type of *Neurospora crassa*. A comparison has also been made between the cytidylyltransferases synthetizing the appropriate cytidine diphosphate esters in rat and *Neurospora crassa*.

## MATERIALS AND METHODS

*Chemicals.* [$^{32}$P]Orthophosphate was the product of the Institute of Nuclear Research (Warszawa, Poland). The [$^{32}$P]phosphate esters of choline, monomethylaminoethanol, dimethylaminoethanol and ethanolamine were prepared by methods used by Riley (1944) and Schmidt (1957) with the modifications of Ansell & Chojnacki (1966). The $^{32}$P-labelled cytidine diphosphate (of the type CMP-$^{32}$P-base) choline, MMAE and DMAE were prepared as described by Ansell & Chojnacki (1966). CMP-$^{32}$PE was prepared by the method of Chojnacki & Metcalfe (1966). CTP was from Sigma Chemical Company (St. Louis, Mo., U.S.A.). Sephadex G-200 was a Pharmacia Ltd. (Uppsala, Sweden) product. Charcoal (Zakł. Elektr. Węgl., Racibórz, Poland) was prepared according to Threlfall (1957).

*Analytical.* Phosphorus, $^{32}$P, and protein were analysed as described previously (Chojnacki, Radomińska-Pyrek & Korzybski, 1967). The determination of $^{32}$P was performed also by measuring the Cerencow radiation in aqueous samples in a Packard Tri-Carb spectrometer. Paper chromatography of phosphoric esters and nucleotides was performed on Whatman no. 1 paper in propan-2-ol - water - trichloroacetic acid - 18 N-$NH_3$ (76:25:5:0.3, v/v/w/v) (Ebel, 1952). The autoradiograms of paper chromatograms were made using the "Foton" (Poland) X-ray plates (44CUK). Extraction of lipids was performed by the method of Folch, Lees & Sloane-Stanley (1957).

*Animals and homogenates.* White Wistar rats, 6 weeks old, weighing 200 - 220 g. were used. The 105 000 *g* supernatant was obtained from rat liver as previously described (Chojnacki *et al.*, 1967).

*Cultures of* Neurospora crassa. Tubes containing 10 ml. of Vogel's minimal medium (Vogel, 1956) supplemented with 1% sucrose and 1.5% agar were inoculated with the wild type strain of *Neurospora crassa* 74 ORA and cultured at 34° for 5 days. Conidia were harvested by using 20 ml. of water and transferred to 1 l. Erlenmayer flask containing 500 ml. of sterile Vogel's minimal medium supplemented with 1% sucrose. After two days of incubation in an orbital shaker at 34°, the mycelia were washed with water, blotted as dry as possible and weighed. The yield was usually 8 g. of wet weight. The mycelium was suspended in about 32 ml. of 0.25 M-sucrose, cut into small fragments with scissors and ground in a cold mortar with 5 ml. of powdered glass for about 10 min. Cell debris and powdered glass were removed by 10 min. centrifugation at 1000 *g*. The obtained homogenate was centrifuged for 1 hr. at 105 000 *g*.

*Gel filtration.* Tree-ml. sample of the 105 000 *g* supernatant of rat liver (15 mg. of protein) or *Neurospora crassa* (12 mg. of protein) was applied to a Sephadex G-200 column (2×60 cm.) equilibrated with 0.145 M-NaCl - 0.005 M-tris-HCl

buffer, pH 7.5, and the same solution was used for elution. Fractions of 1 ml. were collected and assayed for enzymic activities.

*Determination of cytidylyltransferase activities.* The reaction mixture (final volume 0.5 ml.) contained: 20 mM-tris-HCl buffer, the pH values being 7.6 for rat liver and 7.0 for *Neurospora crassa*, 15 mM-magnesium chloride, 0.25 mM-CTP, 0.05 mM-[$^{32}$P]phosphate ester of ethanolamine (sp. act. $5.5\times10^6$ counts/min./μmole), MMAE, DMAE or choline (sp. act. $1.2\times10^6$) and 100 μl. of enzyme preparation from rat liver or *Neurospora crassa*. After 15 min. incubation at 37°, 0.5 ml. of 10% trichloroacetic acid and 1 ml. of an aqueous suspension of charcoal (50 mg./ml.) were added and the labelled nucleotide was estimated by the procedure of Borkenhagen & Kennedy (1957).

## RESULTS

The rates of formation of four cytidine diphosphate esters by enzyme preparations from rat liver and *Neurospora crassa* are presented in Table 1. The values were calculated from experiments in which a linear relationship between the time of incubation and the formation of the product was observed. The rates of incorporation of different *N*-methyl substituted phosphorylaminoethanols into nucleotides in rat liver and *Neurospora crassa* were found to be similar. The rate of the incorporation decreased in the following order: CMP-PE, CMP-PC, CMP-PMMAE and CMP-PDMAE. The appropriate nucleotides after paper chromatography were identified by autoradiography.

The results of Sephadex G-200 fractionation of cytidylyltransferases activities of 105 000 *g* supernatants of rat liver and *Neurospora crassa* are presented in Fig. 1. In the experiment with rat liver (Fig. 1A), PC cytidylyltransferase was eluted to-

Table 1

*Incorporation of the* [$^{32}$P]*phosphate esters of* N-(*mono-, di- or tri-*) *methyl-substituted ethanolamines into cytidine diphosphate esters*

The standard incubation mixture, 0.5 ml. (see Methods) contained the 105 000 *g* supernatant from rat liver (450 μg. of protein) or *Neurospora crassa* (400 μg. of protein). The incubation was carried out for 15 min. at 37°, then the reaction was stopped by the addition of 0.5 ml. of 10% trichloroacetic acid, and labelled nucleotides were estimated after Borkenhagen & Kennedy (1957).

| $^{32}$P-labelled substrate | Rat liver | *Neurospora crassa* |
|---|---|---|
| | (mμmoles of nucleotide/1 mg. of protein/1 min.) | |
| Ethanolamine phosphate | 2.35 | 2.22 |
| Monomethylethanolamine phosphate | 0.56 | 0.57 |
| Dimethylethanolamine phosphate | 0.55 | 0.40 |
| Choline phosphate | 1.00 | 1.23 |

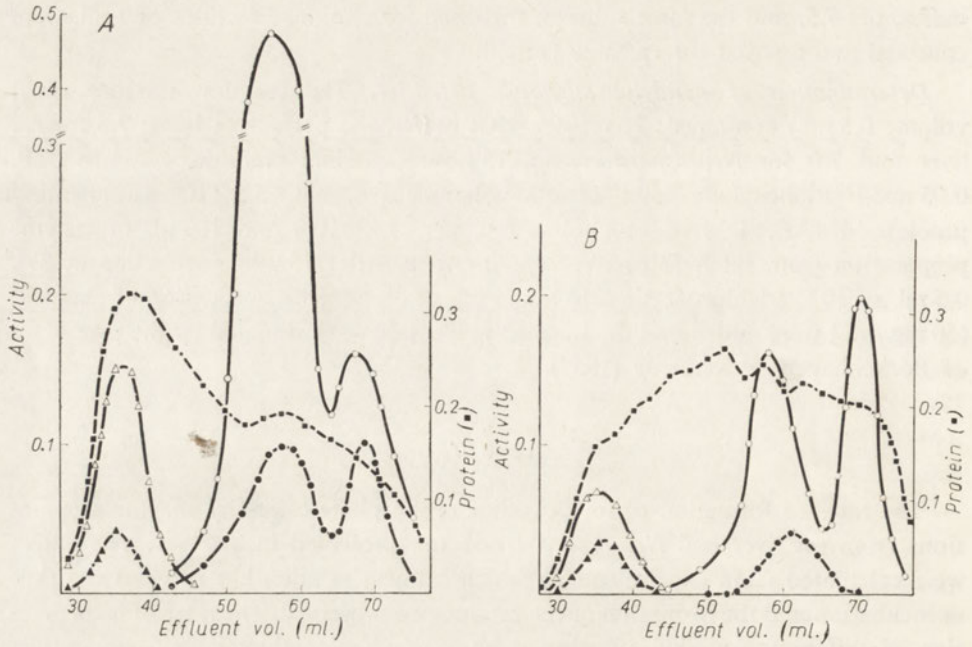


Fig. 1. Sephadex gel filtration of the 105 000 *g* supernatant from *A*, rat liver and *B*, *Neurospora crassa*. The column (2×60 cm.) was eluted with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5. Fractions of 1 ml. were collected and assayed for cytidylyltransferases activity for PE (○), PMMAE (●), PC (△) and PDMAE (▲) by the standard procedure described in the text. The activities are expressed in mμmoles of synthetized nucleotide per 1 ml. per 1 min., and the amount of protein in mg./1 ml. (■).

gether with PDMAE cytidylyltransferase which formed a smaller peak of similar shape. The two peaks of PE cytidylyltransferase eluted subsequently were accompanied by PMMAE cytidylyltransferase. The elution profiles of these two enzymic activities were, however, slightly different. The latter enzyme was eluted in two equal peaks while with the PE enzyme the second peak was only slightly pronounced.

With the material from *Neurospora crassa* (Fig. 1B) the elution volumes of PC- and PDMAE cytidylyltransferases were identical with those of rat liver enzymes. There was, however, a distinct difference between the other two cytidylyltransferases. The PE-specific enzyme was eluted in two peaks, while the PMMAE enzyme in one peak only, that did not correspond with either of those two peaks.

The pH dependence of the activity of *Neurospora crassa* cytidylyltransferases is shown in Fig. 2 For CMP-PC and CMP-PDMAE synthetizing enzymes the curves and optima of pH are similar (Fig. 2B). The PE cytidylyltransferase shows two pH optima, at 6.4 and 7.0, while only one, at pH 7.0, was found for the PMMAE enzyme (Fig. 2A).

The incubation of homogenates of *Neurospora crassa* with each of the four $^{32}$P-labelled cytidine diphosphate esters led to distinct labelling of lipid fraction.

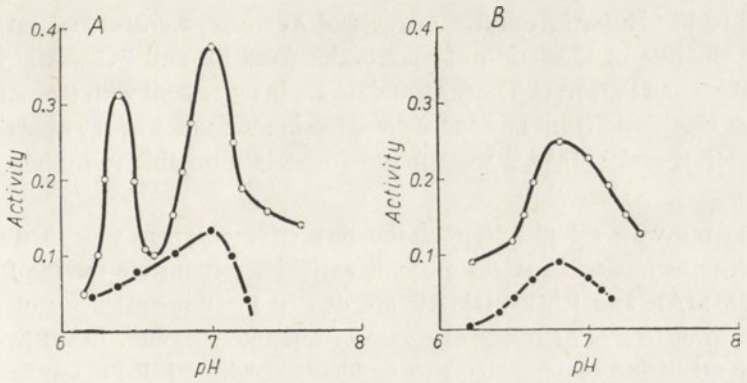


Fig. 2. Effect of pH on the activity of cytidylyltransferases from *Neurospora crassa*. Substrates: *A*, PE (○) and PMMAE (●); *B*, PC (○) and PDMAE (●). The 105 000 *g* supernatant of the homogenate of *Neurospora crassa* was used. The experimental conditions were the same as those of assay method, except that the pH of the system was varied from 5.6 to 8.0 using appropriate sodium phosphate buffers. The pH of the incubation mixture was measured with a Radiometer (Copenhagen) type 22 pH-meter. Activities are expressed in mμmoles of synthetized nucleotide per 1 min. per sample.

The rates of incorporation of $^{32}P$ into phospholipids calculated per gram of wet weight of mycelium were similar to that found in rat liver homogenate (about 0.2 μmole/g./hr.). Column chromatography on aluminum oxide of radioactive lipids formed in homogenates of *Neurospora crassa* from CMP-$^{32}$PE and CMP-$^{32}$PMMAE (Chojnacki & Korzybski, 1964) demonstrated that phosphatidylethanolamine and phosphatidylmonomethylaminoethanol, respectively, became labelled.

## DISCUSSION

Our experiments demonstrate that the cytidine pathway of the formation of lecithin and cephalin is operative in *Neurospora crassa*. This organism has been a classical tool in the studies on the methylation pathway of lecithin formation from cephalin since the finding of Horowitz & Beadle (1943) of the occurrence of choline-deficient mutants of *Neurospora crassa*. These types of mutants accumulate monomethyl- and dimethyl-cephalins because of the lack of one or more phospholipid *N*-methyl transferases. As far as we are aware, there are no data in the literature concerning the formation of lecithin in *Neurospora crassa via* the cytidine mechanism. The only finding that could have suggested the presence of this type of reaction was the detection of CMP-PC in extracts of *Neurospora crassa* by Smith & Wheat (1960).

The formation of monomethylcephalin and dimethylcephalin *via* the respective cytidine diphosphate bases does occur in *Neurospora crassa* similarly as it was found in rat liver and brain by Ansell & Chojnacki (1966). The incorporation of derivatives of ethanolamine, monomethyl-, dimethylethanolamine and choline

was found to be similar in cell-free extracts of *Neurospora crassa* and rat liver. In both cases the rate of formation of nucleotides from PE and PC was higher than from PMMAE and PDMAE. The present data are in agreement with the experiments of Ansell & Chojnacki (1966) on unfractionated homogenates of rat liver and brain where PMMAE and PDMAE were found to be less suitable substrates than PE and PC.

Experiments with gel filtration of the respective enzymes were performed in order to demonstrate whether the enzymic activities responsible for the formation of CMP-PMMAE and CMP-PDMAE are due to the non-specificity of PC- and PE cytidylyltransferases or to the existence of separate enzymes. In rat liver CMP-PDMAE synthetizing enzyme seems to be identical with CMP-PC synthetase. The enzymic activity responsible for the synthesis of CMP-PMMAE was eluted in the region of PE cytidylyltransferase, but in two equal well separated peaks, while in the elution of PE cytidylyltransferase the second enzymic peak was much smaller than the first one. The enzymic activity responsible for synthesis of CMP-PMMAE was due at least in part to an enzyme different from PE cytidylyltransferase.

On comparing the rates of formation of cytidine nucleotides in unfractionated homogenates and eluates from Sephadex G-200, marked losses of cytidylyltransferase activity during gel filtration, greater in *Neurospora crassa* than in rat liver, were observed. A similar loss was noticed in our previous paper (Chojnacki *et al.*, 1967).

In *Neurospora crassa* the synthesis of CMP-PC and CMP-PDMAE is probably due to one enzyme which has the same elution volume as that of liver. The synthesis of CMP-PE is catalysed by two well separated protein fractions while CMP-PMMAE synthetase seems to be a still different enzyme. This may also be concluded from the pH curves.

In an earlier paper from this laboratory (Radomińska-Pyrek, 1969) the occurrence of multiple forms of ethanolaminephosphate cytidylyltransferases has been demonstrated in chicken kidney and gut mucosa; this has now been confirmed in *Neurospora crassa*.

The authors express their gratitude to Dr. Anna Kruszewska for co-operation.

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## BIOSYNTEZA ESTRÓW CYTYDYNODWUFOSFOROWYCH W WĄTROBIE SZCZURA I U *NEUROSPORA CRASSA*

### Streszczenie

1. Biosynteza fosfatydyloetanoloaminy i fosfatydylocholiny u dzikiego szczepu *Neurospora crassa*, podobnie jak w wątrobie szczura, związana jest z tworzeniem cytydynodwufosfoetanoloaminy i cytydynodwufosfocholiny. Fosfomonometyloetanoloamina i fosfodwumetyloetanoloamina są także włączane do fosfolipidów przy udziale CTP.

2. Cytydylilotransferaza katalizująca powstawanie cytydynodwufosfodwumetyloetanoloaminy jest prawdopodobnie identyczna z enzymem syntetyzującym cytydynodwufosfocholinę. Natomiast synteza cytydynodwufosfomonometyloetanoloaminy i cytydynodwufosfoetanoloaminy katalizowana jest przez dwa różne enzymy.

Received 11 June, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

K. TOCZKO

# BASIC PROTEINS OF HOG RENAL CORTEX

## ISOLATION AND CHARACTERIZATION OF HISTONES

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From nucleohistones of hog renal cortex, five histones were isolated. Three of them, histones *f1*, *f2b*, and *f3*, were homogeneous on polyacrylamide gel electrophoresis, whereas histone *f2a* separated into two components. *N*-Terminal amino acids and the amino acid composition of the individual histone fractions were determined.

Histones, the basic proteins which are bound to DNA in the chromatin of animals and higher plants, are believed to play an important role in cell metabolism, especially in the regulation of the transcription process and maintaining of the fine structure of chromatin (Huang & Bonner, 1962; Allfrey, Littau & Mirsky, 1963; Bekhor, Kung & Bonner, 1969; Huang & Huang, 1969). It has been assumed that different organisms and tissues contain specific sets of histones, which could be expected to differ from each other. However, the results of chromatographic and electrophoretic analyses (Hnilica, Johns & Butler, 1962; Hnilica & Bess, 1965; Fambrough & Bonner, 1969) indicate that histones isolated from different organisms are remarkably alike; these results point to the existence of only a limited number of molecular species of histones.

Detailed analyses of histone fractions were performed only for a small number of animals, mainly calf and rat. The present communication describes the isolation and characteristics of histones from hog renal cortex.

## MATERIALS AND METHODS

Hog kidneys were removed immediately after the slaughter of the animals and cooled on ice; they were either processed within 1 hr., or stored at -20°. The time of storage did not exceed one week. For experiments, the renal cortex was isolated.

*Isolation of nucleohistones.* The procedure described by Hnilica (1966) was applied and the preparation obtained from 100 g. of renal cortex, after the last washing with ethanol, was suspended in 300 ml. of acetone and left for 30 min.

with occasional stirring. The suspension was then filtered and the residue washed thrice with 100 ml. portions of acetone, and finally with 200 ml. of peroxide-free diethyl ether.

*Isolation of whole histone.* From the preparation of nucleohistones, histones were extracted with 0.25 N-HCl as described by Johns, Phillips, Simson & Butler (1961). The extract was filtered through a sintered-glass G-4 funnel, dialysed for 16 - 18 hr. against 100 volumes of water and freeze-dried. All manipulations were carried out at 0 - 4°.

*Isolation of individual histones.* From nucleohistones, two fractions, *A* and *B*, were successively isolated according to the procedure 2 of Johns (1964). Fraction *A*, containing histones *f2a* and *f3*, was extracted with 0.25 N-HCl in 80% (v/v) ethanol, concentrated to 1/3 volume under reduced pressure in a rotatory evaporator, filtered through a sintered-glass G-4 funnel, dialysed overnight against 100 vol. of water and freeze-dried. Fraction B, containing histones *f1*, *f2b* and some histone *f3*, was extracted with 0.25 N-HCl, then filtered, dialysed against water and freeze-dried. All manipulations were carried out at 0 - 4°.

The two extracts *A* and *B* were resolved into individual histones by chromatography on CM-cellulose (Serva, Heidelberg, G.F.R.) as described by Hnilica (1966). The elution of proteins was followed by measuring the extinction at 280 mμ in a VSU-1 spectrophotometer. The eluates corresponding to each peak were pooled, dialysed against water and freeze-dried.

Histone *f1* was also isolated by the method 1 of Johns (1964) by direct extraction from nucleohistone with 5% perchloric acid.

*Disc electrophoresis.* Histone fractions were analysed by electrophoresis on polyacrylamide gel by the method of Johns (1967), except that a gel containing 15% of acrylamide, instead of 20%, was applied.

*N-Terminal amino acid analyses.* The fluorodinitrobenzene (DNP) method of Sanger as described by Phillips (1958) and the phenylisothiocyanate (PTH) method of Edman as modified by Eriksson & Sjöquist (1960), were used. The DNP-amino acids were separated and determined quantitatively by the procedure described by Bułhak & Toczko (1966), and PTH-amino acids were identified by thin-layer chromatography as described by Toczko & Szweda (1966).

*Assay methods.* Histone samples (3 - 5 mg.) were hydrolysed in 1 ml. of 5.7 N-HCl in sealed tubes for 24 hr. at 105°, and the amino acid analysis was performed using a Technicon amino acid analyser. Amino nitrogen was determined by the colorimetric method of Yemm & Cocking (1955).

## RESULTS

In preliminary experiments it was found that histone fractions negligibly contaminated by non-chromosomal protein, could be obtained only when nucleohistone was used as starting material.

The yield of whole histone obtained according to Johns *et al.* (1961) from 100 g. of

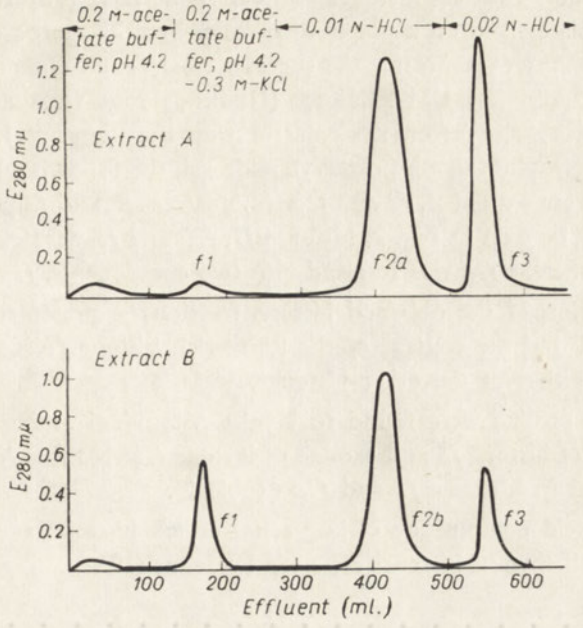


Fig. 1. Elution diagram of hog renal cortex histone extracts *A* and *B* on CM-cellulose column (19×120 mm.). The preparations were obtained by successive extraction of nucleohistones with 0.25 N-HCl in 80% (v/v) ethanol and with 0.25 N-HCl, respectively (method 2 of Johns, 1964). The proteins were eluted as indicated in the diagram; fractions of 6 ml. were collected at a flow rate of 30 ml./hr.

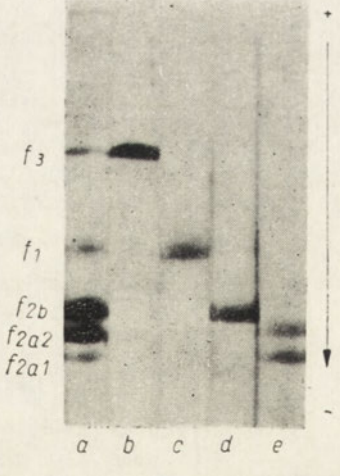


Fig. 2. Electrophoretic patterns of hog renal cortex histone fractions on 15% polyacrylamide gels, pH 2.4; staining with Amido Black. Histone fractions were isolated as described in the text. (*a*), Whole histone; (*b*), histone *f3*; (*c*), histone *f1*; (*d*), histone *f2b*; (*e*) histone *f2a*.

hog renal cortex was 248 mg., S.D. ± 19 mg. (average from 6 preparations). On polyacrylamide gel electrophoresis, at pH 2.4, the whole histone was separated into five components (Fig. 2a).

To identify the individual histones and determine their properties, an attempt was made to isolate them on a preparative scale. For this purpose, basing on the results of preliminary experiments, the successive extraction (Johns, 1964) together with chromatography on CM-cellulose (Hnilica, 1966) were adopted. Elution patterns of extracts *A* and *B* from nucleohistones are shown in Fig. 1. For subsequent analyses and electrophoresis on polyacrylamide gel, the following histone fractions were used: *f2a* from extract *A*, *f1* and *f2b* from extract *B*, and *f3* pooled from both extracts. As may be seen in Fig. 2, histones *f1*, *f2b* and *f3* were electrophoretically homogeneous, whereas *f2a* was resolved into two components, *f2a1* and *f2a2*.

The distribution of the obtained histone fractions is presented in Table 1. It should be noted that the content of the lysine-rich histone *f1* is remarkably low, being about half that of the other histones.

Alanine and proline were found to be the *N*-terminal amino acids of whole histone, alanine of histone *f3* and proline of histone *f2b*. No free *N*-terminal amino acids were found in histones *f1* and *f2a* (Table 2).

The amino acid composition of hog renal cortex histones as well as the data for analogous calf thymus histones reported by Johns (1964) and Hnilica, Edwards

Table 1

*Percentage distribution of histone fractions isolated from hog renal cortex nucleohistones*

The histone fractions were isolated by chromatography on CM-cellulose and submitted to acid hydrolysis; from the content of amino nitrogen, the distribution of the fractions was calculated.

| Analysis no. | Histone fraction (% of the total) | | | |
|---|---|---|---|---|
| | *f1* | *f2a* | *f2b* | *f3* |
| 1 | 10.9 | 33.9 | 29.0 | 26.2 |
| 2 | 11.7 | 34.5 | 27.7 | 26.1 |
| 3 | 12.2 | 35.2 | 26.8 | 25.8 |
| Mean | 11.6 | 34.5 | 27.8 | 26.0 |

Table 2

N-*Terminal amino acids in histones of hog renal cortex*

For details see Materials and Methods.

| Preparation | DNP-method | PTH-method |
|---|---|---|
| Whole histone | Alanine<br>Proline | Alanine<br>Proline |
| Histone *f1* | None | None |
| Histone *f2a* | Traces of alanine* | Traces of alanine |
| Histone *f2b* | Proline | Proline |
| Histone *f3* | Alanine | Alanine |

* Less than one mole per $5 \times 10^5$ g. of protein.

Table 3

*Amino acid composition of hog renal cortex histones and analogous calf thymus histones*

Values for the respective amino acids are expressed as moles/100 moles of all amino acids found, and are uncorrected for losses during acid hydrolysis. Data for calf thymus histones are taken from: I, Johns (1964), and II, Hnilica *et al.* (1966).

| Amino acid | Histone *f1* | | | Histone *f2a* | | | Histone *f2b* | | | Histone *f3* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hog cortex | Calf thymus | | Hog cortex | Calf thymus | | Hog cortex | Calf thymus | | Hog cortex | Calf thymus | |
| | | I | II | | I | II | | I | II | | I | II |
| Lys | 27.1 | 27.5 | 29.3 | 10.0 | 11.2 | 10.7 | 18.3 | 16.4 | 15.8 | 8.7 | 9.8 | 10.1 |
| His | 0.0 | 0.3 | 0.0 | 2.8 | 2.5 | 2.6 | 2.6 | 2.1 | 2.5 | 1.8 | 2.4 | 1.7 |
| Arg | 2.8 | 2.0 | 1.7 | 11.0 | 11.2 | 11.5 | 6.1 | 7.8 | 6.6 | 11.7 | 11.1 | 12.8 |
| Asp | 2.4 | 3.4 | 2.2 | 4.5 | 5.8 | 5.7 | 3.8 | 5.5 | 4.9 | 5.2 | 4.6 | 5.0 |
| Glu | 3.7 | 5.6 | 3.4 | 10.9 | 8.3 | 9.2 | 7.0 | 9.3 | 8.0 | 12.9 | 11.2 | 11.0 |
| Gly | 6.0 | 6.4 | 6.8 | 11.2 | 12.5 | 11.1 | 7.1 | 7.4 | 5.7 | 6.6 | 6.5 | 6.7 |
| Ala | 25.1 | 23.6 | 24.2 | 11.7 | 10.0 | 11.9 | 14.8 | 10.6 | 10.5 | 13.3 | 12.6 | 13.1 |
| Val | 5.2 | 9.8 | 4.5 | 7.1 | 14.1 | 5.8 | 7.0 | 11.2 | 7.0 | 4.9 | 12.6 | 4.9 |
| Leu | 4.2 | | 4.1 | 10.1 | | 10.8 | 5.6 | | 5.0 | 9.8 | | 9.6 |
| Ile | 1.4 | | 0.7 | 5.5 | | 4.4 | 4.5 | | 4.8 | 5.3 | | 4.7 |
| Ser | 6.5 | 5.4 | 6.9 | 2.9 | 3.9 | 3.2 | 7.6 | 7.4 | 11.0 | 4.6 | 4.7 | 3.9 |
| Thr | 5.8 | 5.1 | 6.2 | 4.0 | 5.1 | 5.2 | 5.5 | 5.7 | 6.2 | 6.1 | 6.4 | 6.3 |
| Pro | 8.6 | 9.7 | 8.7 | 2.5 | 3.0 | 3.4 | 5.4 | 5.0 | 4.6 | 4.0 | 4.8 | 4.5 |
| Tyr | 0.4 | 0.2 | 0.4 | 2.5 | 2.7 | 2.3 | 2.8 | 3.1 | 3.8 | 2.1 | 2.3 | 2.0 |
| Phe | 0.6 | 1.0 | 0.5 | 1.9 | 2.0 | 1.3 | 1.8 | 1.9 | 1.5 | 3.0 | 2.6 | 2.5 |
| Met | 0.0 | — | — | + | — | 0.4 | + | — | 1.4 | + | — | 1.1 |
| CySH | 0.0 | — | — | 0.0 | — | — | 0.0 | — | 0.0 | + | — | — |

& Hey (1966), are summarized in Table 3. In hog renal cortex histones, the presence of cysteine was found only in histone *f3* whereas methionine was present in all histones except *f1*. However, the results of these determinations were not quantified because of possible error due to losses of these two amino acids during acid hydrolysis. As shown in Table 3, the histones studied, similarly as the corresponding histones of calf thymus, displayed large differences in amino acid composition. In addition to typical differences in the content of lysine and arginine, the other major differences found were the absence in histone *f1* of histidine and methionine, and a low content of aromatic amino acids. As compared with other histones, *f2a* contained a higher amount of glycine, and histone *f3*, of glutamic acid.

## DISCUSSION

The histones obtained from hog renal cortex nucleohistones, were found to be similar to the homologous histones from calf thymus studied by Johns (1964) and Hnilica *et al.* (1966). The isolated histones had similar electrophoretic and chromatographic properties, identical *N*-terminal amino acids, and showed

remarkable resemblances in their amino acid composition. They differed, however, from calf thymus histones in their quantitative distribution. In hog renal cortex, histone *f1* formed a smaller percentage of whole histone than in calf thymus, and histone *f3* a correspondingly higher percentage, whereas the relative content of histones *f2a* and *f2b* was practically the same.

The presence in different animal species of an identical set of closely similar, if not identical, histones in organs which differ so widely in physiological function as kidney and thymus, seems to indicate that in various tissues the histones perform a similar biological function.

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I am deeply grateful to Professor Irena Chmielewska for her encouragement and continued interest in this work, and for helpful criticism of the manuscript. Thanks are also due to Doc. Dr. Maria Rakowska of the Institute of Food and Nutrition, Warsaw, for carrying out the amino acid analyses.

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## ZASADOWE BIAŁKA KORY NEREK WIEPRZA.
### WYDZIELENIE I CHARAKTERYSTYKA HISTONÓW

Streszczenie

Wykazano, że histony kory nerek wieprza rozdzielają się metodą elektroforezy w żelu poliakrylamidowym na pięć składników. Składniki *f1*, *f2b* i *f3* wydzielono w postaci elektroforetycznie jednorodnej, a pozostałe dwa składniki (*f2a1* i *f2a2*) razem. Poszczególne wydzielone preparaty scharakteryzowano przez oznaczenie ich składu aminokwasowego i aminokwasów *N*-końcowych.

Received 18 June, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

Z. RUCZAJ, DANUTA SAWNOR-KORSZYŃSKA, LIDIA PAŚŚ and KONSTANCJA RACZYŃSKA-BOJANOWSKA

# ON THE RELEASE OF ENZYMES UPON LYSOZYME TREATMENT OF *STREPTOMYCES*

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1. In *Streptomyces*, in contrast to Gram-negative *E. coli*, glucose-6-phosphate and isocitrate dehydrogenases are set free during conversion of mycelium to spheroplasts on lysozyme-EDTA treatment; the release of enzymes is unrelated to the kind of antibiotic produced by the investigated strains. 2. A similar release was observed in Gram-positive *B. subtilis* producing bacitracin. 3. Phosphatases in *Streptomyces* are either completely released as in *E. coli* or retained within spheroplasts to a species-characteristic extent. 4. The evidence from the electron micrographs and liberation of DNA suggest the effect of lysozyme on cytoplasmic membrane, possibly mesosomal tubules.

Spheroplasts have been widely used as an useful tool in biochemical studies on localization of enzymes in micro-organisms. Among the enzymes removing the cell wall, egg-white lysozyme is most frequently used to obtain spheroplasts, which are then subjected to osmotic shock or different washing treatments. In the present work, the release of enzymes during spheroplast formation was investigated in different strains of *Streptomyces*. The experiments concerned: (1), phosphatases, the enzymes most widely studied by this technique of cell fractionation, and (2), NADP-dependent glucose-6-phosphate and isocitrate dehydrogenases, because of their possible participation in regeneration of NADPH for erythronolide biosynthesis and a postulated role of the cell wall in the biosynthesis of antibiotics (Corcoran & Chick, 1966). The results were compared with those obtained with Gram-negative *E. coli* and Gram-positive *B. subtilis*.

## MATERIALS AND METHODS

*Reagents.* Lysozyme (from egg white, 25 units/mg.), glucose-6-phosphate and isocitrate were products of Sigma (St. Louis, Mo., U.S.A.), 2-mercaptoethanol of Light and Co. Ltd. (Colnbrook, Bucks., England) and $NADP^+$ of C. F. Boehringer Soehne (Mannheim, G.F.R.). Tris(hydroxymethyl)aminomethane was obtained from Loba-Chemie (Wien-Fischamend, Austria), glycyl-glycine from Reanal

(Budapest, Hungary) and Difco beef extract from Difco Laboratories (Detroit, Mich., U.S.A.). Other reagents used in this work were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

*Organisms and growth conditions.* The highly productive mutants of *Streptomyces erythreus* and *Streptomyces* sp. producing viomycin were obtained from Drs. B. Ostrowska-Krysiak and M. Tyc of the Division of Microbiology; *S. nursei*, *S. griseus*, *Escherichia coli* B. and *Bacillus subtilis* from the Strain Collection, Institute of Antibiotics, Warsaw.

The *Streptomyces* strains were grown in 50 ml. Erlenmayer flasks on a reciprocating shaker (240 strokes/min.) at 28 - 30° for 24 - 48 hr. on the complex soluble medium of the following composition per 100 ml. of water: glucose, 5.0 g.; soya flour extract, 3.0 g.; NaCl, 0.5 g.; $(NH_4)_2SO_4$, 0.3 g.; $CaCO_3$, 0.6 g.; pH 7.0. The growth medium of *Streptomyces* sp. producing viomycin contained additionally 1% of soya beans oil. *B. subtilis* was grown on the soluble medium composed of: soluble starch, 1.0 g.; peptone, 1.0 g.; Difco beef extract, 0.5 g.; NaCl, 0.25 g.; pH 7.0; and *E. coli* B on the medium containing per 100 ml. of water: peptone, 1.9 g.; Difco beef extract, 0.3 g.; NaCl, 0.5 g.; pH 7.0.

*Lysozyme-EDTA-tris and EDTA-tris treatment.* The 24 - 48 hr. mycelium of *Streptomyces* or the 24 hr. cultures of *E. coli* and *B. subtilis* were spun down at 2000 rev./min. for 15 min. at 0°, washed twice with cold water and suspended in 1.5 or 2.0 vol. of 20% sucrose (w/v) containing 10 mM-2-mercaptoethanol, 20 mM-tris buffer, pH 8.0, lysozyme (1.5 mg./g. wet wt.) and EDTA (9.3 mg./g. wet wt.). In some experiments tris was replaced by glycyl-glycine buffer of the same molarity and pH, and sucrose by mannitol of the same molarity. The suspensions of micro-organisms were agitated for 30 min. at 30° and spheroplast formation was controlled by microscopic examination and extinction measurements at 470 mμ. For determination of enzymic activity the spheroplasts were isolated by centrifuging at 16 000 rev./min. and disintegrated by sonication (M.S.E. sonicator) or by osmotic shock in 0.01 M-phosphate buffer, pH 7.4.

Alternatively, the *Streptomyces* mycelia were treated with EDTA-tris in 20% sucrose solution without lysozyme for 30 min. at 30°, centrifuged, and washed with cold 50 mM-$MgCl_2$ (Nossal & Heppel, 1966); the washings were collected for enzymic, DNA and protein determinations.

*Enzyme assay.* Glucose-6-phosphate dehydrogenase activity was determined spectrophotometrically at 340 mμ according to Kornberg & Horecker (1955) and isocitrate dehydrogenase after Ochoa (1955). The activities of glucose-6-phosphatase, inorganic pyrophosphatase, and alkaline and acid phosphatases were determined as previously described (Paśś & Raczyńska-Bojanowska, 1969).

*Determination of protein and DNA.* Protein was determined by the Lowry method as described by Layne (1955) and DNA according to Burton (1956), DNA being extracted three times with perchloric acid during 20 min.

*Measurement of respiration.* Respiration was measured by the Warburg technique: suspensions (2.5 ml.) of intact mycelium, lysozyme-EDTA treated mycelium or

isolated spheroplasts were respiring for 2 hr. at 30° in 20% sucrose containing 20 mM-tris buffer, pH 8.0, and 10 μmoles of sodium-succinate.

*Electron microscope examinations.* The *S. erythreus* mycelium and spheroplasts were fixed in 3.6% glutaraldehyde in 0.02 M-phosphate buffer, pH 7.4, for 2 hr. at 4° and with 1% osmium tetroxide in 0.02 M-phosphate buffer, pH 7.4, for the next 2 hr., dehydrated and embedded in Epon-812 and Vestopal W. The electron micrographs were taken with the Jem 7 Electron Microscope at the Department of Pathological Anatomy of the Warsaw Medical School.

## RESULTS

The successive stages of spheroplast formation upon the lysozyme-EDTA treatment of *S. erythreus* were followed by the phase-contrast microscopy; the long thread-like mycelium (Fig. 1A) became irregularly shaped (Fig. 1B), then was broken to short segments (Fig. 1C) to give rise finally to spherical bodies — spheroplasts. These easily burst when transferred to hypotonic solution, and sedimented rapidly.

As can be seen from Table 1, glucose-6-phosphate and isocitrate dehydrogenases in *S. erythreus* were totally released during spheroplast formation, similarly as in *B. subtilis* and in contrast to *E. coli* in which under the same conditions both enzymes were retained within spheroplasts. Glucose-6-phosphate dehydrogenase in the spheroplast supernatant fraction of *S. erythreus* was not sedimented upon a 2 hr. centrifugation at 105 000 *g*. The release of both dehydrogenases in this

Table 1

*Localization of glucose-6-phosphate and isocitrate dehydrogenases in* S. erythreus, E. coli *and* B. subtilis

The micro-organisms were treated with lysozyme and EDTA, and the "soluble fraction" was separated by centrifugation at 16 000 rev./min. Before activity determinations, the treated cells and spheroplasts were sonicated. In parentheses, the percentage distribution of activity is given.

| Organism | Enzyme | Activity (μmoles NADP/g. wet wt./min.) | | |
|---|---|---|---|---|
| | | Whole cells treated | "Soluble fraction" | Spheroplasts |
| *S. erythreus* | Glucose-6-phosphate dehydrogenase | 1.02 | 0.95 (98%) | 0.022 (2%) |
| | Isocitrate dehydrogenase | 1.18 | 1.18 (98%) | 0.033 (2%) |
| *E. coli* B | Glucose-6-phosphate dehydrogenase | 0.68 | 0 (0%) | 0.64 (100%) |
| | Isocitrate dehydrogenase | 1.53 | 0 (0%) | 1.75 (100%) |
| *B. subtilis* | Glucose-6-phosphate dehydrogenase | 0.93 | 0.90 (97%) | — |
| | Isocitrate dehydrogenase | 2.46 | 2.10 (85%) | — |

strain was the same with mannitol used instead of sucrose as the stabilizing medium during spheroplast formation. When intact *S. erythreus* mycelium was incubated in 20% sucrose containing 0.03 M-tris and 0.5 mM-EDTA without lysozyme, and then washed with 10 mM-$MgCl_2$ as applied after Neu & Heppel (1964) and Nossal & Heppel (1966), the dehydrogenases were not released and neither protein nor DNA were found in the washings.

In a nystatin producing *S. nursei* and a streptomycin producing *S. griseus*, glucose-6-phosphate dehydrogenase was found in the soluble fraction separated from spheroplasts formed upon EDTA-lysozyme treatment but, in contrast to *S. erythreus*, 50 and 30%, respectively, of this activity remained within the spheroplasts (Table 2). In *S. griseus*, glucose-6-phosphate dehydrogenase could not be detected in the sonicates of the whole mycelium unless sonication was preceded by the lysozyme treatment; the same was true for isocitrate dehydrogenase in *S. erythreus*. *Streptomyces* sp. producing viomycin was completely devoid of the glucose-6-phosphate dehydrogenase activity.

For purpose of comparison, liberation of hydrolases exemplified by phosphatases was studied during spheroplast formation in *Streptomyces*. A wide variation in the activity and extent of liberation of glucose-6-phosphatase, inorganic pyrophosphatase and alkaline and acid phosphatases was observed in the investigated strains (Table 3). Generally a low activity of all the phosphate splitting enzymes was found in *S. erythreus* as contrasted with other species. In *Streptomyces* sp. producing viomycin, these enzymes remained practically exclusively in spheroplasts, in contrast to *S. erythreus* in which alkaline and acid phosphatases were found only in the soluble fraction. Under the same conditions in this species glucose-6-phosphatase remained in spheroplasts. In *S. nursei* and *S. griseus* the phosphatases were set free in 30 - 70% of the total activity found in the lysozyme-treated, sonicated whole cells.

It was found that when spheroplasts were disintegrated by osmotic shock, acid phosphatase and inorganic pyrophosphatase in all the investigated strains, and glucose-6-phosphatase in *S. erythreus* and *S. griseus*, were liberated to the same extent as on ultrasonic treatment. In *S. nursei*, however, only 9% of glucose-6-phosphatase present in spheroplasts could be liberated by osmotic shock. The same holds for alkaline phosphatase which proved to be an enzyme firmly bound in *S. nursei*, *S. griseus* and, as previously shown (Paśś & Raczyńska-Bojanowska, 1968), in *Streptomyces* sp. producing viomycin. In the former two species 60 - 70% of the activity present in spheroplasts remained bound in the structures of the osmotically broken spheroplasts.

To check the biochemical integrity of the spheroplasts formed from *Streptomyces* mycelia, their respiration was measured with succinate as substrate. Respiration was practically unaffected when *S. erythreus* mycelium was treated with EDTA and lysozyme (150 and 142 µl. of $O_2$ taken up/g.wet wt./hr., respectively) but was reduced to one-half (72 µl. $O_2$) on separation of spheroplasts from the soluble fraction.

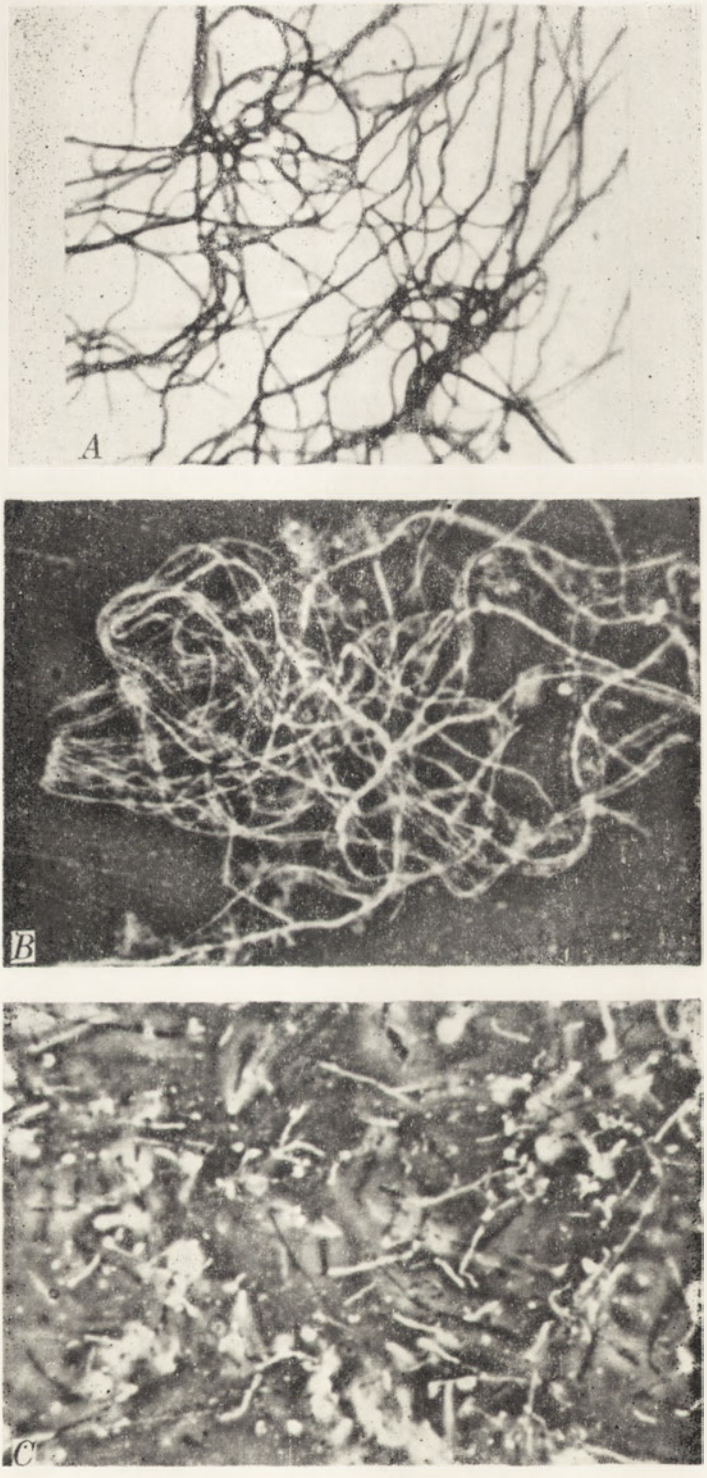


Fig. 1. Successive stages of spheroplast formation by the EDTA-lysozyme treatment of *S. erythreus*; phase contrast microscopy, mycelium stained with Gram reagent. Magnification: 1200×.

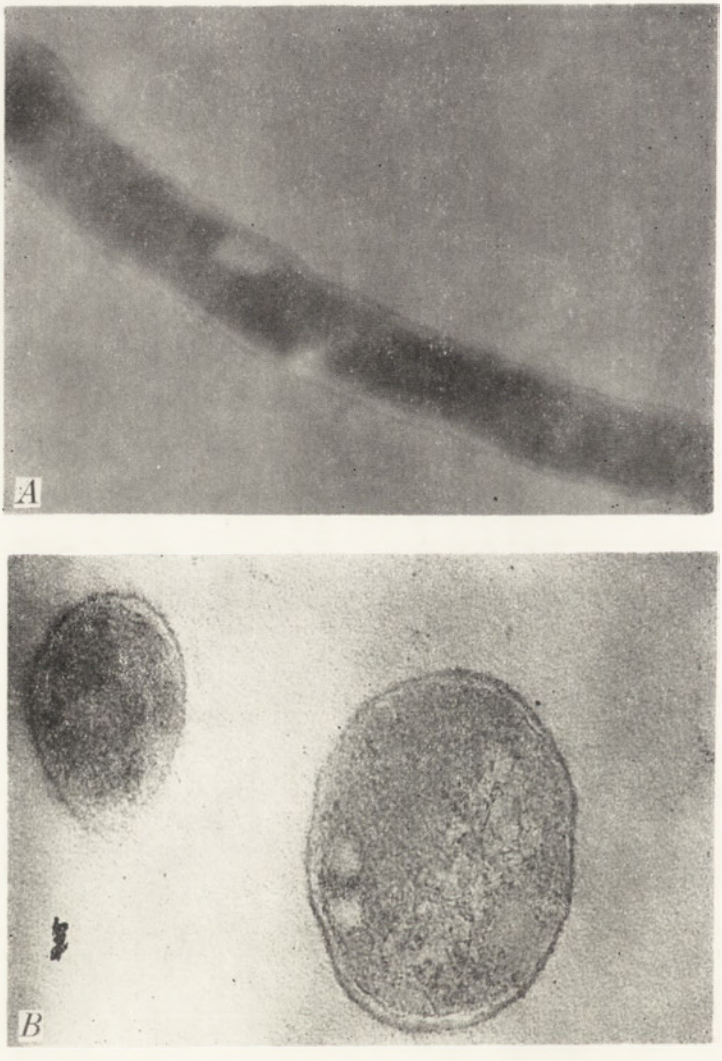


Fig. 2. Electron micrographs of longitudinal and cross sections of intact mycelium of *S. erythreus* fixed with glutaraldehyde and osmium tetroxide. Magnification: *A*, 70 000×; *B*, 122 000×.

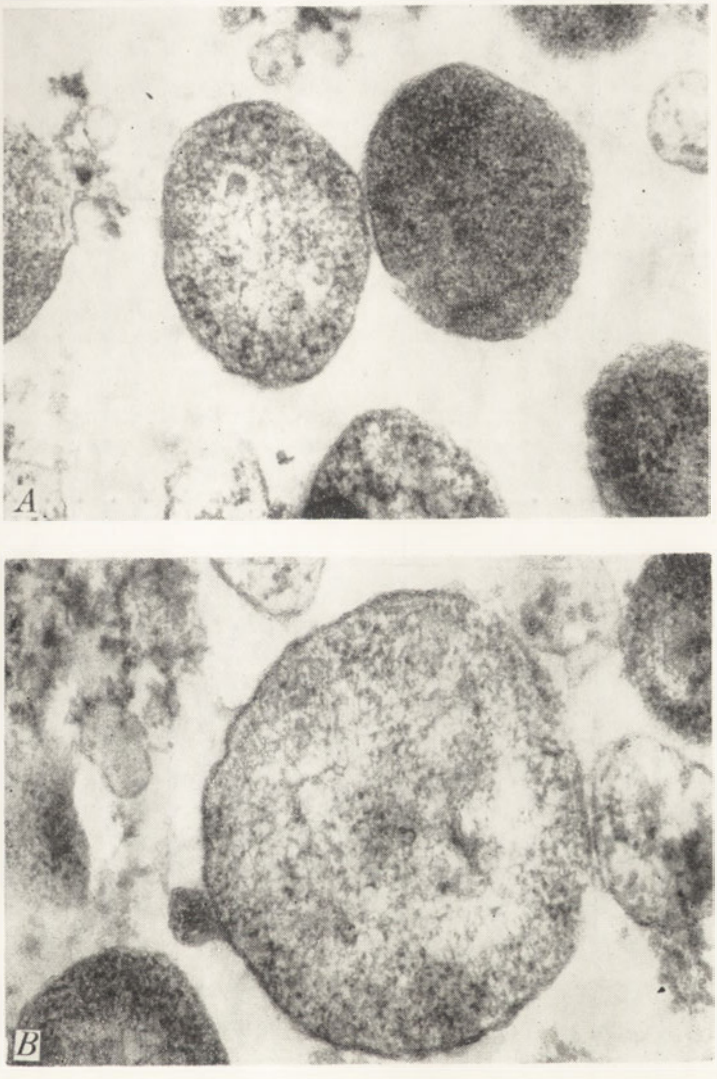


Fig. 3. Electron micrographs of spheroplasts of *S. erythreus* formed upon lysozyme-EDTA treatment, fixed as in Fig. 2. Magnification: 70 000×.

Table 2

*Distribution of glucose-6-phosphate dehydrogenase in* Streptomyces *strains following lysozyme-EDTA treatment*

Whole cells were treated, and spheroplasts and "soluble fraction" were obtained as indicated in Table 1. In parentheses, the percentage distribution of activity is given.

| *Streptomyces*, and antibiotic produced | Activity (μmoles NADP/g. wet wt./min.) | | |
|---|---|---|---|
| | Whole cells treated | "Soluble fraction" | Spheroplasts |
| *S. erythreus*, erythromycin | 1.02 | 0.95 (98%) | 0.022 (2%) |
| *S. nursei*, nystatin | 2.81 | 1.33 (47%) | 1.52 (53%) |
| *S. griseus*, streptomycin | 1.16 | 0.71 (67%) | 0.35 (33%) |
| *S.* species producing viomycin | 0 | 0 | 0 |

The release of enzymic protein on conversion to spheroplasts was associated in *S. erythreus* with an about 30% loss of DNA (19.9 - 21.7 μg./g. wet wt.).

Electron microscopic examination of the longitudinal and cross sections of *S. erythreus* mycelium showed a double-layered structure of the "unit" membrane type (Fig. 2A, B). Spheroplasts appeared as the confined spherical bodies with visible extrusions and irregular contours (Fig. 3A, B).

Table 3

*Distribution of phosphatases in* Streptomyces *strains following lysozyme-EDTA treatment*

The spheroplasts were centrifuged at 16 000 rev./min., and sonicated. The activity in spheroplasts and soluble fraction expressed in μmoles $P_i$/g. wet wt./15 min.; in parentheses the activity in soluble fraction in per cent of the total activity.

| *Streptomyces* fraction | Alkaline phosphatase | Acid phosphatase | Inorganic pyrophosphatase | Glucose-6-phosphatase |
|---|---|---|---|---|
| *S. erythreus* | | | | |
| spheroplasts | 0.02 | 0 | 1.38 | 0.71 |
| soluble fraction | 0.32 (94%) | 0.11 (100%) | 1.45 (57%) | 0.03 (4%) |
| *S. nursei* | | | | |
| spheroplasts | 3.92 | 5.32 | 5.31 | 1.82 |
| soluble fraction | 1.65 (30%) | 4.25 (45%) | 2.95 (35%) | 1.85 (50%) |
| *S. griseus* | | | | |
| spheroplasts | 8.32 | 3.68 | 5.14 | 2.14 |
| soluble fraction | 10.70 (46%) | 5.36 (68%) | 8.09 (65%) | 5.11 (83%) |
| *S.* species producing viomycin | | | | |
| spheroplasts | 6.03 | 1.24 | 11.60 | 1.93 |
| soluble fraction | 0.79 (12%) | 0 (0%) | 0.25 (2%) | 0.20 (9%) |

## DISCUSSION

None of the criteria available for localization of the enzymes in micro-organisms are rigorous (Pollock, 1962; Heppel, 1967) and the release of the enzymes upon lysozyme treatment cannot be considered as an unequivocal and comparable criterion in these studies. The use, however, of this bacteriolytic enzyme gives valuable information about distribution and binding of the enzymes to the subcellular structures.

Malamy & Horecker (1961, 1964) regard the enzymes released on conversion of Gram-negative *E. coli* to spheroplasts as the periplasmic enzymes, i.e. confined to the space between protoplasmic membrane and the wall layers. An additional proof for this localization is an easy washing out of the periplasmic enzymes by 0.1 - 10 mM-$MgCl_2$ solution from the cells subjected to cold water osmotic shock (Neu & Heppel, 1965; Nossal & Heppel, 1966). So far only hydrolases: asparaginase (Cedar & Schwartz, 1967) and the phosphate splitting enzymes including ribonuclease and deoxyribonuclease (Heppel, 1967) have been found in the supernatant separated from spheroplasts following the lysozyme treatment of *E. coli*. No DNA and 10 - 20% of protein were released under these conditions. The periplasmic localization of alkaline phosphatase (Done, Shorey, Loke & Pollak, 1965), cyclic phosphodiesterase and acid phosphatase (Spicer, Wetzel & Heppel, 1966) was confirmed histochemically.

The release of nucleosidase and nuclease was observed also upon conversion to spheroplasts of Gram-positive *B. subtilis* (Momose, Nishikawa & Katsuya, 1964; Birboim, 1966) and that of glycosidases during protoplasting of fungi (Villenueva, 1966). Liberation of phosphate splitting enzymes was found also in our experiments with Gram-positive *Streptomyces*, organisms phylogenetically intermediate between eubacteria and fungi. In this respect the observed variation in the kind and extent of liberation of phosphatases is noteworthy in connection with a similar phenomenon reported for yeasts (Villenueva, 1966).

The release of dehydrogenases upon lysozyme treatment of *Streptomyces* and *B. subtilis* may be explained by the resemblance of the structure of these Gram-positive micro-organisms. In contrast to Gram-negative bacteria, they are characterized by the extensive membraneous components continuous with the plasma membrane (Glauert & Hopwood, 1960; Ryter, 1968). These mesosomal structures are thought to be a site of oxido-reductive processes in bacteria and are connected with the nucleoid material (Ryter, 1968). Exposure of Gram-positive bacteria to hypertonic medium induces extrusion of the mesosomes from cytoplasm into a space between the membrane and the cell wall and their subsequent release into the medium when the cell wall is digested by lysozyme (Ryter & Landman, 1964; Fitz-James, 1965). The release of mesosomes is probably due to the combined action of various factors: differences in mesosomal configuration (Ryter, 1968), varied carbohydrate content (Salton, 1968) and other factors such as inorganic ion concentration. Rogers, Reaveley & Burdet (1968) found that the stability of

mesosomes of *B. licheniformis* depends on Mg concentration in stabilizing medium and found a correlation between the liberation of $K_3Fe(CN)_6$ reductase into the supernatant fluid and the liberation of mesosomes as evidenced by electron microscopy.

It seems that the release of glucose-6-phosphate and isocitrate dehydrogenases in *Streptomyces* are connected with the lability of the membraneous mesosomal structure during protoplasting with lysozyme and involve leakage of DNA. The low recovery of dehydrogenases in the sonicates from mycelia non-treated with lysozyme, and their retention within the cell under conditions of "cold water shock" suggest binding of these enzymes with the subcellular structures similar to that observed for membraneous ATPase (Munoz, Nachbar, Schor & Salton, 1968).

The release of glucose-6-phosphate dehydrogenase in *S. erythreus* treated with lysozyme and the intraspheroplast localization of glucose-6-phosphatase — the enzymes regulating glucose metabolism — and a similar separation of metabolically interlinked X-amidinotransferase and alkaline phosphatase in *Streptomyces* sp. producing viomycin (Paśś & Raczyńska-Bojanowska, 1969) may indicate the role of compartmentalization of these enzymes in metabolic regulations in *Streptomyces*.

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Our thanks are due to Dr. W. Biczysko and Mr. M. Walski, M.S., for the electron micrographs of *Streptomyces* mycelium and spheroplasts.

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## UWALNIANIE ENZYMÓW PRZEZ LIZOZYM U PROMIENIOWCÓW

### Streszczenie

1. U promieniowców, przeciwnie niż u *E. coli* (gram-ujemne), dehydrogenazy glukozo-6-fosforanowa i izocytrynianowa są uwalniane podczas przekształcania grzybni w sferoplasty pod wpływem lizozymu i EDTA; właściwość tę wykazują wszystkie badane szczepy promieniowców niezależnie od rodzaju wytwarzanego antybiotyku.

2. Podobne uwalnianie dehydrogenaz obserwowano w tych samych warunkach u *B. subtilis* (gram-dodatnie) wytwarzającego bacytracynę.

3. Fosfatazy u promieniowców są uwalniane w całości, jak u *E. coli*, lub pozostają w sferoplastach w stopniu charakterystycznym dla danego gatunku.

4. Zdjęcia z mikroskopu elektronowego sferoplastów oraz uwalnianie DNA sugerują wpływ lizozymu na membranę cytoplazmatyczną, prawdopodobnie na jej część mezozomalną.

Received 20 June, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

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# THE ROLE OF A SOLUBLE FRACTION AND RIBOSOMES IN THE FIDELITY OF POLY U TRANSLATION IN BACTERIAL CELL-FREE SYSTEMS

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1. Washing of *Bacillus stearothermophilus* ribosomes with 0.5 M-$NH_4Cl$, dissociation into subunits and purification with 0.2 M-sucrose leads to several-fold increase of ambiguity in poly U translation. 2. Addition of the wash restores the accuracy of translation. 3. A heterologous system containing washed *B. stearothermophilus* ribosomes and *E. coli* supernatant, translates poly U with high fidelity. This is due to the presence in the *E. coli* supernatant of a thermolabile factor(s) involved in the accuracy of translation. 4. The fidelity of poly U translation in the studied systems is dependent on the presence of substances which occur both in ribosomes and in the soluble fraction.

Ambiguity of polyuridylic acid (poly U) translation in an *E. coli* cell-free system was first demonstrated by Nirenberg and coworkers (Matthaei, Jones, Martin & Nirenberg, 1962) and Bretscher & Grunberg-Manago (1962). They have shown that poly U can stimulate incorporation not only of phenylalanine but also of leucine and valine. Ambiguity of translation has also been demonstrated for other synthetic polynucleotides (Friedman & Weinstein, 1964, 1965; Davies, 1966; Nishimura, Harada & Hirabayashi, 1969), and it is known to be dependent on environmental conditions, such as temperature, $Mg^{2+}$ concentration (Szer & Ochoa, 1964), addition of ethanol (So & Davie, 1965), polyamines (Friedman & Weinstein, 1964), aminoglycoside antibiotics (Davies, Gilbert & Gorini, 1964), concentration of sRNA, and pH of the medium (Grunberg-Manago & Dondon, 1965). Ambiguity of translation may be dependent also on the mode of preparation of the cell-free system (Lamfrom & Grunberg-Manago, 1967), and the origin of the isolated system (Friedman, Berezney & Weinstein, 1968). Preparations obtained from different bacteria translate synthetic polynucleotides with different fidelity. The system from *B. stearothermophilus* (Friedman & Weinstein, 1966) translates poly U with a much higher ambiguity than does a system from *E. coli* under the same conditions

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(Szer & Ochoa, 1964). This suggests that the accuracy of translation is dependent on the presence of components involved in protein biosynthesis, characteristic of a given organism.

In the present work it has been demonstrated that ribosomes from *B. stearothermophilus* contain substances the removal of which enhances the ambiguity of poly U translation. The addition of a supernatant from *E. coli* to these ribosomes abolishes the ambiguity. A preliminary account of this work has been published (Perzyński, Chomczyński & Szafrański, 1969).

## MATERIALS AND METHODS

*Reagents.* The chemicals used were purchased from the following sources: yeast extract from Difco Lab. (Detroit, Mich., U.S.A.); Proteobak from Bacutil (Warsaw, Poland); L-[U-$^{14}$C]phenylalanine (sp. act. 21 c/mole), L-[U-$^{14}$C]leucine (sp. act. 40 c/mole) and L-[4,5-$^{3}$H]-leucine (sp. act. 200 c/mole) from the Radiochemical Centre (Amersham, Bucks., England); [$^{12}$C]amino acids and creatine phosphokinase from Calbiochem (Los Angeles, Calif., U.S.A.); phosphocreatine, ATP and GTP from Sigma Chem. Corp. (St. Louis, Mo., U.S.A.); polyuridylic acid from Miles Chem. Co. (Elkhart, Ind., U.S.A.); deoxyribonuclease (ribonuclease-free) from Worthington (Freehold, N.J., U.S.A.); 2,5-diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazol-2-yl)-benzene (POPOP), hydroxide of hyamine 10-X from Packard Instrument Co. Inc. (Downers Grove, Ill., U.S.A.), and Sephadex G-25 from Pharmacia (Uppsala, Sweden).

*Growth of bacteria. Bacillus stearothermophilus* was obtained from the Department of Microbiology of the Warsaw University. The bacteria were grown at the optimum temperature (60°) in a medium containing in 1 litre: 5 g. of yeast extract, 10 g. of Proteobak, 5 g. of glucose and 5 g. of NaCl, and adjusted to pH 7.2 with 1 N-NaOH. The bacteria grown on agar slants (2% agar and the above growth medium) were suspended in 30 ml. of the medium and after 12 hr. transferred to 300 ml. of the medium and cultured until the extinction at 650 mμ reached a value of 0.6. Then the bacterial suspension was transferred to 4 litres of the medium and shaken for about 2 hr. until $E_{650}$ reached a value of 0.4 (the mid-phase of logarithmic growth). The culture was then cooled by addition of solid carbon dioxide, the cells harvested by centrifugation, washed twice with TNM buffer (10 mM-tris-HCl, pH 7.8 - 70 mM-$NH_4Cl$ - 14 mM-magnesium acetate - 6 mM-2-mercaptoethanol) and stored at -40° until used.

*Escherichia coli* B (from the collection of the State Institute of Hygiene in Warsaw, Poland) was grown at 32° and *Pseudomonas fluorescens* PCM 500 (from the collection of The Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland) was grown at 20° as described by Perzyński & Szafrański (1967).

*Preparation of the 122 000 g supernatant and ribosomes.* The bacteria frozen in solid carbon dioxide were disrupted in an Eaton press (Eaton, 1962) and suspended

in two volumes of TNM buffer. The suspension was adjusted to pH 7.4 with 1 M-tris, added with deoxyribonuclease (2 μg./ml.) and centrifuged at 30 000 *g* for 30 min. The supernatants (fraction S-30) were collected and preincubated in a mixture prepared according to Nirenberg & Matthaei (1961), the preincubation time being for *E. coli* 60 min. at 37°, for *B. stearothermophilus* 10 min. at 60° and for *Ps. fluorescens* 20 min. at 20°. All further steps of the procedure were carried out at 4°. The preincubated S-30 fractions were centrifuged for 2.5 hr. at 122 000 *g*. The upper three-quarters of the supernatant was collected, centrifuged again under the same conditions, then dialysed overnight against 100 volumes of TNM buffer and stored at -40° (supernatant S-122).

The sedimented ribosomes were suspended in 0.5 M-$NH_4Cl$-tris buffer, pH 7.8 (0.5 M-$NH_4Cl$, neutralized to pH 7 - 0.01 M-tris-HCl, pH 7.8 - 0.014 M-magnesium acetate - 0.006 M-2-mercaptoethanol) and centrifuged for 15 min. at 14 000 *g* and then for 2.5 hr. at 122 000 *g*. The ribosomes were suspended in a small volume of the same 0.5 M-$NH_4Cl$-tris buffer, centrifuged for 15 min. at 14 000 *g*, and dialysed overnight against TNM I buffer (70 mM-$NH_4Cl$ - 0.1 mM-magnesium acetate - 10 mM-tris-HCl buffer, pH 7.8). The dissociated ribosomes were applied on a layer of 0.2 M-sucrose in TNM I buffer and centrifuged for 4 hr. at 170 000 *g*. The pellet of ribosomes was suspended in TNM buffer, at a concentration of about 20 mg. per 1 ml. ($E^{1\%}_{1\,cm.} = 150$) and, after centrifuging off the undissolved material for 15 min. at 14 000 *g* stored at -40° in small portions.

*Preparation of sRNA. B. stearothermophilus* and *E. coli* sRNA's were obtained by the method of Zubay (1962).

*Acylation of sRNA. E. coli* sRNA was acylated with [$^{14}$C]phenylalanine or [$^{3}$H]leucine after Zubay (1962) in a mixture containing in a volume of 2.5 ml.: 100 mM-cacodylic buffer, pH 7.0, 10 mM-magnesium acetate, 70 mM-$NH_4Cl$, 5 mM-ATP, 0.5 mM-[$^{14}$C]phenylalanine or 0.5 mM-[$^{3}$H]leucine, nineteen [$^{12}$C]amino acids (each at 0.5 mM concn.), S-122 supernatant from *E. coli* (2.2 mg. of protein), and sRNA from *E. coli* (10 mg.). After 15 min. at 37°, the reaction mixture was shaken with an equal volume of freshly distilled phenol for 20 min. at 4°. The aqueous phase was collected and the phenol layer was shaken again with an equal volume of 1 mM-cacodylic buffer, pH 6.0. The pooled aqueous phases were applied to a Sephadex G-25 column and eluted with the above buffer. The acylated sRNA depleted of free amino acids was collected and freeze-dried.

*Incorporation of amino acids.* In the experiments, the technique of double labelling with $^{14}$C and $^{3}$H (De Wachter & Fiers, 1967) was used. The incubation mixture contained in a volume of 0.25 ml.: ribosomes (0.1 mg. of protein), S-122 supernatant (0.4 mg. of protein), 50 mM-tris-HCl buffer, pH 7.8, 70 mM-$NH_4Cl$, 14 mM-magnesium acetate, 2 mM-ATP, 0.5 mM-GTP, 10 mM-phosphocreatine, 0.012 mg. of creatine phosphokinase, 6 mM-2-mercaptoethanol, 0.1 mg. of *E. coli* sRNA, 0.025 mg. of poly U, 50 mM-[$^{14}$C]phenylalanine, 50 mM-[$^{3}$H]leucine, and eighteen [$^{12}$C]amino acids (each at 50 mM concn.). In each experiment a mixture containing no poly U was used as a reference sample. After 40 min. of incubation at 37°, 1 mg.

of albumin was added, and then 5 ml. of 5% trichloroacetic acid (TCA) containing 2% casein hydrolysate. The centrifuged sediment was washed with 5% TCA, dissolved in 0.2 ml. of 1 N-KOH, precipitated with 5 ml. of 10% TCA, centrifuged, suspended in 5% TCA, heated for 15 min. at 90°, and centrifuged again. For radioactivity determination, the sediment was washed with ethanol - ether (3:1, v/v) mixture, then with ether, dissolved in 0.4 ml. of hyamine and transferred to the scintillation vial containing PPO and POPOP solution in toluene. The measurements were done in a Packard Tri-Carb liquid scintillation spectrometer. In the technique of double counting, the efficiency for $^{14}C$ and $^{3}H$ was 26% and 28%, respectively.

*Protein determination.* The method of Lowry, Rosebrough, Farr & Randall (1951) was used.

## RESULTS

*The effect of mode of preparation of ribosomes on the ambiguity of translation.* The effect of the procedure applied for the preparation of *B. stearothermophilus* ribosomes on the ambiguity of poly U translation in a homologous system is shown in Table 1. The ambiguity is expressed as percentage of leucine to phenylalanine net incorporation. The experiments presented in Table 1 were performed by the technique of double labelling. In some parallel experiments, [$^{14}C$]phenylalanine or [$^{14}C$]leucine was used separately, and practically the same results were obtained. Washing of the ribosomes with 0.5 M-$NH_4Cl$ did not influence significantly the fidelity of poly U translation as compared with that of the preincubated fraction S-30. Additional purification in 0.2 M-sucrose caused an increase in ambiguity to 11%. Washing of the ribosomes with 1 M-$NH_4Cl$ raised the ambiguity to 14%.

Table 1

*The effect of mode of preparation of ribosomes on the ambiguity of poly U translation in the system from* B. stearothermophilus

The S-30 fraction was preincubated for 10 min. at 60°, the ribosomes were sedimented at 122 000 *g* and treated as indicated in the Table. The conditions of incorporation are described in Methods. The ambiguity is expressed as percentage of leucine to phenylalanine net incorporation.

| Mode of ribosomes preparation | Amino acid incorporation (μμmoles/mg. ribosomal protein) | | Ambiguity (%) |
|---|---|---|---|
| | Phe | Leu | |
| Fraction S-30 (control) | 970 | 40 | 4 |
| Washing with 0.5 M-$NH_4Cl$-tris buffer | 920 | 55 | 6 |
| Washing with 1 M-$NH_4Cl$-tris buffer | 93 | 13 | 14 |
| Washing with 0.5 M-$NH_4Cl$-tris buffer and 0.2 M-sucrose | 830 | 88 | 11 |
| Washing with 0.5 M-$NH_4Cl$-tris buffer and dissociation into subunits | 460 | 170 | 37 |
| Washing with 0.5 M-$NH_4Cl$-tris buffer, dissociation, and washing with 0.2 M-sucrose | 570 | 300 | 53 |

The system containing ribosomes washed with 0.5 M-$NH_4Cl$ and dissociated at low $Mg^{2+}$ concentration, exhibited an ambiguity of 37%, whereas additional purification of ribosomes in 0.2 M-sucrose resulted in a still greater ambiguity (53%). These results suggest that the components which were washed out from the ribosomes during the purification procedure, are involved in the accuracy of poly U translation.

*The effect of the wash on the fidelity of poly U translation.* The 170 000 *g* supernatant obtained after centrifugation of the dissociated *B. stearothermophilus* ribosomes in 0.2 M-sucrose solution, contained protein and nucleic acids. Its effect on poly U-directed incorporation of phenylalanine and leucine was studied in *B. stearothermophilus* system (Table 2). When the wash was added to the system, the ambiguity decreased to a half of the value obtained in its absence.

Table 2

*The effect of the wash from* B. stearothermophilus *ribosomes on the fidelity of poly U translation*

To the incubation mixture containing the preparation of dissociated and washed ribosomes, the wash (56 μg. protein) was added. The conditions of incorporation were as described in Methods.

| Expt. no. | Incubation mixture | Amino acid incorporation (μμmoles/mg. ribosomal protein) | | Ambiguity (%) |
|---|---|---|---|---|
| | | Phe | Leu | |
| 1 | Control | 457 | 210 | 46 |
| | Supplemented with wash | 127 | 30 | 21 |
| 2 | Control | 325 | 157 | 48 |
| | Supplemented with wash | 141 | 31 | 22 |

Table 3

*Translation of poly U in cross experiments with systems derived from* E. coli *and* B. stearothermophilus

The ribosomes, washed, dissociated and purified with sucrose solution, were used to study the incorporation of amino acids in the presence of homologous and heterologous S-122 supernatants. The conditions of incorporation were as described in Methods.

| Source of ribosomes | Source of S-122 supernatant | Amino acid incorporation (μμmoles/mg. ribosomal protein) | | Ambiguity (%) |
|---|---|---|---|---|
| | | Phe | Leu | |
| *B. stearothermophilus* | *B. stearothermophilus* | 426 | 224 | 53 |
| | *E. coli* | 1910 | 20 | 1 |
| *E. coli* | *E. coli* | 6103 | 241 | 4 |
| | *B. stearothermophilus* | 5450 | 420 | 8 |

*The fidelity of poly U translation in mixed systems.* To study the effect of the S-122 supernatant on the accuracy of poly U translation, cross experiments were carried out with systems derived from *B. stearothermophilus* and *E. coli* (Table 3). High ambiguity of translation, 53%, was observed only in the homologous system from *B. stearothermophilus*. Substitution of the S-122 supernatant from *B. stearothermophilus* by the supernatant from *E. coli* lowered the ambiguity to 1%. The homologous system from *E. coli* showed only an ambiguity of 4%, which with *B. stearothermophilus* supernatant increased to 8%. In the case of *B. stearothermophilus* ribosomes, the accuracy of poly U translation was also restored by the S-122 supernatant from *Ps. fluorescens*. It should be noted that the homologous cell-free system from *Ps. fluorescens* showed a high fidelity of poly U translation, similar to that observed in the *E. coli* system.

The results obtained in the above experiments were not dependent on the origin of sRNA; the same fidelity of poly U translation was observed with sRNA derived either from *E. coli* or *B. stearothermophilus*. Also the use of *E. coli* sRNA previously acylated with [$^{14}$C]phenylalanine or [$^{3}$H]leucine, gave the same results.

The presented experiments indicate that the ribosome-free S-122 supernatants influence the accuracy of poly U translation in the bacterial systems studied. An additional proof for this suggestion was obtained in the experiment in which gradual substitution of *B. stearothermophilus* supernatant by *E. coli* supernatant caused a decrease in the ambiguity of translation from 56% to 8% (Fig. 1). In subsequent

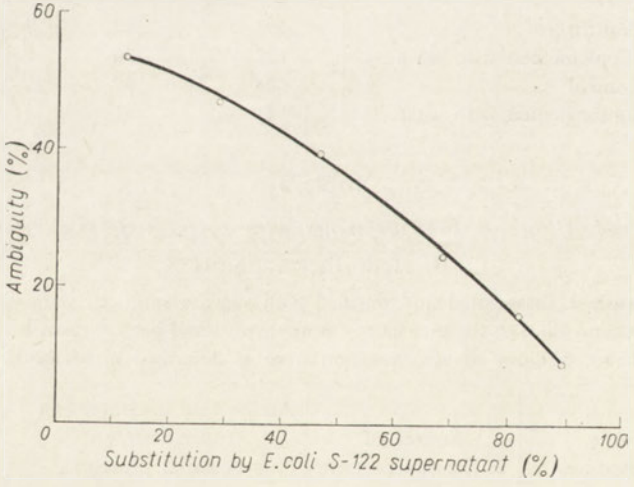


Fig. 1. The effect of gradual substitution of S-122 supernatant in *B. stearothermophilus* system by *E. coli* supernatant, on precision of poly U translation. Incorporation of [$^{14}$C]phenylalanine and [$^{3}$H]leucine was carried out as described in Methods.

experiments the effect of heating of the *E. coli* supernatant to 100° was checked (Table 4). The assays were performed with two different amounts of *B. stearothermophilus* supernatant, the same amount of *E. coli* supernatant being added

Table 4

*The effect of heating of the* E. coli *S-122 supernatant on the fidelity of poly U translation in the system from* B. stearothermophilus

To the incubation mixture containing the dissociated and washed ribosome preparations from *B. stearothermophilus*, the indicated amounts of S-122 supernatants were added. The *E. coli* supernatant protein was denatured by heating for 5 min. at 100°.

| S-122 supernatant from | | Amino acid incorporation (μμmoles/mg. ribosomal protein) | | Ambiguity (%) |
|---|---|---|---|---|
| *B. stearothermophilus* (μg. protein) | *E. coli* (μg. protein) | Phe | Leu | |
| 400 | None | 301 | 170 | 55 |
| 220 | 315, native | 84 | 18 | 21 |
| 110 | 315, native | 56 | 10 | 18 |
| 220 | 315, denatured | 198 | 99 | 50 |
| 110 | 315, denatured | 172 | 71 | 41 |

to either system. The native *E. coli* supernatant decreased the ambiguity by a half, while the heated supernatant had no effect. These results indicate that the components involved in the fidelity of poly U translation, present in the *E. coli* supernatant, are thermolabile.

## DISCUSSION

The effect of factors involved in the fidelity of translation of messenger RNA (mRNA) has been studied in several laboratories and some of the experiments suggest an active role of ribosomes in codon recognition during protein synthesis (Davies *et al.*, 1964; Lamfrom & Grunberg-Manago, 1967; Kaji, 1967; Friedman *et al.*, 1968; Rosset & Gorini, 1969). It is assumed that the correct translation of the matrix depends on the complementation between the corresponding base triplets according to the genetic code. A number of factors, however, mentioned in the introduction, influences the mRNA - tRNA - ribosome complex leading to ambiguity in translation of the matrix. The effect of these factors differs depending on the origin of the ribosomes. A classical example is the binding of streptomycin to ribosomes, resulting in a high ambiguity only in streptomycin-sensitive bacterial systems (Davies *et al.*, 1964). Lamfrom & Grunberg-Manago (1967) who studied the translation of poly U in a cell-free system from reticulocytes, postulated that the mode of preparation of ribosomes affected the precision of the codon-anticodon interaction. In our experiments it has been demonstrated that washing of *B. stearothermophilus* ribosomes with 0.5 M-$NH_4Cl$, dissociation into subunits, and then purification with 0.2 M-sucrose, leads to a several-fold higher poly U-directed incorporation of leucine, as compared with unwashed ribosomes. The system from *E. coli*, containing ribosomes prepared in the same way as those of *B. stearothermo-*

*philus*, gave in our experiments but a slight degree of ambiguity. This might indicate that in the case of *E. coli* ribosomes the factors essential for the accuracy of poly U translation, were not eluted. It seems possible that such factors exist also in *E. coli* ribosomes but are more tightly bound than those of *B. stearothermophilus* ribosomes.

The role of the S-122 supernatant in the accuracy of poly U translation has also been demonstrated. In the *B. stearothermophilus* system, substitution of the homologous supernatant by *E. coli* supernatant caused a decrease in ambiguity from 53 to 1%; a similar result was obtained when the supernatant from *Ps. fluorescens* was used. This effect was found to be due to the presence in those supernatants of some thermolabile substances which are involved in the accuracy of poly U translation. Preliminary experiments on the fractionation of *E. coli* supernatant at pH 5 indicate that these factors exist mainly in the post pH 5 fraction. On the basis of presented results we conclude that the fidelity of poly U translation is dependent on the presence of some substances which may occur both in ribosomes and in the soluble fraction. Work on isolation and characterization of these substances is in progress.

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This work was supported in part by Grant FG-PO 232 from the U.S. Department of Agriculture.

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## ROLA ROZPUSZCZALNEJ FRAKCJI I RYBOSOMÓW W WIERNOŚCI TRANSLACJI POLI U W BEZKOMÓRKOWYCH UKŁADACH BAKTERYJNYCH

Streszczenie

1. Płukanie rybosomów *Bacillus stearothermophilus* 0.5 M-$NH_4Cl$, dysocjacja na podjednostki i oczyszczanie 0.2 M-sacharozą powoduje kilkakrotne zwiększenie dwuznaczności translacji poli U.

2. Dodanie materiału wypłukanego z rybosomów do układu bezkomórkowego przywraca dokładność translacji.

3. Układ mieszany zawierający płukane rybosomy z *B. stearothermophilus* i supernatant z *E. coli*, odczytuje poli U z wysoką wiernością. Jest to spowodowane obecnością w supernatancie z *E. coli* termolabilnych czynników wpływających na precyzję procesu translacji.

4. Wierność translacji poli U w badanych układach zależna jest od obecności pewnych substancji, które występują zarówno w rybosomach, jak i we frakcji rozpuszczalnej.

Received 3 July, 1969.

ACTA BIOCHIMICA POLONICA

Vol 16 1969 No. 4

# RECENZJE KSIĄŻEK

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS (R. T. Holman, ed.) vol. 10, part 1. Pergamon Press, 1967; str. 150, cena $ 7.75.

Omawiany tom przynosi jedną monograficzną pracę C. C. Lucasa i J. H. Ridout poświęconą zjawisku stłuszczenia wątroby i czynnikom lipotropowym. Autorzy zajmują się jedynie stłuszczeniem wątroby spowodowanym niedoborem czynników pokarmowych, pomijają natomiast zagadnienie stłuszczenia wątroby pochodzenia toksycznego. Książka składa się z siedmiu rozdziałów, w których omówiono powstawanie stłuszczenia wątroby u zwierząt laboratoryjnych, a także towarzyszące mu inne zjawiska patologiczne. W wielu miejscach książka ma charakter pracy eksperymentalnej; na przykład w rozdziale drugim podano obszerne zestawienia i omówienia różnych metod ekstrakcji lipidów z materiału biologicznego. Większa część monografii traktuje o najważniejszym czynniku lipotropowym — cholinie. Opisano również przemiany grupy metylowej z uwzględnieniem literatury do roku 1964.

Książka zawiera wiele ciekawych danych odnośnie funkcji wątroby w metabolizmie tłuszczy. Warto zaznaczyć, że autorami omawianej monografii są znani specjaliści, którzy w początkach lat trzydziestych rozpoczęli wraz z H. C. Bestem w Toronto badania nad czynnikami lipotropowymi.

Książka stanowić będzie cenną lekturę tak dla biochemików jako literatura podstawowa o roli metabolicznej choliny, jak i dla fizjologów i lekarzy klinicystów jako szczegółowa monografia omawiająca mechanizm stłuszczenia wątroby.

*Tadeusz Chojnacki*

SYMPOSIUM ON MODERN METHODS IN THE INVESTIGATION OF PROTEIN STRUCTURE (F. B. Straub & P. Friedrich, eds.) Akadémiai Kiadó, Budapest 1967; str. 93.

Omawiana książka zawiera sześć artykułów dotyczących nowoczesnych metod stosowanych w badaniach struktury białek. Artykuły te przedstawiają treść referatów wygłoszonych przez wybitnych znawców tematu na międzynarodowym sympozjum, które odbyło się w Budapeszcie w okresie od 6 do 8 października 1965 r.

Artykuł H. Hansona i S. Fittkau jako jedyny w tym tomie jest napisany w języku niemieckim. Autorzy przedstawiają w nim swoje doświadczenia w zakresie analizy struktury I-rzędowej peptydów na drodze enzymatycznej. Znajdujemy tu zarówno ogólne uwagi dotyczące tego tematu, jak też i dane metodyczne oraz wyniki przykładowych analiz.

W. Mejbaum-Katzenellenbogen w swoim opracowaniu przedstawia możliwości zastosowania taniny do oznaczania produktów degradacji polipeptydów. Artykuł ten zawiera cenne dane metodyczne oraz wyniki przykładowych analiz przeprowadzonych na tej drodze.

Dwa dalsze artykuły dotyczą metod fizyko-chemicznych stosowanych obecnie do oceny struktury białek. W obydwu referatach jest mowa o możliwościach zastosowania analizy własności optycznych białek do oceny ich struktury. Praca B. K. Vainshteina daje przegląd współczesnej analizy białek przy zastosowaniu promieni X, badań nad dyfrakcją elektronową i na drodze mikroskopii elektronowej. Uwzględnił on też odpowiednie odniesienia do ujęć starszych i, jak się wydaje, błędnych.

Dwa pozostałe artykuły omawiają zmiany aktywności enzymów na tle zmienionej ich struktury. Treść tych artykułów zawiera ciekawe dane oraz sugestie, które zbliżają czytelnika do zrozumienia

na poziomie molekularnym niektórych zjawisk związanych z procesami życia. Zarówno artykuł G. Szabolosi, jak i A. E. Braunsteina rozpatrują aktywność enzymów w świetle wpływu modyfikacji chemicznych i zmian strukturalnych w łańcuchach bocznych i centrach aktywnych.

Cała książka jest bogato ilustrowana wykresami oraz schematami, co w znacznym stopniu ułatwia odbiór przedstawionej treści.

*Leopold Myszkowski*

CHEMICAL BIOLOGY OF INFLAMMATION. Proceedings of a Conference Held at Brook Lodge, Augusta, Michigan, 1967 (J. C. Houck & B. K. Forscher, eds.) Pergamon Press 1968; str. 334.

Ta bardzo starannie wydana książka jest zbiorem referatów i doniesień wygłoszonych na międzynarodowej konferencji na temat zapalenia.

Zasadniczym celem konferencji było nawiązanie kontaktów i przerzucenie mostów między cytologami a biochemikami zajmującymi się reakcją ustroju na uraz, gdyż tym jest w swej istocie zjawisko zapalenia.

Kluczem do całej książki są wstępne rozważania Houcka. Według najnowszych poglądów istotą zapalenia jest wylanie zawartości wnętrza komórki do przestrzeni pozakomórkowej. Wewnątrzkomórkowo zlokalizowane czynne ciała, jak histamina, serotonina, wpływają na mikrokrążenie. Proteolityczne enzymy atakują wyściółkę naczyń, zwiększając ich przepuszczalność. Proteazy wewnątrzkomórkowe aktywują kalikreinę, która zwalnia z kolei brydykininę z $\alpha_2$-globulin, a ta uwalnia kininy, również zwiększające przepuszczalność ścian naczyniowych, co powoduje nagromadzenie się białek w miejscu reakcji zapalnej. Peptydowe produkty działania proteaz na pozakomórkowe substraty wywierają działanie chemotaktyczne na leukocyty, szczególnie granulocyty, następuje rozluźnienie struktury łącznotkankowej, kolagenoliza, płytki ulegają agregacji. Pojawia się enzym przekształcający fibrynogen w fibrynę (nie trombina), następuje polimeryzacja fibryny, zaczopowanie naczyń, ischemia, anoksja i lokalna kwasica. Odczyn zapalny jest konsekwencją zewnątrzkomórkowego działania wewnątrzkomórkowych enzymów na lepiszcze komórek, a efektem tych działań jest zmiana mikrokrążenia tkankowego. Celem biologicznym zapalenia jest odnowa i powrót funkcji, ale tymi sprawami zupełnie nie zajmowano się na konferencji, gdyż jeszcze tak daleko wiedza nie sięga.

Zapalenie przewlekłe w odróżnieniu od zapalenia ostrego wydaje się być proliferacją monocytów w odpowiedzi na przewlekle działający bodziec, zwykle natury immunologicznej. Uszkodzenie ma być wynikiem powstawania i zwalniania autoantygenów. Takim antygenem mogą być sekwestrowane glikoproteidy.

W konferencji brało udział 50 uczestników, którzy przedstawili 28 referatów z bardzo różnych dziedzin. Wśród uczestników był Selye, który przedstawił tylko dość generalizującą koncepcję chorób wieloprzyczynowych. Materiałem dowodowym były ogólnoustrojowe i lokalne odczyny zapalne, wywołane przez różne czynniki, "conditioner" i "challenger", i ich wzajemną zależność oraz hamowanie odczynu zapalnego przez różnorodne przeciwzapalne środki. W toku dyskusji podkreślono ogólnikowość jego danych.

Wiele referatów przedstawiało badania cytologiczne i histochemiczne, m. in. nad rolą leukocytów: bazofilów, monocytów, komórek tucznych, limfocytów, różnych populacji limfocytów, długo i krótko żyjących, nad ultrastrukturą leukocytów w różnych etapach rozwoju, w fagocytozie, w przemianie limfocytów w makrofagi w zapaleniu u ludzi, nad chemotaksją leukocytów (warunkiem chemotaksji jest zachowana zdolność syntezy białka w leukocytach), agregacją płytek krwi, rolą płytek krwi w wywoływaniu uszkodzeń mięśnia sercowego i nerek, płytkowym czynnikiem przepuszczalności naczyń. Agregacja płytek jest hamowana przez przeciwzapalnie działające środki, iak kortyzon i fenylbutazon. Kilka referatów poświęcono znaczeniu krzepnięcia w ostrym zapaleniu, fibrynolizie i jej roli w pozapalnej odnowie tkanki.

Kilka prac, bardzo interesujących, było czysto biochemicznych. Dotyczyły one badań nad lizozomami zapalnymi, wykazując ich odmienność w stosunku do lizozomów tkanki zdrowej

i pozakomórkową aktywność lizozomów rozpadłych po dezintegracji komórek. Lizozomy wstrzyknięte dostawowo wzbudzają proces zapalny nie tylko przez zawartą w nich aktywność hydrolazową, ale i przez ich zdolność endocytozy. Przedstawiono również badania nad kolagenolizą, której towarzyszy nagromadzenie się fukoglikoproteidów; skład płynu wysiękowego; proteazy komórek tucznych i antyproteazy w środowisku zapalnym. Szereg prac poświęcono zmienionemu biochemizmowi leukocytów w zapaleniu. Modelem badawczym zapalenia było badanie wpływu na zapalenie środków immunosupresyjnych, leków przeciwzapalnych, hormonów i t.d.

Kilka prac było typu immunologicznego: omawiały one badania nad rolą antygenów jądrowych i przeciwciał tych antygenów w procesie zapalenia; w wysięku zapalnym opóźnionej reakcji immunologicznej znaleziono przewagę leukocytów zasadochłonnych.

Pearson w słowie zamykającym podkreślił, że w zjawisku tak złożonym jak zapalenie znamy rolę i umiejscowienie kilku zaledwie czynników; podczas konferencji przedstawiono wyniki poszukiwań oderwanych faktów z różnych dziedzin w jakiś sposób biorących udział w zapaleniu, lecz dalecy jesteśmy od poznania wzajemnej zależności i kolejności, czy znaczenia, poszczególnych ogniw tego mechanizmu, innymi słowy jesteśmy na analitycznym etapie badania zapalenia.

Konferencja ta stanowi przykład rozszerzania się agend biochemii, której metodyka znajduje zastosowanie w badaniu starych, znanych od stuleci zjawisk, przyczyniając się do ich głębszego poznania.

Kto będzie zainteresowany tą książką? Patolodzy, fizjopatolodzy, biochemicy kliniczni, cytolodzy, histochemicy, koagulolodzy i immunolodzy; natomiast klinicyści, ze względu na teoretyczno-doświadczalny charakter badań, chyba z tej książki korzystać nie będą.

*Leszek Tomaszewski*

OXYGEN TRANSPORT IN BLOOD AND TISSUE (D. W. Lübbers, U. C. Luft, G. Thews & E. Witzleb, eds.) G. Thieme Verlag, Stuttgart 1968; str. 264; cena 54 DM.

Książka obejmuje 26 referatów przedstawionych na 7 Międzynarodowej Konferencji w Bad Oeynhausen. Problematyka konferencji była bardzo rozległa.

Przedstawiono współczesne poglądy na zagadnienia wymiany gazowej w płucach w oparciu o zmodyfikowane równanie pojemności dyfuzyjnej (Thews), nowe dane o reakcji gazów z hemoglobiną i mioglobiną (Kreuzer, Hutten), rzucające interesujące światło na klasyczną teorię Adaira i nową hipotezę Niesela (encompassment hypothesis). Szeroko omówiony jest transport i wymiana tlenu poprzez łożysko ludzkie (Fischer, Moll), dyfuzja tlenu w tkance mózgowej (Liers, Diemer, Lübbers), w siatkówce (Bernards i Horsten), mięśniu sercowym (Fabel, Schmidt i Nielsen), mięśniach szkieletowych (Kunze), nerkach (Deetjen, Weiss) i wątrobie (Peters, Kessler). Badania autorów oparte są o najnowsze współczesne techniki, jak mikrospektrofotometria "*in situ*" lub mikroelektrody tlenowe w układzie platyna - teflon o rozmiarach rzędu 1 mikrona. Referaty przynoszą wiele nowych danych, często modyfikujących dotychczasowe poglądy (np. badania Hammersena nad ultrastrukturą naczyń włosowatych w mięśniach szkieletowych). Interesujące jest ogólne ujęcie zaopatrzenia tlenowego przez Grunewalda w oparciu o układy modelowe i koncepcję systemów przeciwprądowych w sieci naczyń włosowatych. Poważny wkład do konferencji wnieśli morfolodzy, przedstawiając nowe wyniki w zakresie stereogeometrii naczyń włosowatych mięśnia sercowego (Hort, Arndt), mięśni szkieletowych (Hammersen) oraz krążenia obocznego w mięśniu sercowym (Schaper i Jageneau).

Szeroki zakres referatów, wyniki oparte o najnowszą metodykę i nienagannie opracowane ilościowo, z zastosowaniem techniki komputerowej, ujęcie zagadnienia z wielu punktów widzenia od strony fizjologii, biochemii, biofizyki, morfologii — czynią z książki cenną monografię godną polecenia wszystkim fizjologom, biochemikom, morfologom i klinicystom zainteresowanym problematyką transportu i dyfuzji tlenu.

*Andrzej Trzebski*

K. F. Weinges, GLUCAGON. G. Thieme Verlag, Stuttgart 1968; str. 112, cena 39.60 DM.

Książka Kurta F. Weingesa stanowi dalszą pozycję z serii monografii wydawanych pod ogólnym hasłem "Biochemie und Klinik", a redagowanych przez G. Weitzela i N. Zöllnera. Obecne wydawnictwo jest monograficznym opracowaniem wielu zagadnień związanych z glukagonem. W krótkim wprowadzeniu autor przedstawia najważniejsze fakty historyczne dotyczące odkrycia i wyizolowania tego hormonu; dalsze rozdziały poświęcone są nader szczegółowemu omówieniu chemicznych własności glukagonu i jego budowy, możliwościom jego wykrywania i ilościowego oznaczania, następnie omówiono miejsca wytwarzania glukagonu w ustrojach żywych, wydzielanie wewnętrzne i regulację tego wydzielania. Wreszcie autor zajmuje się szczegółami działania omawianego hormonu na metabolizm i mechanizmem tego działania, w którym to rozdziale cytuje szczegółowo wyniki własnych doświadczeń. Monografię zamykają cztery krótkie rozdziały przedstawiające badania nad wpływem glukagonu na elektrolity osocza i funkcję nerek, omawiające wpływ na przewód pokarmowy, znaczenie glukagonu w cukrzycy oraz możliwości diagnostycznego i terapeutycznego zastosowania hormonu. Końcowym dodatkiem jest przepis radioimmunologicznego oznaczania glukagonu.

Materiał przedstawiony w monografii jest zebrany bardzo systematycznie i udokumentowany licznymi cytatami oraz wynikami konkretnych doświadczeń. Poza wnikliwą i często dość szczegółową dyskusją przedstawianych wyników, książka zawiera sporo uogólniających schematów, które są interesujące, ułatwiają czytanie i prześledzenie myśli autora. Mimo wielkiej staranności opracowania, wkradły się do niego niewielkie pomyłki formalnej raczej natury; np. na str. 49, w schemacie rys. 24 przedstawiono działanie fosfodwuesterazy na cykliczny 3′,5′-AMP tak, jak gdyby w wyniku reakcji powstawał nieorganiczny fosforan, co jest oczywistą pomyłką. Oceniającemu wydaje się, że byłoby lepiej, gdyby autor — zgodnie z zaleceniem Komisji Enzymowej Międzynarodowej Unii Biochemicznej — używał skrótu NADP zamiast dawnego TPN, co ma miejsce w całej książce. Wyrażenie użyte na str. 38 w wierszu 18: „...Glykolyse über den Pentosephosphatweg..." nie wydaje się także właściwe, gdyż powszechnie przyjęto nazywać mianem glikolizy rozpad glukozy lub glikogenu do kwasu mlekowego poprzez kwas pirogronowy.

Wielkim walorem omawianej książki, poza ułatwieniem dostępu do dotychczasowych wiadomości o glukagonie, jest zebranie i przedstawienie w formie pełnego wykazu, wraz z tytułami, 670 pozycji cytowanej literatury. Alfabetyczny skorowidz rzeczowy na końcu monografii ułatwi niewątpliwie każdemu odszukanie interesującego go zagadnienia. Książka zainteresuje zarówno biochemików, jak i klinicystów, fizjologów, farmakologów i innych specjalistów stykających się z endokrynologią. Jest to cenna pozycja monograficzna.

*Mariusz Żydowo*

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS (R. T. Holman, ed.) vol. 9 part 3. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1968; str. 349 - 452, cena $ 5.50.

Kolejny tom tego seryjnego wydawnictwa zawiera trzy rozdziały poświęcone wielonienasyconym kwasom tłuszczowym. W rozdziale „Wodorowanie tłuszczy" H. J. Dutton omawia złożone mechanizmy chemiczne procesów wodorowania nienasyconych kwasów tłuszczowych oraz podaje charakterystyki katalizatorów stosowanych do wodorowania. Zwraca uwagę fakt. że zagadnienie tzw. utwardzania olei, będące początkowo głównie domeną empirycznej technologii przemysłowej, jest obecnie przedmiotem badań nowoczesnej chemii organicznej. W rozdziale „Utlenianie wielonienasyconych kwasów tłuszczowych" W. O. Lundberg i Pentti-Järvi omawiają autokatalityczne procesy utleniania nienasyconych kwasów tłuszczowych oraz metody oznaczania produktów procesów utleniania. W rozdziale tym przedstawiono mechanizmy katalitycznego utleniania lipidów, opisano różne typy syntetycznych katalizatorów i inhibitorów procesów utleniania oraz podano przykłady biologicznych katalizatorów utleniania i przeciwutleniaczy. Omówiono również biologiczne efekty wywołane przez produkty utleniania wielonienasyconych kwa-

sów tłuszczowych. W rozdziale trzecim O. S. Privett zajmuje się zagadnieniami preparatyki wielonienasyconych kwasów tłuszczowych. Autor podaje obszerne zestawienie materiałów biologicznych dla preparatyki około 120 rodzajów nienasyconych kwasów tłuszczowych i omawia zarówno starsze techniki izolacji tych związków (metody destylacji i krystalizacji), jak i nowsze metody chromatograficzne.

Książka ta z pewnością stanowić będzie wartościową pozycję przede wszystkim dla pracowników przemysłu tłuszczowego, a także dla biochemików zajmujących się lipidami.

*Tadeusz Chojnacki*

W. Gerok, PRIMÄRE TUBULOPATHIEN. G. Thieme Verlag, Stuttgart 1969; Str. 256; cena 64 DM.

Jest to monografia z serii „Biochemia i Klinika" wydawanej przez G. Weitzela i N. Zöllnera. Jak wszystkie dotychczas wydane książki z tego zakresu (np. *Glukagon*, *Enzymy osocza krwi*, *Biochemia hormonów nadnerczy*, *Płyn pozakomórkowy* i inne), monografia Geroka jest wszechstronnie opracowanym studium poświęconym mechanizmowi zaburzeń wrodzonych w procesie transportu substancji w kanalikach nerkowych z uwzględnieniem podstaw biochemicznych tych procesów, objawów klinicznych, diagnostyki i terapii.

Książka podzielona jest na dwie części: I. Fizjologia i biochemia, oraz II. Patologia transportu w kanalikach nerkowych. W pierwszej części autor omawia mechanizm transportu i resorpcji zwrotnej glikozy, wpływ hormonów i metabolitów oraz wpływ stężenia cukrów na ten proces. Dalsze rozdziały dotyczą transportu aminokwasów, fosforanów, jonów wodorowych z uwzględnieniem regulacji kwasowo-zasadowej oraz mechanizmu zagęszczania i rozcieńczania moczu. Część druga (14 rozdziałów) poświęcona jest systematycznemu omówieniu poszczególnych wad wrodzonych, jak glikozuria, cystynuria, glicynuria, syndromy Rowley'a i Rosenberga, Lowry'ego, Fanconiego, rodzinna gliko-glicynuria, wrodzone zaburzenia zwrotnej resorpcji kwasu moczowego i inne. W opisie poszczególnych zaburzeń uwzględniono objawy kliniczne, biochemiczne, zmiany morfologiczne, patogenezę, zagadnienia endokrynologiczne, tło genetyczne i wreszcie terapię. Poszczególne samoistne tubulopatie omawiane są w powiązaniu ze zmianami towarzyszącymi w innych, poza nerką, organach, szczególnie w kośćcu, w układzie nerwowym, skórze oraz w przewodzie pokarmowym. Ustępy dotyczące procesów biochemicznych w różnych zaburzeniach potraktowane są może zbyt wycinkowo i w sumie nie dają pełnego obrazu przemian w nerce w omawianych przypadkach patologicznych. Jednak obszerne piśmiennictwo (975 pozycji), zebrane do r. 1967 włącznie, uwzględnia szeroko również zagadnienia biochemiczne.

W sumie książka Geroka jest cenną pozycją zarówno dla klinicystów, jak i biochemików interesujących się patologią tego niezwykle obciążonego pracą narządu, jakim jest nerka.

*Włodzimierz Ostrowski*

H. G. Schlegel, ALLGEMEINE MIKROBIOLOGIE. G. Thieme Verlag. Stuttgart 1969; str. XII+431, cena 14.80 DM.

„Duża dostępność drobnoustrojów, ich szybki wzrost, duża zdolność przystosowania się i wiele innych cech sprawiły, że stały się one głównymi obiektami badań w dziedzinie biochemii i genetyki". Tymi słowy rozpoczyna autor przedmowę do swej zwięzłej pracy zatytułowanej *Mikrobiologia ogólna*. Praca ma służyć jako źródło podstawowych wiadomości z mikrobiologii ogólnej nie tylko dla mikrobiologów, ale i dla studiujących botanikę, zoologię, farmację, rolnictwo, medycynę, chemię i fizykę. Autor położył nacisk na fizjologię drobnoustrojów. Mimo to, zdaniem recenzenta, bardzo szczęśliwie udało mu się znaleźć korzystny stosunek między zakresem aspektu fizjologicznego i biochemicznego. Powiązanie tych dwóch aspektów jest daleko idące, każdy niemal rozdział rozpatrywany jest jednocześnie w aspekcie fizjologii i biochemii.

Treść *Mikrobiologii ogólnej* można podzielić na trzy zasadnicze, mniej więcej równej objętości, części. Pierwsza, złożona z 6 rozdziałów, stanowi biologię drobnoustrojów, druga (8 rozdziałów) omawia przemiany chemiczne zachodzące w świecie drobnoustrojów, ostatnia wreszcie (3 rozdziały) zawiera opis wyższych funkcji, regulacje przemiany materii i mechanizmy genetyczne.

Książkę zaopatrzono w wykaz literatury odnoszącej się do poszczególnych rozdziałów. Wykaz ten uwzględnia podręczniki i artykuły przeglądowe, a także niektóre ważniejsze artykuły oryginalne. Szczegółowy indeks haseł bardzo ułatwia korzystanie z książki jako podręcznego źródła podstawowych informacji z mikrobiologii ogólnej. Czytelnik z pożytkiem skorzysta również z alfabetycznego wykazu fachowych terminów pochodzących z języka łacińskiego, greckiego, angielskiego i francuskiego.

Przy przedstawianiu ciągów metabolicznych w postaci schematów autor zastosował interesujący sposób wyróżniania grup chemicznych lub posiłkowych związków chemicznych za pomocą druku barwnego lub umieszczenia na zaciemnionym tle o różnym kształcie. W ten sposób losy tych grup lub związków w przemianach stają się czytelne już przy pierwszym rzucie oka. Fotografie i rysunki są na dobrym technicznym poziomie. Ważną cechą książki jest jej zwięzłość. Dlatego też autor osiągnął dużą koncentrację treści w stosunkowo małej objętości. Książka może z powodzeniem służyć dla osób chcących zapoznać się z podstawami mikrobiologii, a także jako podręczne źródło informacji w tej dziedzinie.

*Tadeusz Korzybski*