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INFLUENCE OF NATRIUM FLUORIDE ON CITRATE CONTENT IN RAT BONE

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1. Natrium fluoride given to rats reduced the citrate content in bone. This reduction was found to be caused by the diminished uptake and increased release of citrate due to the heteroionic exchange with fluoride ion on the accessible surface of the bone mineral.

Dickens (1941) found a high concentration of citrate in the skeleton and showed that 70% or more of the citric acid of the body was located in the bones. Later, it was proved by Kuyper (1945) that citrate may coprecipitate with calcium phosphate. Armstrong & Singer (1956) demonstrated that dry fat-free bone, the mineral part of bone, as well as synthetic hydroxyapatite, take up citrate from solution.

These findings and the occurrence of citrate in tissue fluids suggested that citrate found in bone could be of non-skeletal origin. It has been shown, however, that the bone contains appreciable amounts of enzymes involved in formation and oxidation of citric acid (Dixon & Perkins, 1952; Van Reen, 1959).

Zipkin, Lee, Schraer & Schraer (1961) reported that fluoride diminishes the citrate content in the skeleton. The significance of this finding is difficult to evaluate because the role of citrate in bone metabolism as yet has not been elucidated.

The experiments described in this paper were aimed at investigating the mechanism responsible for the decrease of citrate content in bone tissue after fluoride administration.

MATERIAL AND METHODS

Animals. The experiments were carried out on male Wistar rats weighing 20 - - 200 g. The animals were kept on a normal diet consisting of corn (wheat, rye, oats), bread, carrot, lettuce, milk and water. For experiments on the effect of fluoride *in vivo*, fluoride was added to drinking water (20 mg. NaF/100 ml.) and the daily intake was recorded. The animals were killed by bleeding in ether anaesthesia.

Bone samples. Immediately after killing, the femurs were removed and cleaned from soft tissues and bone marrow, dissected longitudinally into two halves and

weighed. Then they were used for the *in vitro* experiments and for determination of calcium and citrate.

Fresh-bone "common pool". Cleaned femurs were chopped into pieces about 0.5 mm. in diameter and mixed. Weighed amounts of this mixture were used for experiments.

Defatted and dehydrated bone. Bone pieces about 2 mm. in diameter were kept for 4 hr. in 96% ethanol (25 ml. per 500 mg. of bone) exchanged twice during this time. Then the sample was placed in ethyl ether for 16 hr., dried at 100° to constant weight, ground to powder and sieved. The fraction 0.25 - 0.50 mm. in diameter was used.

Mineral part of bone. This was prepared from rat femurs and tibias by extracting them with ethylenediamine in Soxhlet apparatus according to Williams & Irvine (1954). The carefully washed and dried mineral part was ground and sieved. The fraction 0.25 - 0.50 mm. in diameter was used.

Bone extracts and determination of isocitrate dehydrogenase. To 4 ml. of 0.9% NaCl at 0°, 100 mg. of fresh cleaned bone tissue was added and crushed in a glass homogenizer for 15 min. The homogenate was centrifuged at 3000 rev./min. and the clear supernatant used for determination of isocitrate dehydrogenase activity according to Grafflin & Ochoa (1950). The protein of the bone extracts was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Determination of citrate. Cleaned bones were ground in a mortar with 20 ml. of a mixture of 20% CCl_3COOH and 20% H_2SO_4 (1 : 1, by vol.) and left for 24 hr. The sample was then centrifuged and citrate estimated by the micromethod of Taylor (1953).

Determination of calcium. The bone sample was kept for 24 hr. in 2.5% HCl, then centrifuged and in the supernatant calcium was estimated manganometrically.

Determination of glycolysis. Weighed amounts of fresh-bone "common pool" were incubated with glucose in Krebs-Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1964). After 4 hr. the amount of lactic acid was assayed according to Barker & Summerson (1941).

Determination of fluorine. Fluorine content in rat bone was determined by the spectrophotometric method according to Steiger & Mervine (1956).

Radioactivity measurements were carried out with a mica window G. M. counter.

Chemicals. All reagents used were of standard analytical quality, supplied by Biuro Obrotu Odczynnikami, Gliwice, Poland. Radioactive phosphorus in form of $K_2H^{32}PO_4$ was produced in our Institute in Świerk. *p*-Hydroxydiphenyl was supplied by BDH (Poole, England).

RESULTS

In normal rat bone the amount of citrate was proportional to the content of calcium (Fig. 1). In young rats, fluoride (NaF) administered in drinking water for 14 days diminished significantly the citrate content in bone, to about 65% of normal value, whereas the concentration of citrate in blood serum remained unaffected (Table 1).

Table 1

Effect of NaF *intake on citrate content in bone and blood serum of young rats*

Rats weighing 20 - 40 g. were given NaF in drinking water for 14 days. Total dosis was about 18 mg. Results are given as means ± S. D. Numbers of examined animals are given in parentheses.

Rats	Bone (mg. citrate/100 g. fresh tissue)	Blood (mg.%)
Normal	235.6 ± 29.3 (20)	5.59 ± 0.75 (10)
NaF-treated	152.5 ± 22.7 (20)	5.61 ± 0.70 (10)

Table 2

Effect of NaF in vitro *on citrate content in bone*

Halves of the femur were incubated for 4 hr. at 37° in 20 ml. of the Krebs-Ringer bicarbonate solution containing glucose at a concentration of 0.5 mg. per ml. Citrate was estimated in whole samples i.e. together in bone and solution. Results of separate experiments are given.

Expt.	Citrate (mg. per 100 mg. of fresh bone)	
	Incubation without NaF (control)	Incubation in solution containing 0.12 mM-NaF
1	342	348
2	247	223
3	353	359
4	219	248
5	357	330
6	347	351
7	255	265
8	346	337
9	258	262
10	370	356

Table 3

Effect of NaF *on the activity of isocitrate dehydrogenase in bone tissue*

The enzyme activity was determined according to Grafflin & Ochoa (1950) and expressed as micromoles of oxidized isocitrate per mg. protein per hour; mean values ± S. D. are given.

Bone extracts from	No. of animals	Activity
Normal rats (control)	10	2.4 ± 0.2
NaF-treated rats (18 mg. in 14 days)	4	2.3 ± 0.1
Normal rats; extracts incubated with NaF at final concn.: 10^{-4} M	4	2.5 ± 0.1
10^{-3} M	4	2.5 ± 0.2
10^{-2} M	4	2.6 ± 0.2

Table 4

Effect of sodium fluoride on lactic acid formation in bone in vitro

Equal amounts of pooled bone pieces, 0.5 mm. in diameter, were incubated for 4 hr. in 15 ml. Krebs-Ringer bicarbonate solution containing additionally glucose at a concentration of 0.5 mg./ml. In the incubation solution lactic acid was determined.

Control (without NaF)			Proper sample (with 0.1 mg. NaF)			Inhibition (%)
Bone sample (mg.)	Lactic acid (μg. in solution)	Lactic acid (μg./mg. of bone)	Bone sample (mg.)	Lactic acid (μg. in solution)	Lactic acid (μg./mg. of bone)	
71	232	3.27	71	186	2.62	20
81	132	3.26	81	125	3.08	6
86	250	2.91	86	143	1.66	43
79	250	3.16	79	234	2.95	7
60	64	1.07	60	46	0.77	28
57	100	1.75	57	94	1.65	6
82	108	1.32	82	92	1.12	15
82	152	1.85	82	92	1.12	39
88	90	1.02	88	62	0.70	31
61	70	1.15	61	56	0.92	20

Table 5

Effect of citric acid on fluoride uptake by the mineral part of bone

A sample of 60 mg. of mineral part of bone (particle size 0.25 - 0.50 mm.) was equilibrated successively in two media consisting each of 50 ml. of a neutral solution (11.35 g. KCl and 1.815 g. of diethylbarbituric acid in 1-1. volume, adjusted to pH 7.4) and the additions indicated in the Table. Time of equilibration: 48+48 hr. Then the sample was rinsed with water, dried and fluorine content determined.

First equilibration (additions)	Second equilibration (additions)	F^- content in mineral part (μmoles/50 mg.)
1.2 mg. of NaF	1.5 mg. of citrate	5.0
1.2 mg. of NaF	None	6.3
1.5 mg. of citrate	1.2 mg. of NaF	5.2

No effect of 0.12 mM-NaF on citrate formation was found in bone *in vitro* (Table 2). Since bone contains enzymes which participate in oxidation of citrate, it seemed advisable to examine the activity of isocitrate dehydrogenase both in the bone of rats treated with NaF for 14 days, and in bone of normal rats incubated with NaF (Table 3). The differences between control and the proper samples, evaluated by the use of Student's *t*-test, were not significant. In experiments *in vitro* it was found that NaF inhibits the formation of lactic acid (Table 4).

Defatted and powdered bone as well as the mineral part of bone, after equilibration for 24 hr. in 20% citrate solution, were incubated in solutions containing various concentrations of phosphate, with or without the addition of NaF, and the amount of bone citrate was determined (Fig. 2). It was found that, parallelly with the increasing concentration of phosphate, the bone lost greater amounts of citrate, the loss being enhanced by the presence of NaF.

Displacement of ^{32}P from the mineral part of the bone to the solution during 18 hr. incubation in citrate solutions of various concentrations, is shown in Fig. 3.

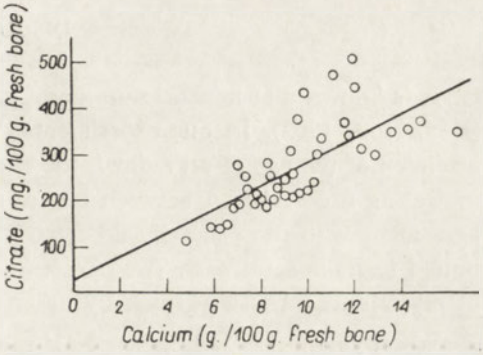

Fig. 1. Relation between citrate and calcium contents in bones of 40 normal young rats, weighing 20 - 200 g.

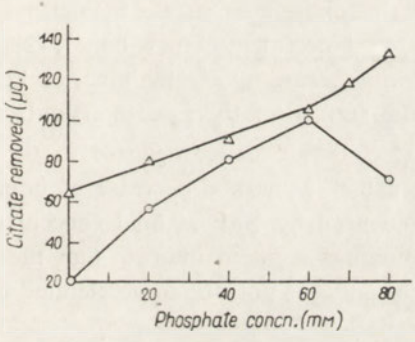

Fig. 2

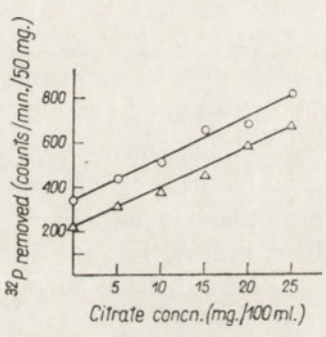

Fig. 3

Fig. 2. Effect of NaF and increasing phosphate concentration on the loss of citrate by fat-free bone. Powdered defatted bone (particle size 0.25 - 0.50 mm.) was equilibrated for 24 hr. in 25% citrate solution, pH 7.4, at 20°. Then it was washed carefully, dried and assayed for citrate content which amounted to 245 µg./50 mg. bone sample. A 50 mg. sample of this material was incubated in 50 ml. of a solution containing various concentrations of phosphate, with or without 0.6 mg. NaF added, adjusted with KCl to ionic strength I = 0.165 and buffered with barbiturate to pH 7.4. After 5 hr. incubation, the samples were washed and citrate was assayed in the solution. (○), Without NaF; (△), with NaF added.

Fig. 3. Effect of NaF and increasing concentration of citrate on the release of ^{32}P. The mineral part of bone (particle size 0.25 - 0.50 mm.) was labelled *in vitro* in $K_2H^{32}PO_4$ solution for 24 hr. at pH 7.4. Washed and dried sample (50 mg., 1550 counts/min.) was incubated for 18 hr. at 37° in 25 ml. of citrate solution of various concentration (pH 7.4), with or without NaF added. The radioactivity in bone was assayed and the results related to 1 hr. (○), Citrate alone; (△), 1.2 mg. NaF added.

The bone material was labelled prior to the experiment by 24 hr. incubation in $K_2H^{32}PO_4$ solution. The data indicate that the displacement of ^{32}P increased with the concentration of citrate in the solution. The presence of NaF reduced the loss of ^{32}P from the mineral part of the bone.

To examine the influence of citrate on the behaviour of fluorine in the mineral part of the bone, samples of this material were equilibrated successively in two solutions of different composition, and fluorine content was measured. The results presented in Table 5 indicate that in the presence of citrate the fluorine content in bone was diminished.

DISCUSSION

It is known that mineralization and calcium content in bone are age-dependent (Dickerson, 1962). In our experiments, when the calcium content was taken as an index of the age of an animal, the citrate level was found to increase with age, indicating a correlation between citrate and mineral deposits in bone. This is in agreement with the generally accepted view that nearly all citric acid in bone is bound to its mineral part. The citrate ion, being present on the accessible surfaces of crystals, can be substituted by other anions (Hendricks, 1955).

When young rats were given NaF in drinking water for two weeks, a decrease in bone citrate was observed. This could have been induced by: (1), inhibition of formation of endogenous or uptake of exogenous citrate by normal growing and mineralizing bone (dilution of citrate by increment of new bone); (2), physico-chemical phenomena, viz. the inhibition of citrate uptake by bone, or heteroionic substitution of citrate by fluoride at the surface of the apatite crystal.

Our experiments demonstrated that isocitrate dehydrogenase in the bone is unaffected by the dose of NaF which causes a marked decrease of bone citrate. The activity in homogenates is not influenced by NaF at final concentrations as high as 10^{-4}, 10^{-3} or 10^{-2} M. These findings seem to indicate that the decrease in citrate content of bone in fluoride poisoning is not due to accelerated oxidation of citrate.

We were unable to demonstrate any influence of NaF on the *in vitro* formation of citrate in bone. One of the possible explanations of this failure may be very low formation of citrate in bone and insufficient sensitivity of the analytical method used. It was found by Borle, Nichols & Nichols (1960) that under anaerobic conditions and in the presence of glucose (similarly as in our experimental conditions) the amount of citrate was 1.4% of the amount of lactate formed. Under similar experimental conditions as for citric acid formation *in vitro*, NaF inhibited the lactic acid formation in bone, as it does in other tissues.

The sensitivity of the enzymes of the citric acid cycle to fluoride is not known, but there are some data which can throw light on the mechanism of the decrease of citrate in bone in fluoride poisoning. When rats were given large doses of NaF, a very small increase in the amount of bone ash and a distinct decrease of bone citrate were observed by Zipkin, Schraer, Schraer & Lee (1963); this may indicate

that the reduced content of bone citrate is not the result of the dilution by formation of new bone. Our results indicate that doses of NaF which decrease the citrate content in bone do not cause a decrease in the citrate content in blood.

The experiments with the mineral part of the bone demonstrated that citrate may be exchanged with phosphate ion. The amount of the released citrate increased in the presence of F^- ions. It may be suggested that F^- ions substitute citrate ions and compete with phosphate ions for the same site on the crystal surface.

Armstrong & Singer (1956) found that NaF diminishes the uptake of citrate from solution by fat-free bone. Recent studies of Zipkin, Posner & Eanes (1962) have shown that fluorides improve the crystallinity of bone crystals. The improvement is connected with the partial exclusion of citrate from the surface of bone crystallites. These findings, as well as the presented results, permit to assume that the main mechanism involved in the decrease of citrate in bone accompanying fluoride poisoning, is a diminished uptake of citrate by the mineral part and the release of citrate present in hydroxyapatite from the accessible crystal surfaces by exchange with fluorine ions.

We are greatly indebted to Professor Dr. E. Kowalski in whose Department this work was carried out, for his continued advice and encouragement. Thanks are due to Miss Z. Długoborska and Miss I. Ausz for their technical assistance. We express our thanks to Mr. W. Rutkowski, B. Sc., from the Analytical Department of our Institute for carrying out the analysis of fluorine in bone samples.

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WPŁYW FLUORKU SODU NA ZAWARTOŚĆ CYTRYNIANU W KOŚCI SZCZURA

Streszczenie

1. Fluorek sodu podawany szczurom powodował zmniejszenie zawartości cytrynianów w kości. Jest to wynikiem zmniejszonego pobierania cytrynianów przez kość oraz zwiększonego uwalniania cytrynianów z dostępnej powierzchni części mineralnej kości, na drodze wymiany heterojonowej z jonem F^-.

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CORRELATION BETWEEN THE BINDING OF CALCIUM AND ATP BY G-ACTIN *

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1. The rates of the release of actin-bound ATP and actin-bound Ca were examined. It was found that during incubation of G-actin with Dowex 1 the release of bound Ca follows that of bound ATP, and, similarly, during incubation with Dowex 50 the release of bound ATP follows that of bound Ca. The same concentrations of free ATP and free Ca decrease the rate of the release of bound ATP and bound Ca to about the same extent. 2. The results obtained support the view of the existence of close correlation between bound ATP and bound Ca in actin molecule. A scheme for the mechanism of dissociation of G-actin - Ca - ATP complex is proposed in which the existence of labile intermediates G-actin - ATP and G-actin - Ca is postulated.

It has been established that a part of ATP in G-actin preparations is bound to actin in proportion of one mole per mole of the protein; the presence of bound ATP is necessary for the maintenance of all specific properties of actin and the excess of free ATP prevents G-actin from slow spontaneous inactivation (Straub & Feuer, 1950; Mommaerts, 1951, 1952; Ulbrecht, Grubhofer, Jaisle & Walter, 1960; Asakura, 1961; Grubhofer & Weber, 1961; Barany, Nagy, Finkelman & Chrambach, 1961; Martonosi & Gouvea, 1961; Strohman & Samorodin, 1962). It was also found that the anion exchange resin Dowex 1 removed from G-actin solutions only the free nucleotides without touching the bound ATP (Tsuboi & Hayashi, 1959; Ulbrecht *et al.*, 1960; Asakura, 1961).

It was later shown that, in addition to ATP, G-actin solutions contained calcium and that a part of it, which was also necessary for maintenance of the activity of this protein, was bound to actin in proportion similar to that of ATP (Chrambach, Barany & Finkelman, 1961; Maruyama & Gergely, 1961; Maruyama, 1962; Tonomura & Yoshimura, 1961; Barany, Finkelman & Therattil-Antony, 1962; Oo-

* Some parts of the present work were presented at the Annual Meetings of the Polish Biochemical Society (Łódź, September 1963, Abstr. Commun. p. 15, and Gdańsk, June 1964, Abstr. Commun. p. 73).

sawa *et al.*, 1962). Like ATP, free Ca can be quantitatively removed by cation exchange resin, Dowex 50 (Barany *et al.*, 1962).

Asakura (1961), Grubhofer & Weber (1961) and Maruyama (1962) used Dowex 1 for the kinetic studies on the process of inactivation of G-actin. They determined, however, mainly the final effect, i.e. the loss of polymerizability.

In the present work, by use of Dowex 1 and Dowex 50, the release of bound ATP and bound Ca from G-actin as well as the effect of free ATP and free Ca on these processes was examined in detail. The comparison of the rates of the release of bound ATP and bound Ca in various conditions was expected to provide more information concerning the correlation between the binding of these two compounds by G-actin, recently suggested by various authors (Tonomura & Yoshimura, 1961, 1962; Barany *et al.*, 1962; Maruyama, 1962).

The treatment with Dowex 1 or Dowex 50 are now commonly used for the removal of free nucleotides and free bivalent cations from actin solutions. The procedures applied by various authors differ, however, in details. In some papers even the conditions of the treatment have not been described. Therefore, in the present study it seemed necessary to establish also the proper conditions for the removal of free ATP and free Ca from G-actin solutions by ion exchangers.

The preliminary experiments concerning some aspects of the present work were started in 1960 in Dr. Gergely's Laboratory in Boston as a part of an extensive study on the kinetics of the dissociation of actin-ATP complex and of the exchange of actin-bound ATP (Gergely, Drabikowski & Kuehl, 1963). Subsequently the investigations on various aspects of the whole problem have been continued independently in Boston by Dr. M. Kuehl and in our Laboratory. Accidentally, some of the observations of the present work concerning the effect of pH, of the amount of Dowex 1 and of some other factors on the release of bound ATP were made also by Dr. Kuehl (private communication). It should be emphasized that all these results are in good agreement.

MATERIALS AND METHODS

Crude actin was extracted from acetone-dried muscle powder with deionized water at 0° for 30 min. as described previously (Drabikowski & Gergely, 1962). It was polymerized with 0.1 M-KCl and purified by ultracentrifugation according to Mommaerts (1952). F-Actin pellets were suspended in 0.2 mM-ATP and 2 mM-tris-HCl buffer, pH 7.5, homogenized with a teflon homogenizer and then dialysed against the ATP-tris buffer solution for 36 hr. at 2°. The resultant G-actin solution, containing 3 - 5 mg. protein/ml., was clarified by 20 min. centrifugation at 28 000 *g*.

G-Actin containing bound [^{14}C]ATP was prepared by 15 min. incubation with 0.05 - 0.1 mM-[^{14}C]ATP at 2° (Martonosi, Gouvea & Gergely, 1960) after previous removal of free ATP by Dowex 1 treatment. Similarly, G-actin containing bound ^{45}Ca was prepared by 15 min. incubation with 0.05 - 0.1 mM-$^{45}CaCl_2$ at 2° (Barany *et al.*, 1962) after previous removal of free bivalent cations by Dowex 50 treatment.

Protein concentration was determined by the biuret method (Gornall, Bardawill & David, 1949) calibrated by micro-Kjeldahl nitrogen determination.

For the measurements of radioactivity samples of G-actin solutions containing ^{45}Ca or [^{14}C]ATP were dried in aluminium planchets under an infrared lamp and counted in a Chicago Nuclear Corporation gas-flow counter with "Micromil" window. To avoid correction for self-absorption the same amounts of the protein, not higher than 2 mg., were present in the counted samples throughout each experiment.

pH of the solutions was measured with a "Radiometer" pH-meter (Copenhaven).

Crystalline ATP (disodium salt) was purchased from Pabst Laboratories (Milwaukee, Wisc., U.S.A.), [^{14}C]ATP (disodium salt) from Schwarz BioResearch (Orangeburg, N.Y., U.S.A.) and $^{45}CaCl_2$ from the Radiochemical Centre, Amersham, England.

For the removal of free nucleotides and free bivalent cations Dowex 1-X8 (200 - 400 mesh) and Dowex 50 (W-X4 or W-X2, 200 - 400 mesh), were used respectively. Both resins were products of J. T. Baker Chemical Co. (Phillipsburg, Pa., U.S.A.).

Before use, commercial Dowex 1 was washed successively with 10 - 20 vol. of 2 N-NaOH, exhaustively with deionized water, with 10 - 20 vol. of 2 N-HCl and again with deionized water up to the neutral reaction of the effluent. Finally, the resin was equilibrated with 2 mM-tris-HCl buffer, pH 8.0. During storage of the suspension of the resin in tris buffer a slow increase of absorbancy in UV as well as some acidification of the solution due to extraction of some compounds from the resin took place; for that reason, directly before use, the resin was washed exhaustively with 2 mM-tris-HCl buffer of the required pH.

Dowex 50 was purified in a similar way as Dowex 1 except that the washing with HCl preceded that with NaOH.

To avoid metal contaminations the solutions used throughout this work were prepared using deionized water, and polyethylene vessels were used instead of glassware.

RESULTS

The removal of free Ca *and free ATP from G-actin solutions*

Most authors used a few minutes time treatment with Dowex 1 for the removal of free ATP from G-actin. On the other hand, Barany *et al.* (1962) employed at least two successive treatments with Dowex 50 at 4°, each for 10 min., to remove free Ca.

To check the time course of the removal of free Ca, G-actin containing equally labelled free and bound ^{45}Ca was stirred with Dowex 50. As one can see in Fig. 1A, a very sharp decrease in the radioactivity of G-actin solution took place in the first 1 - 2 min. of stirring with Dowex 50. The further decrease of radioactivity proceeded at a much smaller rate. This suggests that all the free Ca originally pre-

sent in G-actin solution was adsorbed by the resin very quickly and the further decrease of the radioactivity of the solution was due to the removal by Dowex 50 of Ca slowly released from its complex with the protein. The parallelly determined amounts of ^{45}Ca remaining in the solution after repeated treatment with Dowex 50 according to Barany *et al.* (1962) lay on the same curve. This indicates that in the procedure of Barany *et al.* the removal of not only free Ca but also of some part of the initially bound Ca takes place.

Figure 1B shows that the first sharp decrease of radioactivity, corresponding to the removal of free Ca, was the same at 0° and at 21°; however, further removal of ^{45}Ca by Dowex 50 proceeded faster at 21°, as it could be expected for the temperature-dependent process of the release of bound Ca from G-actin.

A similar pattern was obtained when G-actin containing free and bound [^{14}C]ATP of the same specific activity was incubated with Dowex 1. The decrease of radioactivity due to the release of bound ATP proceeded, however, slower than that of bound Ca; thus, during 1 hr. incubation with Dowex 1 at 0° the radioactivity

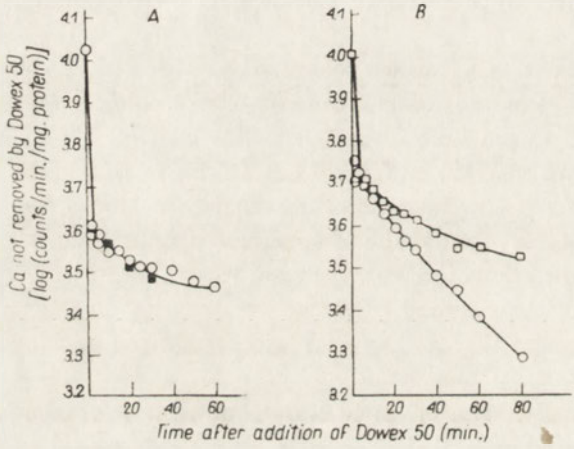


Fig. 1

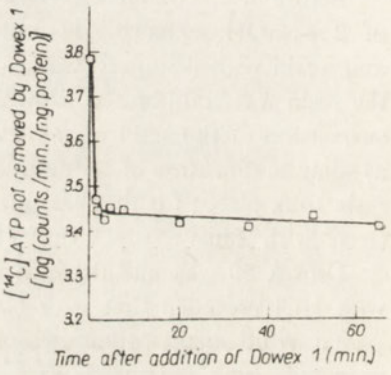


Fig. 2

Fig. 1. The removal of ^{45}Ca from G-actin solution by Dowex 50. *A*. G-Actin solution (3.1 mg./ml.) in 0.1 mM-ATP and 2 mM-tris-HCl, final pH 7.1, after 15 min. preincubation with 0.05 mM-$^{45}CaCl_2$, was stirred with 1/14 volume of wet Dowex 50 at 0°. At time intervals indicated on the abscissa samples were withdrawn, the resin was immediately centrifuged off, and in the supernatants radioactivity was determined (○). Another part of the solution, after 10 min. treatment with Dowex 50 and the removal of the resin, was stirred again with a new portion of Dowex 50 for another 10 min. This treatment was repeated once more. After each removal of Dowex, samples were taken off for radioactivity and protein determinations (■). *B*. G-Actin solution (2.9 mg./ml.) in 0.1 mM-ATP and 2 mM-tris-HCl, pH 7.1, after 15 min. preincubation with 0.05 mM-$^{45}CaCl_2$, was stirred with 1/14 volume of wet Dowex 50 at 0° (□) and 21° (○). At time intervals indicated on the abscissa samples were withdrawn, the resin was immediately centrifuged off, and in the supernatants radioactivity was determined.

Fig. 2. The removal of [^{14}C]ATP from G-actin solution by Dowex 1 at 0°. G-Actin solution (3.1 mg./ml.) in 2 mM-tris-HCl, pH 7.1, after 15 min. preincubation with 0.05 mM-[^{14}C]ATP, was stirred with 1/14 volume of wet Dowex 1 at 0°. Further procedure as described in the legend of Fig. 1B.

remaining in the solution decreased by only 4% of that found after the first 5 min. of Dowex 1 treatment (Fig. 2).

The amount of Ca or ATP remaining in the solution after short-time treatment (1 - 3 min.) of G-actin with the appropriate resin was found to be the same in the pH range between 7 and 8. It was also observed that the amount of Ca or ATP remaining in the solution after 1 or 2 min. treatment at 21° decreased only slightly with the increase of the amount of the resin from 1/56 to 1/7 of the volume of actin solution. As can be seen from Fig. 3 and 4, only after a longer time of treatment

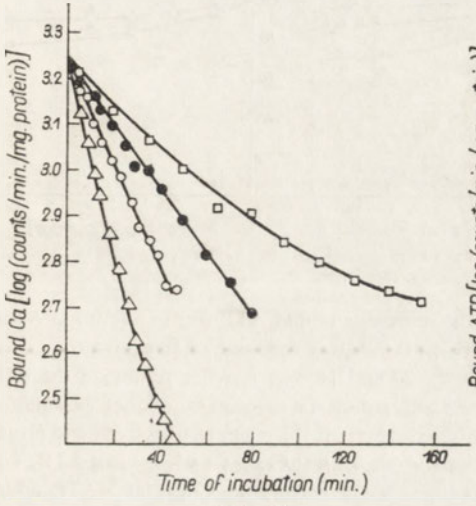


Fig. 3

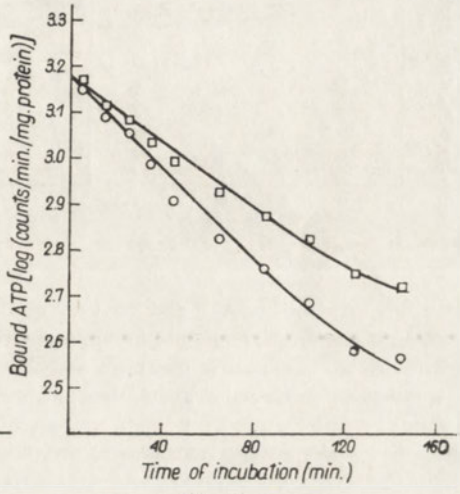


Fig. 4

Fig. 3. The release of bound Ca from G-actin incubated in the presence of various amounts of Dowex 50. G-Actin - ^{45}Ca solution (3.0 mg./ml) in 4 mM-tris-HCl, final pH 8.0, deprived of free bivalent cations and of free ATP by successive treatment with Dowex 50 and Dowex 1, was incubated at 21° in the presence of various amounts of Dowex 50 with constant stirring. Further procedure as described in the legend of Fig. 1B. The amounts of Dowex 50 in the incubation mixture (volume of wet resin per one volume of G-actin solution): (□), 1/56; (●), 1/28; (○), 1/14; (△), 1/7.

Fig. 4. The release of bound ATP from G-actin incubated in the presence of various amounts of Dowex 1. G-Actin - [^{14}C]ATP solution (3.1 mg./ml.) in 4 mM-tris-HCl, final pH 8.0, was deprived of free bivalent cations by treatment with Dowex 50. After removal of the resin the solution was incubated at 21° in the presence of 1/20 (□) and 1/10 volume of wet Dowex 1 (○). Further procedure as described in the legend of Fig. 1B.

with Dowex 50 or Dowex 1 the differences become significant due to the release of bound Ca or bound ATP from G-actin, proceeding at a rate greatly depending on the amount of the resin in the solution.

On the basis of the above results, in the present work free Ca and free ATP were removed from actin solutions by stirring with the appropriate resin for 2 - 3 min. The resins were used at the ratio of about 1/15 volume of wet resin per volume of actin solution, as in the previous paper (Drabikowski & Gergely, 1963). Depending on the conditions of the particular experiment the pH was in the range between 7 and 8.

The spontaneous release of bound Ca *and bound ATP from G-actin*

As can be seen from Fig. 5 and 6, G-actin deprived of free bivalent cations and of free nucleotides lost both its bound Ca and bound ATP. This process, however, proceeded slowly, especially at 0°, and its rate diminished with time; this could be attributed to the protective effect of Ca and ATP liberated to the solution during

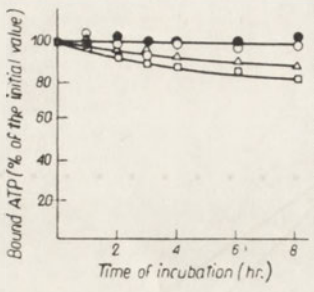
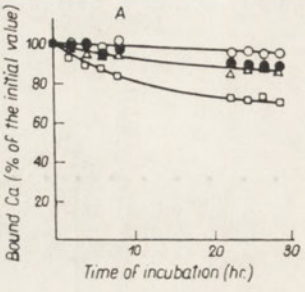


Fig. 5



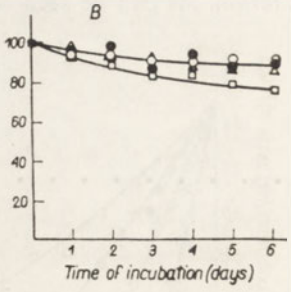


Fig. 6

Fig. 5. The effect of free ATP and free Ca^{2+} on the release of bound ATP from G-actin. G-Actin - -[^{14}C]ATP solution (2.9 mg./ml.) in 4 mM-tris-HCl, pH 7.9, was deprived of free bivalent cations and free ATP by successive treatment with Dowex 50 and Dowex 1. After removing the resins the solution was incubated at 21°. At time intervals indicated on the abscissa samples of the solutions were treated with Dowex 1 for 3 min. and, after centrifuging off the resin, radioactivity and protein concentration in the supernatants were determined. (△), 0.1 mM-$CaCl_2$; (●), 0.1 mM-ATP; (○), 0.1 mM-$CaCl_2$ and 0.1 mM-ATP, added at zero time; (□), control, no $CaCl_2$ and ATP added.

Fig. 6. The effect of free ATP and free Ca^{2+} on the release of bound Ca from G-actin. G-Actin - -^{45}Ca solution (2.6 mg./ml.) in 4 mM-tris-HCl, pH 7.9, was deprived of free ATP and free bivalent cations by successive treatment with Dowex 1 and Dowex 50. After removal of the resins the solution was incubated at 21° (*A*) or at 0° (*B*). At time intervals indicated on the abscissa samples of the solutions were treated with Dowex 50 for 3 min. and, after centrifuging off the resin, radioactivity and protein concentration in the supernatants were determined. (△), 0.1 mM-$CaCl_2$; (●), 0.1 mM-ATP; (○), 0.1 mM-$CaCl_2$ and 0.1 mM-ATP, added at zero time; (□), control, no $CaCl_2$ and ATP added.

incubation. For those reasons it was rather difficult to determine the half-life times for the combinations of Ca and ATP with G-actin. From the slope of the curves at the initial period of incubation the values of the order of 20 hr. were obtained. $CaCl_2$ or ATP at 0.1 mM concentration added to actin solutions at zero time greatly reduced or almost completely prevented any change in the content of both bound Ca and bound ATP.

The release of bound Ca *and bound ATP from G-actin during incubation in the presence of Dowex* 1 *or Dowex* 50

The release of both bound Ca and bound ATP during incubation of G-actin in the continuous presence of either Dowex 50 or Dowex 1 was studied. It might be assumed that in the case of incubation with Dowex 50 any amount of Ca liberated

from the complex with G-actin was instantaneously removed by the resin and thus the protective effect of free Ca was eliminated. In these series of experiments the effect of free ATP on the release of both bound Ca and bound ATP was examined. Similarly, in the presence of Dowex 1 the immediate removal of any liberated ATP was supposed to take place. In this case the addition of free Ca to the incubation mixture made possible to examine its effect on the release of bound Ca and bound ATP. All these experiments enabled us to compare the rates of the release of bound Ca and bound ATP from G-actin in various conditions and to examine separately the influence of free Ca and free ATP on the release of both bound Ca and bound ATP.

It was found that the increase of pH of the medium from 7.0 to 8.0 distinctly enhanced the rate of the release of both bound Ca and bound ATP. Therefore all experiments of each series were performed at one pH value. Similarly, the actin concentration as well as the ratio of the resin to actin were kept on the same level and the same lot of each resin was used throughout all further experiments.

Figure 7A shows the release of bound Ca from G-actin incubated in the presence of Dowex 50 at 21° ± 1° and pH 8.0 after removal of free ATP from the solution. Parallelly the release of bound ATP in these conditions was examined (Fig. 8A). In the latter case the samples of the incubation mixture, immediately after withdrawing Dowex 50, were treated with Dowex 1 to remove free ATP. The results of experiments in which G-actin was incubated in the presence of Dowex 50 with 0.1 mM-ATP are shown in Fig. 7B and Fig. 8B. As can be seen, in all cases there was a linear relationship between G-actin-bound Ca or bound ATP content, presented in a logarithmic scale, and the time of incubation, at least up to the release of about half of the initial amount of bound Ca or ATP. This indicates that the release of bound Ca and ATP followed the kinetics of a first-order reaction. The upwards deviation from the linearity observed when the incubation was prolonged, especially in the case of determinations of the bound Ca, was probably due to the protective effect of ATP being released from its complex with actin and accumulated in the incubation mixture, and possibly also due to some lowering of pH which took place during prolonged incubation. Such a lowering of pH of the solution (by about 0.2 - 0.3 pH units) was observed when Dowex 50 or Dowex 1 was stirred in 4 mM-tris-HCl buffer, pH 8, for a longer period of time, even in the absence of the protein; however, during about half an hour of stirring there was practically no change of pH of the solution. The decrease in pH was not observed in the experiments started at pH 7.

The release of bound ATP and bound Ca from G-actin incubated at 21° ± 1° and pH 8.0 in the presence of Dowex 1 after removal of free bivalent cations from the solution is shown in Fig. 9A and 10A, respectively. To follow the changes in the content of bound Ca the samples of the incubation mixture, immediately after withdrawing Dowex 1, were treated with Dowex 50 for removal of free Ca. In the experiments shown in Fig. 9B and Fig. 10B the effect of 0.1 mM-$CaCl_2$ added at zero time is presented.

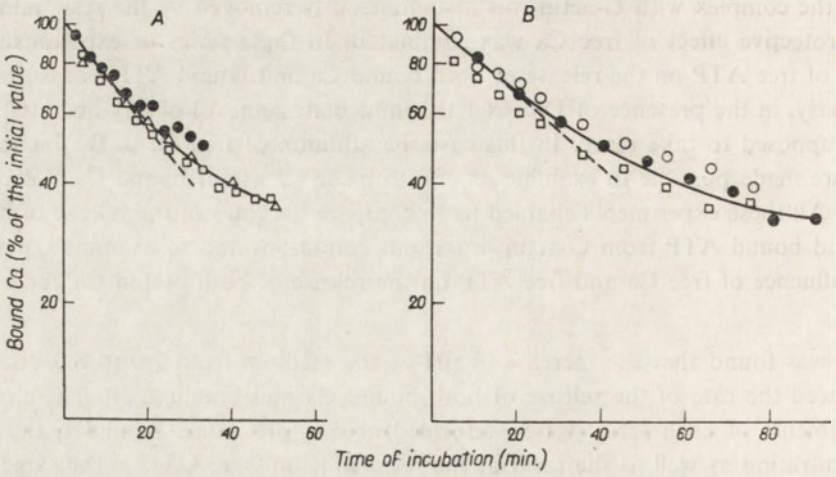


Fig. 7. The release of bound Ca from G-actin incubated in the presence of Dowex 50. G-Actin - ^{45}Ca solution (2.9 - 3.1 mg./ml.) in 4 mM-tris-HCl, pH 8.0, deprived of free ATP by Dowex 1 treatment, was stirred with 1/14 volume of wet Dowex 50 at 21°. In series (*A*) no ATP was added; in series (*B*) 0.1 mM-ATP was added at zero time. Further procedure as described in the legend of Fig. 1B. Zero time corresponds to the sixth minute after addition of Dowex 50 and therefore the initial level of ^{45}Ca in the solution is not shown. Different symbols denote the results of separate experiments.

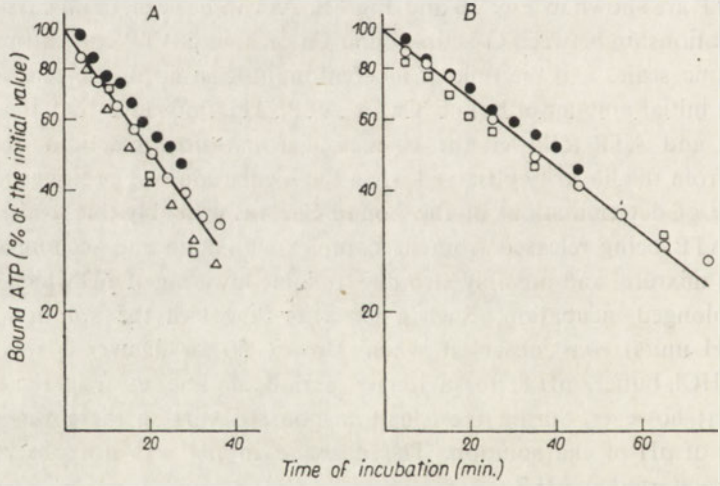


Fig. 8. The release of bound ATP from G-actin incubated in the presence of Dowex 50. G-Actin - - [^{14}C]ATP solution (2.9 - 3.1 mg./ml.) in 4 mM-tris-HCl, pH 8.0, deprived of free ATP by Dowex 1 treatment was stirred with 1/14 volume of wet Dowex 50 at 21°. In series (*A*) no ATP was added; in series (*B*) 0.1 mM-ATP was added 10 min. before starting the incubation. At time intervals indicated on the abscissa samples of the incubation mixture were freed of the resin by 1 min. centrifugation and the supernatants were shaken with Dowex 1 for 3 min. to remove ATP released during incubation. After centrifuging off the resin, radioactivity and protein concentration were determined in the supernatants. Zero time corresponds to the sixth minute after addition of Dowex 50. Different symbols denote the results of separate experiments.

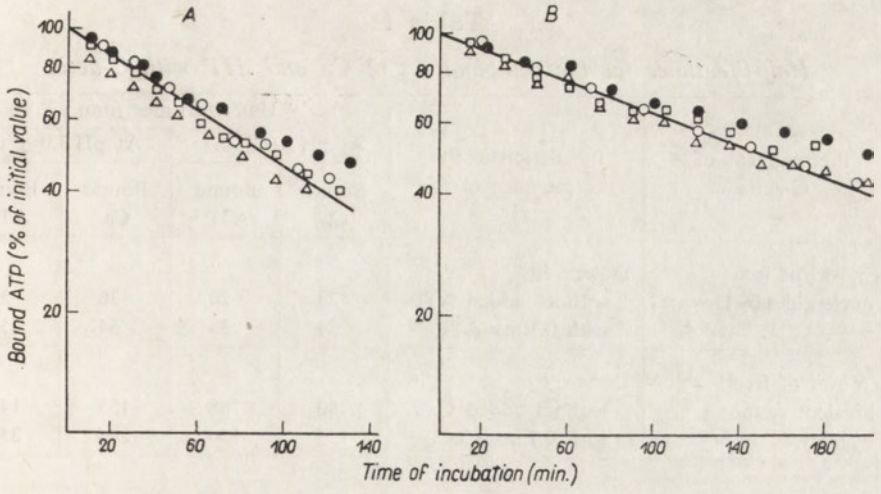


Fig. 9. The release of bound ATP from G-actin incubated in the presence of Dowex 1. G-Actin - - [^{14}C]ATP solution (3.0 - 3.2 mg./ml.) in 4 mM-tris-HCl, pH 8.0, deprived of free bivalent cations by Dowex 50 treatment, was stirred with 1/14 volume of wet Dowex 1 at 21°. In series (*A*) no $CaCl_2$ was added; in series (*B*) 0.1 mM-$CaCl_2$ was added at zero time. Further procedure as described in the legend of Fig. 1B. Zero time corresponds to the sixth minute after addition of Dowex 1 and therefore the initial level of [^{14}C]ATP in the solution is not shown. Different symbols denote the results of separate experiments.

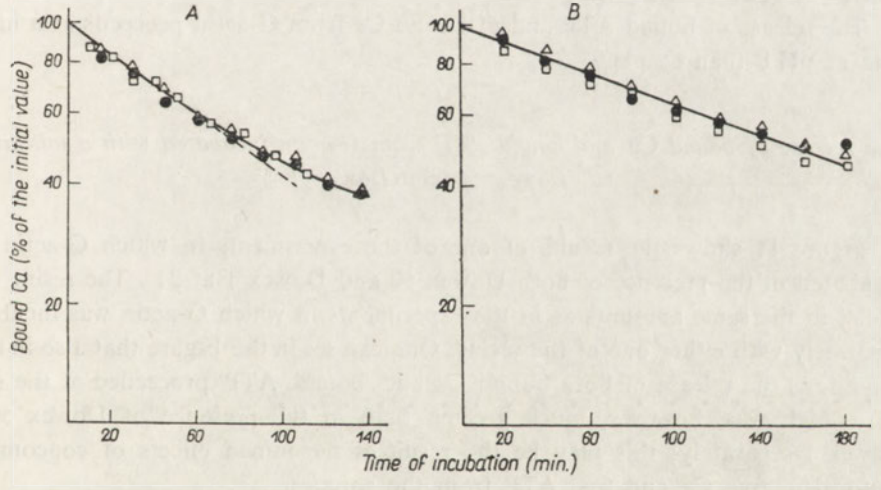


Fig. 10. The release of bound Ca from G-actin incubated in the presence of Dowex 1. G-Actin - - ^{45}Ca solution (2.9 - 3.2 mg./ml.) in 4 mM-tris-HCl, pH 8.0, deprived of free bivalent cations by Dowex 50 treatment, was stirred with 1/14 volume of wet Dowex 1 at 21°. In series (*A*) no $CaCl_2$ was added; in series (*B*) 0.1 mM-$CaCl_2$ was added 15 min. before starting the incubation. At time intervals indicated on the abscissa samples of the incubation mixture were freed of the resin by 1 min. centrifugation and the supernatants were shaken with Dowex 50 for 3 min. to remove Ca released during incubation. After centrifuging off the resin, radioactivity and protein concentration were determined in the supernatants. Zero time corresponds to the sixth minute after addition of Dowex 1. Different symbols denote the results of separate experiments.

Table 1

Half-life times for the combinations of Ca *and ATP with G-actin*

Pretreatment of G-actin	Incubation in the presence of	Half-life times (min.)			
		At pH 8.0 ± 0.1		At pH 7.0 ± 0.1	
		Bound Ca	Bound ATP	Bound Ca	Bound ATP
Removal of free nucleotides by Dowex 1	Dowex 50				
	without added ATP	23	20	36	29
	with 0.1 mM-ATP	34	34	64	58
Removal of free bivalent cations by Dowex 50	Dowex 1				
	without added Ca	80	88	155	146
	with 0.1 mM-$CaCl_2$	154	150	296	330

On the basis of the results of all experiments with Dowex 1 at pH 8 (Figs. 7 - 10) as well as of similar experiments performed at pH 7, average half-life time values of the combination of Ca and of ATP with G-actin were calculated (Table 1). For calculations only the initial period of incubation with the resins, in which the reaction was linear with time, was taken into account. In every case the release of bound Ca parallels the release of bound ATP. The results show that free Ca ions and free ATP decrease the rates of the release of both bound Ca and bound ATP to about the same extent.

The release of bound ATP and of bound Ca from G-actin proceeds at a higher rate at pH 8 than at pH 7.

The release of bound Ca *and bound ATP from G-actin incubated with a mixture of Dowex* 50 *and Dowex* 1

Figure 11 shows the results of one of the experiments in which G-actin was incubated in the presence of both Dowex 50 and Dowex 1 at 21°. The resins were added in the same amounts as in the experiments in which G-actin was incubated separately with either one of the resins. One can see in the Figure that also in these conditions the release of both bound Ca and bound ATP proceeded at the same rate which was, however, much greater than in the presence of Dowex 50 or Dowex 1 separately; this may be the result of the joined effects of concomitant removal of free Ca and free ATP from the solution.

DISCUSSION

The exchange of G-actin-bound ATP with free ATP, the spontaneous release of bound ATP in the absence of free ATP and the protective effect of free ATP against inactivation of G-actin led to the conclusion that an equilibrium between bound and free ATP exists in G-actin solutions (Martonosi *et al.*, 1960; Asakura,

1961; Strohman & Samorodin, 1962; Tsuboi & Hayashi, 1963). The existence of an analogous equilibrium between bound and free Ca was suggested by Barany *et al.* (1962) on the basis of the rapid and complete exchange of G-actin-bound Ca with free Ca ions.

In the recent years a close relationship between the binding of ATP and Ca by G-actin was demonstrated. Several agents known to remove bound ATP appeared to lead also to the release of bound Ca. On the other hand, chelating agents caused not only the removal of bound Ca but also a liberation of bound ATP. On the

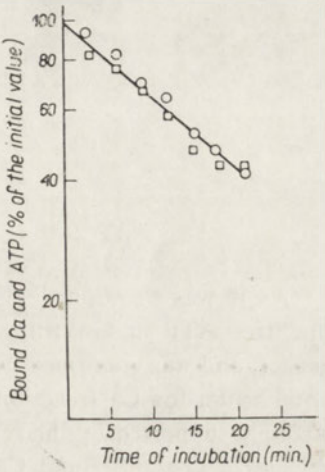


Fig. 11. The release of bound Ca and bound ATP from G-actin incubated with a mixture of Dowex 50 and Dowex 1. G-Actin solution (3.0 mg./ml.) in 2 mM-tris-HCl, pH 7.5, was divided into two portions; one was preincubated with 0.05 mM-$^{45}CaCl_2$ (□), the second with 0.05 mM-[^{14}C]ATP (○). Both solutions were then stirred at 21° with 1/14 volume of wet Dowex 50+1/14 volume of wet Dowex 1. Further procedure as described in the legend of Fig. 1B.

basis of the above observations the participation of Ca in the binding of ATP by G-actin was suggested (Martonosi & Gouvea, 1961; Strohman & Samorodin, 1962; Barany *et al.*, 1962; Maruyama, 1962; Tonomura & Yoshimura, 1962). All the results of the present work confirm the close relationship between the binding of ATP and Ca by G-actin and show, moreover, that not only Ca stabilizes the binding of ATP but also ATP stabilizes the binding of Ca to G-actin.

Before the role of Ca in actin molecule was known, the scheme of inactivation of G-actin was proposed as follows (Asakura, 1961; Grubhofer & Weber, 1961):

$$\text{G-ATP} \rightleftharpoons \text{G} + \text{ATP}$$
$$\downarrow$$
$$\text{I}$$

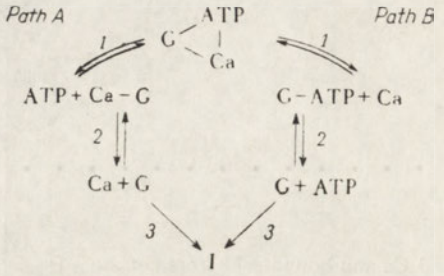
where G was a molecule of active and I, that of inactive G-actin. This scheme was later on extended by Maruyama (1962), who suggested the following reaction as the first step of the inactivation process:

$$\text{G} - \text{ATP} \rightleftharpoons \text{G-ATP} + \text{Ca}$$
$$\diagdown\!\diagup$$
$$\text{Ca}$$

In the complex of G-actin with ATP and Ca, according to the previous suggestions (Martonosi & Gouvea, 1961; Strohman & Samorodin, 1962; Maruyama, 1962; Tonomura & Yoshimura, 1962), ATP has at least two-point binding site: one to the

protein through the purine ring and the other to Ca through the pyrophosphate group. Consequently, Ca should also have two binding sites: one to ATP and the other to the protein.

One may assume that the dissociation of G<ATP/Ca complex can proceed in two ways, *A* or *B*, both leading to the same final effect, i.e. to the formation of inactive actin (I) according to the following scheme.



As it was mentioned above, the existence of an equilibrium between bound and free ATP in G-actin solutions (reaction *1* of Path *A*) had been established earlier, and was confirmed in the present work. The existence of similar dissociation equilibrium for Ca (reaction *1* of Path *B*), postulated by Barany *et al.* (1962), is strongly supported by the results of the present work showing the inhibition of the release of G-actin-bound Ca by free Ca ions and the acceleration of this process by Dowex 50.

The equilibrium of both reactions, *1A* and *1B*, appears to be shifted towards the formation of the complex G<ATP/Ca because the dissociation of this complex proceeds very slowly even after the removal of free nucleotides and free bivalent cations from the solution (see Figs. 5 and 6). When free ATP or free Ca is continuously removed from the solution, which is the case during incubation of G-actin in the presence of Dowex 1 or Dowex 50, respectively, the dissociation equilibria determined by the reactions *1A* and *1B* in the presented scheme are disturbed and the inactivation proceeds much faster than in the absence of the resins. One can suppose that in the presence of Dowex 1 inactivation of G-actin proceeds through the Path *A*, and in the presence of Dowex 50 through the Path *B*; in the first case G-Ca and in the second one G-ATP are formed as intermediates. The appearance of G-ATP, strongly suggested by the experiments of Strohman & Samorodin (1962) and Maruyama (1962), would explain the inhibition of the release of both bound Ca and bound ATP by free ATP during incubation of G-actin with Dowex 50. Similarly, the existence of G-Ca would explain the inhibition of the release of bound Ca and bound ATP by free Ca when G-actin is incubated with Dowex 1.

During incubation with Dowex 1 at 21° the changes in the content of bound Ca appeared to proceed at the same rate as the release of bound ATP (see Figs. 9

and 10). Similarly, in the case of incubation with Dowex 50, the rate of the release of bound ATP was found to be the same as that of bound Ca (see Figs. 7 and 8). This indicates that, at least in the conditions of our experiments, the intermediates G-Ca and G-ATP are very easily split.

In our experiments the release of bound Ca during incubation of G-actin with Dowex 50 was found to proceed faster than the release of bound ATP during incubation with Dowex 1. It seems, however, that this fact alone cannot serve as an evidence for the difference between the values of dissociation constants of the reaction *1* of the Path *A* and that of the Path *B*. We tried to maintain the same conditions (pH, protein concentration, ratio of the amount of the resin to actin) in the experiments with Dowex 50 and Dowex 1, nevertheless, such factors as the exchange capacity, the pore size, crossing and perhaps some other properties of the resin could also influence the rates of the release of bound Ca and bound ATP. Therefore even the rates of the release of the same compound, Ca or ATP, are not comparable in the experiments in which different lots of the resin were used (e.g. Fig. 3 and Fig. 7A or Fig. 4 and Fig. 9A). For the same reasons it is difficult to compare the values of half-life times obtained in the present work with those determined by Asakura (1961) and Grubhofer & Weber (1961) because all the determinations were performed in different conditions.

The results of the present paper show that the release of the bound Ca and bound ATP from G-actin proceeds faster at pH 8 than at pH 7. It is known that in the more alkaline medium (pH $\sim$ 10) G-actin undergoes rapid denaturation (Martonosi & Gouvea, 1961; Mihashi & Ooi, 1965; Drabikowski & Stahl, 1966). One may assume that already at pH about 8 some conformational changes in the G-actin molecule occur, which promote the release of bound Ca and ATP.

As it was mentioned in the introduction, it seemed necessary to establish proper conditions for the quantitative removal of free ATP and free Ca from G-actin without liberation of bound ATP and bound Ca. The obtained results clearly show that a few minutes treatment with Dowex 50 is sufficient for the removal of free Ca, similarly to the removal of free ATP by Dowex 1. Longer incubation with Dowex 50 or Dowex 1, apart from some quantitative differences between these two resins (see Figs. 1 and 2), leads to the release of some part of bound Ca or bound ATP, respectively. Although the rates of the release of bound Ca and bound ATP greatly depend on the ratio of the amount of the resin to actin (see Fig. 3 and Fig. 4), all curves of the release of each of these compounds, extrapolated to zero time, give the same point on the ordinate. This point most probably corresponds to the real value of the originally bound Ca or ATP. Since, however, the values obtained after the first few minutes of treatment with Dowex 50 or Dowex 1 are fairly close to the extrapolated zero time value they can serve in practice as the measure of the amount of the originally bound Ca or ATP.

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WSPÓŁZALEŻNOŚĆ MIĘDZY WIĄZANIEM WAPNIA I ATP PRZEZ G-AKTYNĘ

Streszczenie

1. Badano szybkość uwalniania związanego ATP i związanego Ca z ich kompleksu z G-aktyną. Stwierdzono, że podczas inkubacji G-aktyny w obecności Dowexu 1 uwalnianiu związanego ATP towarzyszy zachodzące z podobną szybkością uwalnianie związanego Ca. Analogicznie podczas inkubacji G-aktyny w obecności Dowexu 50 równocześnie z uwalnianiem związanego Ca zachodzi uwalnianie związanego ATP. Wolny ATP i wolny Ca, użyte w jednakowym stężeniu, w podobnym stopniu zmniejszają szybkość uwalniania związanego ATP i związanego Ca. Uzyskane wyniki potwierdzają pogląd o istnieniu ścisłej współzależności między wiązaniem ATP i Ca przez G-aktynę.

2. Przedstawiono schemat mechanizmu dysocjacji kompleksu G-aktyna - Ca - ATP, w którym postulowane jest istnienie labilnych intermediatów G-aktyna - ATP i G-aktyna - Ca.

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THE EFFECT OF PHENYLALANINE ADMINISTRATION ON THE ACTIVITIES OF PHENYLALANINE HYDROXYLASE, SOME AMINOTRANSFERASES AND DECARBOXYLASES IN ADULT RATS

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Dedicated to Professor Józef Heller in honour of his 70th birthday

1. After intraperitoneal administration to adult rats of L-phenylalanine (0.5 m-mole/100 g. body wt.), a transient increase of phenylalanine was observed in blood (maximum after 1 hr.) and in the liver (max. after 4 hr.); the activity of phenylalanine hydroxylase in the liver extract decreased simultaneously to 30% of the normal value. 2. Phenylalanine aminotransferase activity in the liver extract was not affected 4 hr. after the injection of phenylalanine, whereas the treatment with tyrosine or tryptophan increased the activity 2 to 3-fold. The activities of tryptophan and tyrosine aminotransferases were enhanced several times after the administration of the aromatic amino acids studied. 3. The activity of DOPA decarboxylase in kidney and liver increased twofold at 2 hr. after the injection of phenylalanine or tyrosine, and at 4 hr. in kidney, liver and brain it decreased to half of the initial values.

In phenylketonuria (*Oligophrenia phenylpyruvica*), an inborn error of metabolism, the primary defect is the impairment of phenylalanine hydroxylase (Mitoma, Auld & Udenfriend, 1957; Wallace, Moldave & Meister, 1957) which catalyses the conversion of phenylalanine to tyrosine. This leads to an increased content of phenylalanine in blood and urine, and to the occurrence of phenylpyruvate, *o*-hydroxyphenylacetate, phenyllactate and phenylacetylglutamate in urine. These characteristic features of phenylketonuria were also observed in rats fed for several weeks a diet containing added phenylalanine (Auerbach, Waisman & Wyckoff, 1958; Wang & Waisman, 1961; Boggs & Waisman, 1964) and in rats after injection of phenylalanine (Goldstein, 1961).

Phenylketonuria in children is accompanied by mental defect but the mechanism of this phenomenon has not yet been elucidated. No correlation was found between the degree of intellectual impairment and the content of phenylalanine or phenylpyruvate in blood (Borek, Brecher, Jervis & Waelsch, 1950). It can be assumed that in phenylketonuria the primary metabolic defect leads to secondary biochemical disturbances which have a toxic effect on the brain.

In the present paper, the effect of intraperitoneal injection of phenylalanine to adult rats on the content of phenylalanine in blood and liver, and on the activity of phenylalanine hydroxylase is reported. The effect of phenylalanine on transamination of aromatic amino acids was also studied as well as the activity of decarboxylase, in connection with the known physiological role of amines in the brain. The effect of tyrosine and tryptophan administration is also described.

A preliminary report has been presented (Bełżecka, 1965).

MATERIALS AND METHODS

Animals. White rats of either sex 4 - 5 months old, weighing 200 - 230 g., were used for experiments. A single dose of 7% aqueous solution of L-phenylalanine, L-tyrosine or L-tryptophan was administered intraperitoneally (0.5 m-mole per 100 g. body weight). Following the injection, the animals were deprived of food but received drinking water *ad libitum*.

Homogenates and tissue extracts. The animals were stunned and killed by decapitation. The blood was collected into a vessel containing sodium citrate, centrifuged immediately, and plasma was separated.

Liver, kidneys and brain were isolated, immediately cooled and the subsequent procedures were carried out at 0 - 4°. Liver and kidney before homogenization were disintegrated by passing them through a fine sieve. The conditions of homogenization in all-glass Potter-type apparatus, and centrifugation are described below for the respective methods. The supernatants obtained were freed of a thin layer of fat and used as tissue extracts for determinations of enzymic activity.

Determination of phenylalanine. Blood plasma and liver homogenates (1 g. in 2 vol. of 0.01 M-Na-K-phosphate buffer, pH 7.0) were deproteinized with an equal volume of an ethanol-acetone mixture (1 : 1, v/v), centrifuged, and the content of phenylalanine in the supernatant was determined by the method of Berry (1962). In this method, after chromatographic separation on Whatman no. 1 paper in the solvent system: butan-1-ol - ethanol - water (7 : 2 : 3, by vol.) the spots are located with 0.2% ninhydrin solution in acetone, and after 24 hr. the amount of phenylalanine is determined by comparison with the intensity of the standard spots. The accuracy of this method in our hands was ±10% when the content of phenylalanine in blood plasma did not exceed 5 mg.%, and ±5% for higher content of phenylalanine in plasma and in liver.

Phenylalanine hydroxylase activity. The liver was homogenized with 2 vol. of 0.9% KCl and centrifuged for 60 min. at 0° and 13 000 *g*. The extract obtained from 1 g. of the tissue contained 100 - 120 mg. of protein. The enzyme assay was performed after Udenfriend & Cooper (1952) and the incubation mixture contained in a volume of 3.75 ml.: the extract from 0.5 g. of liver (1.5 ml.), 1.65 μmoles of NAD, 15 μmoles of nicotinamide, 0.01 M-Na-K-phosphate buffer, pH 7.0 and 6 μmoles of L-phenylalanine. However, in a number of experiments, different amounts of phenylalanine were used as indicated in the Results. At zero time and after 30 min.

incubation at 37° (with shaking), the 1.8 ml. samples were withdrawn, deproteinized with 0.85 ml. of 15% trichloroacetic acid, and tyrosine was determined in the supernatant by the colorimetric method of Ceriotti & Spandrio (1957). The activity was expressed as μmoles of tyrosine formed per 1 g. of liver per 30 min.

An aqueous extract from rat liver deproteinized by heating according to Kaufman (1958) was used as a source of natural phenylalanine hydroxylase cofactor.

Activity of phenylalanine, tyrosine and tryptophan aminotransferase(s). The liver was homogenized with 3 vol. of 0.2 M-Na-K-phosphate, pH 7.0, and centrifuged for 15 min. at 0° and 10 000 *g*. In the extract, the activities of the aromatic amino acids - α-oxoglutarate aminotransferases were determined according to Lin, Pitt, Civen & Knox (1958) by preincubating the extract for 5 min. at 37° with 0.05 μmole of pyridoxal phosphate, then adding alternatively 6 μmoles of L-phenylalanine, 6 μmoles of L-tyrosine or 60 μmoles of L-tryptophan, and 40 μmoles of α-oxoglutarate and 0.2 M-Na-K-phosphate buffer, pH 7.0, to a final volume of 1.75 ml. For tyrosine aminotransferase determination, 5 μmoles of diethyldithiocarbamate was additionally applied. After 10 min. incubation at 37°, the mixture was deproteinized with metaphosphoric acid, centrifuged, and in the supernatant the aromatic α-oxoacid was determined. The extinction of enol borate tautomer of phenylpyruvate was measured at 300 mμ, of *p*-hydroxyphenylpyruvate at 310 mμ and of indolepyruvate at 328 mμ. The activity was expressed as μmoles of aromatic oxoacid formed per 10 min. per 100 mg. of extract protein.

Activity of phenylalanine, tyrosine and DOPA[1] *decarboxylation.* The liver, kidney and brain were homogenized with 3 vol. of 0.2 M-Na-K-phosphate buffer, pH 7.0, and centrifuged for 15 min. at 0° and 10 000 *g*. In the supernatant, decarboxylase activity was determined manometrically. After a 20 min. preincubation at 37° of the tissue extract with 0.7 μmole of pyridoxal phosphate and 0.2 M-Na-K-phosphate buffer, pH 7.0, in the Warburg flask, 12 μmoles of substrate was added from the side arm. The readings were taken at 5-min. intervals, and after the production of CO_2 had ceased, 0.2 ml. of 6 N-H_2SO_4 was added to liberate the total amount of CO_2, and the readings were taken after another 15 min. The activity was expressed as μmoles CO_2 per 100 mg. protein of the extracts studied.

Formation of the phenylalanine and tyrosine decarboxylation products was also checked chromatographically. After a 60 min. incubation at 37°, the reaction mixture was adjusted with acetic acid to pH 5 and heated for 3 min. in a boiling water bath. Then 0.3 ml. of the deproteinized supernatant was applied on Whatman no. 4 paper and the chromatograms were developed in the system: butan-1-ol - - acetic acid - water (4 : 1 : 5, by vol.). The spots were located after Schwick & Adams (1959) with 0.5% solution of diazonium salt of sulphanilic acid in 10% Na_2CO_3.

Protein determination. The tannin method of Mejbaum-Katzenellenbogen (1955) was used.

Special reagents. 3,4-Dihydroxyphenylalanine, 3,4-dihydroxyphenylethylamine, phenylacetic acid and phenylpyruvic acid (Koch-Light Lab., Colnbrook, Bucks.,

[1] DOPA, 3,4-dihydroxyphenylalanine.

England); L-tyrosine (Zakłady Farmaceutyczne Polfa, Warszawa, Poland); α-oxoglutaric acid and NAD (Nutr. Biochem. Corp., Cleveland, Ohio, U.S.A.); L-tryptophan (Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland); L-phenylalanine and pyridoxal phosphate (Fluka A. G., Buchs, Switzerland); *p*-hydroxyphenylpyruvic acid and nialamide [1,2-(benzylcarbamyl)ethyl-2-isonicotinoyl-hydrazine] were Sigma Chem. Comp., St. Louis, Mo., U.S.A., products; α-nitroso-β--naphthol, trichloroacetic acid and sulphanilic acid (Fabryka Odczynników Chemicznych, Gliwice, Poland); diethyldithiocarbamate (B.D.H., Poole, England); diazonium salt of sulphanilic acid was prepared by treating sulphanilic acid with sodium nitrite.

RESULTS

Phenylalanine in blood plasma and liver

In a normal rat the content of phenylalanine in blood plasma amounted to 1 mg.% and in the liver to 15 mg. per 100 g. of fresh tissue. After intraperitoneal injection of 0.5 m-mole of phenylalanine per 100 g. rat body weight, the content of phenylalanine increased reaching in blood plasma at 1 hr. the maximum, 45 mg.%,

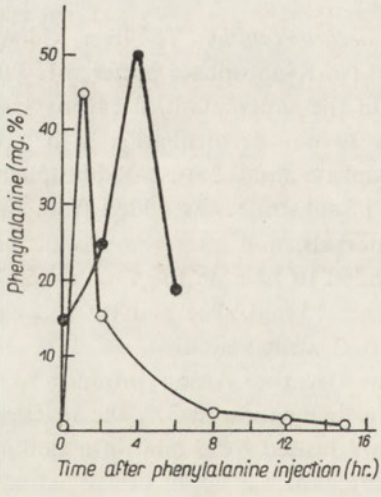

Fig. 1

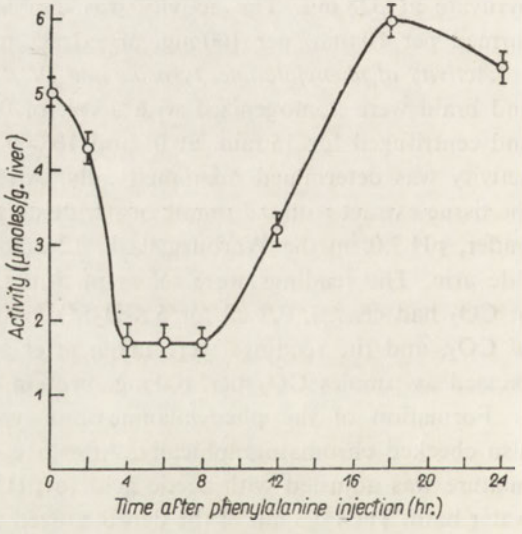

Fig. 2

Fig. 1. The effect of intraperitoneal administration to rats of 0.5 m-mole of L-phenylalanine per 100 g. body wt., on the content of phenylalanine in (○), blood serum (mg./100 ml.) and (●), liver (mg./100 g. fresh wt.).

Fig. 2. Activity of phenylalanine hydroxylase in rat liver after intraperitoneal administration of 0.5 m-mole of phenylalanine per 100 g. body wt. The incubation mixture contained in a volume of 3.75 ml.: 6 μmoles of L-phenylalanine, 1.65 μmoles of NAD, 15 μmoles of nicotinamide, 1.5 ml. of liver extract (50 - 60 mg. protein) and 0.01 M-Na-K-phosphate buffer, pH 7.0. The activity is expressed as μmoles of tyrosine formed per 1 g. of liver per 30 min. Average values from 3 experiments are given.

whereas in the liver the maximum content, 50 mg.%, was observed after 4 hr. Later its content in blood plasma and liver gradually decreased (Fig. 1).

In the urine, the test with 10% $FeCl_3$ for the presence of phenylpyruvate was negative before the injection, positive at 2 hr. after the injection, and after another 2 hr. the presence of phenylpyruvate could not be detected.

Hydroxylation of phenylalanine in liver

The relation between the activity of phenylalanine hydroxylase and the time interval since the administration of phenylalanine, is shown in Fig. 2. The incubation mixture contained 12 μmoles of the substrate per 1 g. of liver, as in the experiments of Udenfriend & Cooper (1952). In the liver of the normal rat, the activity amounted to 5 μmoles of tyrosine formed per 1 g. of liver. After administration of phenylala-

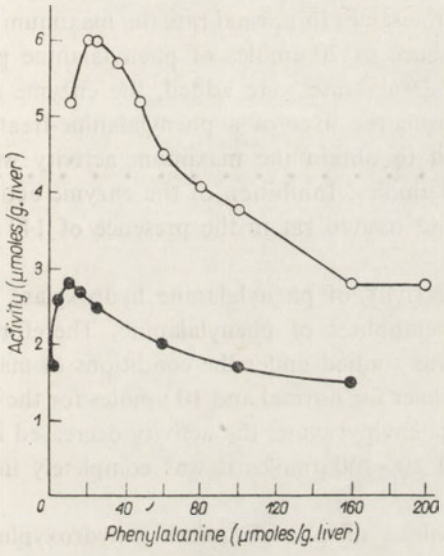

Fig. 3

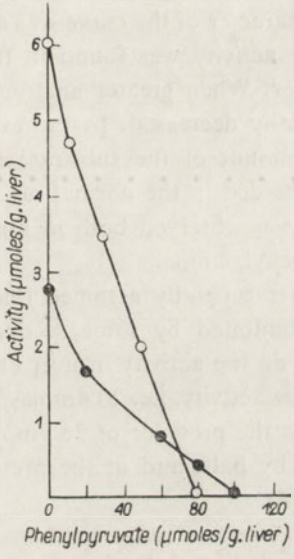

Fig. 4

Fig. 3. The effect of substrate concentration *in vitro* on the activity of phenylalanine hydroxylase in the liver (○), of a control rat, and (●), at 4 hr. after administration of L-phenylalanine. The incubation mixture was as described for Fig. 2, except that different amounts of phenylalanine were applied. The activity is expressed as μmoles of tyrosine formed per 1 g. liver per 30 min.

Fig. 4. The effect of phenylpyruvate *in vitro* on the activity of phenylalanine hydroxylase in the liver (○), of a control rat and (●), at 4 hr. after administration of L-phenylalanine. The composition of the incubation mixture was as described for Fig. 2 except that for the extract from the normal rat 10 μmoles of phenylalanine was applied, and for that from the treated rat, 5 μmoles. The activity is expressed as μmoles of tyrosine formed per 1 g. of liver per 30 min.

nine, the activity decreased reaching at 4 hr. 1.7 μmoles; this 70% inhibition was maintained for a further 4 hr., then the activity increased, being after 18 hr. somewhat higher than that found in the normal rat, and at 24 hr. it returned to a normal value.

The considerable decrease of hydroxylase activity after phenylalanine administration was not due to a lack of cofactor as the addition of the crude cofactor preparation (Kaufman, 1958) had no effect on the activity.

Udenfriend & Cooper (1952) demonstrated *in vitro* that phenylalanine hydroxylase is inhibited by the excess of substrate. It was supposed therefore that the decreased activity in treated rat was due to the increased amount of phenylalanine in liver, and reactivation of the inhibited enzymic activity by dialysis was attempted. It was found, however, that a 3 hr. dialysis at 0 - 3° with shaking, against a continuously flowing solution of 0.9% KCl in 0.01 M-Na-K-phosphate buffer, pH 7.0, did not change the hydroxylase activity of the liver extract, either from the normal or phenylalanine-treated rats.

The dependence of hydroxylase activity on substrate concentration was studied and the result of a typical experiment is shown in Fig. 3. This experiment was repeated several times; the results obtained ranged within the limits of ±10% and the character of the curve was always the same. In normal rats the maximum hydroxylase activity was found in the presence of 20 μmoles of phenylalanine per 1 g. of liver. When greater amounts of the substrate were added, the enzyme activity gradually decreased. In the extract from the liver of a phenylalanine-treated rat, the amount of the substrate required to obtain the maximum activity was half that needed in the normal rat, i.e. 10 μmoles. Inhibition of the enzyme activity by 50% was observed both in normal and treated rat in the presence of 140 μmoles of phenylalanine.

It is generally assumed that the activity of phenylalanine hydroxylase can be also inhibited by some secondary metabolites of phenylalanine. Therefore their effect on the activity in liver extract was studied under the conditions of maximum enzyme activity, i.e. 20 μmoles/1 g. of liver for normal and 10 μmoles for the treated rat. In the presence of 35 μmoles of phenylpyruvate, the activity decreased in both cases by half, and in the presence of 80 - 100 μmoles it was completely inhibited (Fig. 4).

The two other secondary metabolites of phenylalanine: *p*-hydroxyphenylpyruvate and phenylacetate at concentration 10 and 20 μmoles per 1 g. of liver, had no effect on the hydroxylase activity in the liver extract from the normal rat.

Transamination of phenylalanine, tyrosine and tryptophan in liver

In the control rat, the activity of phenylalanine aminotransferase amounted to 1.0 μmole of phenylpyruvate formed per 100 mg. of protein of liver extract; 4 hr. after the administration of phenylalanine the activity remained unchanged, whereas the injection of the same amount of tyrosine or tryptophan increased the activity two- and threefold, respectively (Fig. 5).

The activity of tyrosine aminotransferase was 3.5 μmoles of *p*-hydroxyphenylpyruvate, and after the administration of each of the three aromatic amino acids used it increased 6 - 10 fold, the greatest increase being found after the injection of tyrosine.

In normal rat liver, the activity of tryptophan aminotransferase was 2.2 μmoles of indolepyruvate formed. After the treatment with phenylalanine, tyrosine or tryptophan, the activity was enhanced 4 - 5 fold.

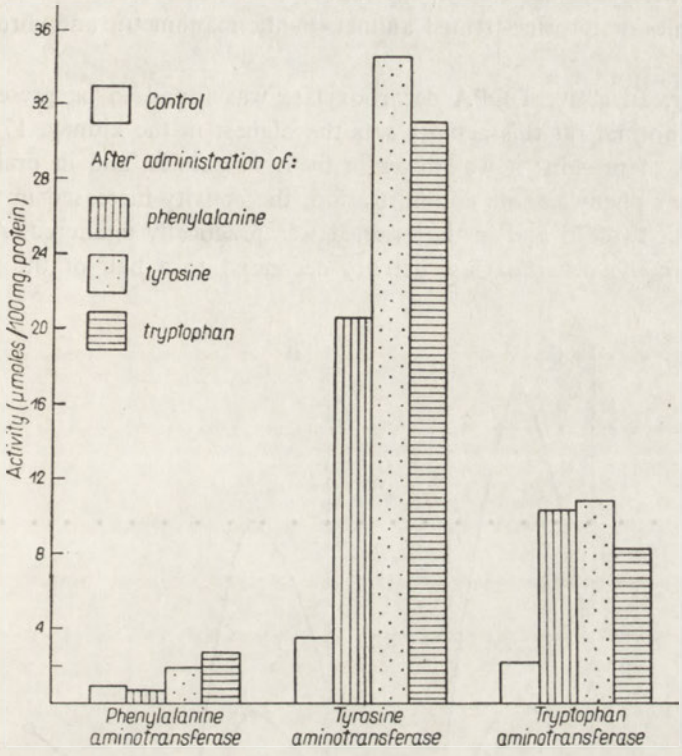

Fig. 5. The effect of administration of phenylalanine, tyrosine, or tryptophan on the activity of aromatic amino acids : α-oxoglutarate aminotransferases in rat liver. For determinations of activities of the three aminotransferases in the liver of control rats, and for determination of phenylalanine aminotransferase in treated rats, 0.3 ml. of the extract (9.0 - 13.2 mg. protein) was used, and for other determinations, 0.1 ml. of the extract (3.0 - 4.4 mg. protein). The extract was preincubated for 5 min. at 37° with 0.05 μmole of pyridoxal phosphate. Then was added 6 μmoles of L-phenylalanine, or 6 μmoles of L-tyrosine or 60 μmoles of L-tryptophan, and 40 μmoles of α-oxoglutarate and 0.2 M-Na-K-phosphate buffer, pH 7.0, to a final volume of 1.75 ml. For tyrosine aminotransferase determination, 5 μmoles of diethyldithiocarbamate was additionally applied. The activity is expressed as μmoles of α-oxoacid formed per 10 min. per 100 mg. extract protein. Average values from 3 - 5 experiments are given (± 10%).

Decarboxylation of DOPA in kidney, liver and brain

To avoid the possible interference of monoamine oxidase in the manometric measurements of the decarboxylase activity, nialamide, a known inhibitor of monoamine oxidase, was added to the incubation mixture (final concn. 0.62 mM). The effect of nialamide was also studied *in vivo*; 25 mg./100 g. body weight was injected intraperitoneally either separately or simultaneously with 0.5 m-mole of phenyl-

alanine or tyrosine. In both kinds of experiments nialamide had no effect on the determination of decarboxylase activity.

Under the experimental conditions used, no phenylalanine or tyrosine decarboxylase activities were detected in the kidneys, liver and brain either of normal or phenylalanine- or tyrosine-treated animals by the manometric and chromatographic techniques.

However, an active DOPA decarboxylase was found to be present. In tissue extracts of normal rat this activity was the highest in the kidney, 17 μmoles CO_2 per 100 mg. of protein; it was lower in liver, 11 μmoles, and in brain, 6 μmoles. At 2 hr. after phenylalanine administration, the activity increased in the liver and kidney about twofold and in the brain it was practically unaltered. At 4 hr. after the injection, the decarboxylase activity decreased to a half of the initial values

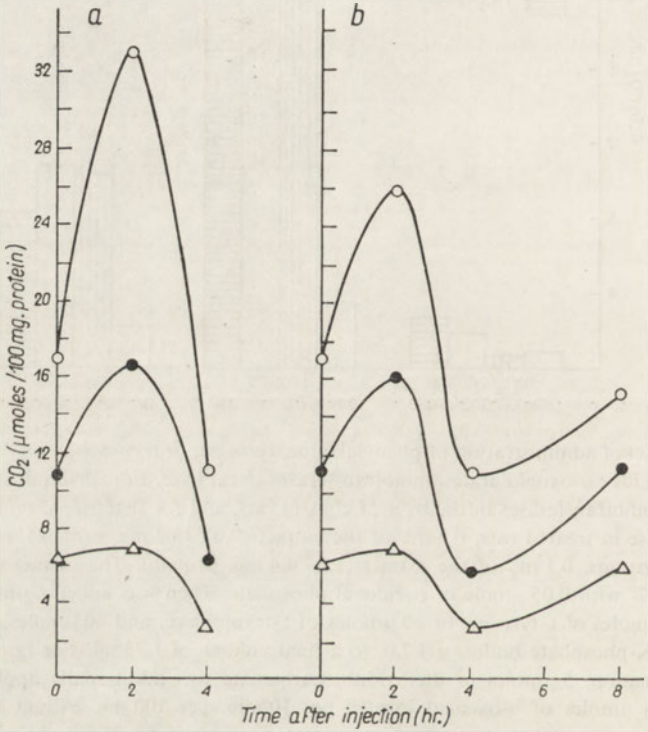

Fig. 6. Activity of DOPA decarboxylase in rat tissues: (○), kidney; (●), liver; and (△), brain, after administration of (*a*), L-phenylalanine and (*b*), L-tyrosine. The Warburg flask contained 0.2 ml. (0.7 μmole) of pyridoxal phosphate, 1.0 ml. of kidney extract (20 - 27 mg. protein) or 1.0 ml. of liver extract (35 - 42 mg. protein) or 1.5 ml. of brain extract (12 - 15 mg. protein) and 0.2 M-Na--K-phosphate buffer, pH 7.0, to a volume of 2.5 ml. The side arm contained 0.5 ml. (12 μmoles) of DOPA. After 20 min. preincubation at 37°, the substrate was tipped in and the readings were taken at 5-min. intervals. Liberation of CO_2 ceased after 15 min. for the extracts of kidney and liver, and after 5 min. for the brain extract. Then 0.2 ml. of 6 N-H_2SO_4 (final volume 3.2 ml.) was introduced through the side arm and the readings were taken after another 15 min. The activity is expressed as μmoles CO_2 per 100 mg. protein of the extract.

(Fig. 6a). The effect of tyrosine administration *in vivo* was similar to that found after phenylalanine treatment (Fig. 6b).

The decrease observed in DOPA decarboxylation 4 hr. after phenylalanine or tyrosine administration could be due to inhibition by the accumulating decarboxylation product (DOPAmine). Therefore extracts from liver and kidney of a control rat were preincubated for 20 min. at pH 7.0 with DOPAmine before the determination of decarboxylase activity. Inhibition of activity by 50% was obtained in

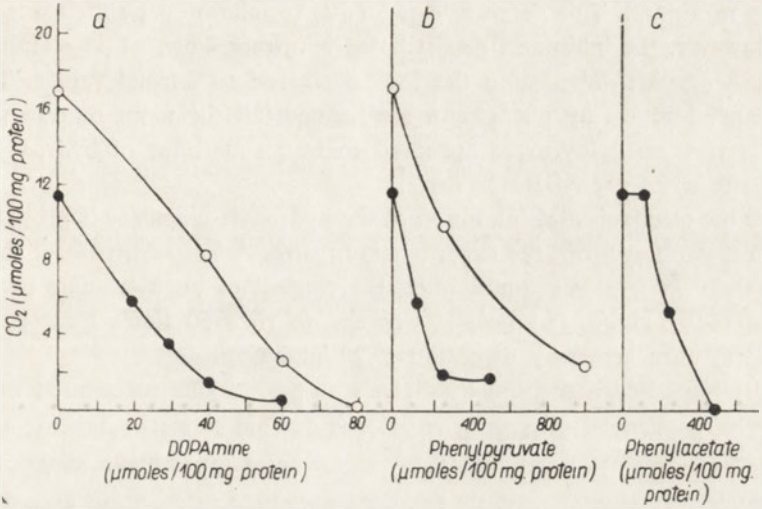

Fig. 7. The effect *in vitro* of (*a*), DOPAmine, (*b*), phenylpyruvate, and (*c*), phenylacetate, on DOPA decarboxylase activity in (○), kidney and (●), liver of a normal rat. The enzyme extract was preincubated in a Warburg vessel with pyridoxal phosphate and inhibitor; the substrate was added after 20 min. and the activity was determined manometrically as described for Fig. 6. The activity is expressed as μmoles CO_2 per 100 mg. of protein of the extract.

the liver using 20 μmoles DOPAmine per 100 mg. of extract protein, and in the kidney using 40 μmoles. In the presence of 60 μmoles of DOPAmine the activity was practically completely inhibited in the liver, and with 80 μmoles in the kidney as well (Fig. 7a).

Decarboxylase activity could be also inhibited by phenylpyruvate and phenylacetate. (These two compounds, as it has been found, did not undergo decarboxylation under the conditions applied). Phenylpyruvate inhibited the DOPA decarboxylase activity by 50% in concn. of respectively, 120 μmoles and 350 μmoles/100 mg. of protein of the liver and kidney extracts (Fig. 7b). Phenylacetate inhibited DOPA decarboxylase by 50% in liver in concn. of 250 μmoles, a complete inhibition being observed with 500 μmoles (Fig. 7c).

DISCUSSION

By intraperitoneal administration of phenylalanine to rats (0.5 m-mole/100 g. body weight) it was possible to obtain transient phenylketonuria, characterized by an almost 50-fold increase in the content of phenylalanine in blood, excretion

of phenylpyruvate in urine, and inhibition by 70% of the activity of phenylalanine hydroxylase in liver.

Recently Freedland, Krakowski & Waisman (1964) reported that in rats kept for 4 weeks on a diet supplemented with phenylalanine, the hydroxylase activity decreased by 50%.

In our experiments, the greatest decrease in activity was observed 4 hr. after phenylalanine injection, at the time when the content of phenylalanine in the liver was at its maximum. This decrease can be due to inhibition by the excess of substrate. However, the inhibition persisted for a further 4 hr., while simultaneously the content of phenylalanine in the liver decreased to normal values. This may indicate an inhibition by phenylpyruvate formed, all the more that in the experiments *in vitro* phenylpyruvate appeared to be an inhibitor of hydroxylase four times as strong as phenylalanine itself.

In vivo the mechanism of inhibition of the hydroxylase activity is probably different from that observed in the experiments *in vitro*. A 70% decrease of the hydroxylase activity *in vivo* was found when the content of phenylalanine in the liver amounted to 0.5 mg./g. (3 μmoles), whereas *in vitro* 50 times greater amounts (140 μmoles) were necessary to obtain 50% inhibition.

The attempts at reactivation by dialysis of the enzyme inactivated *in vivo* by treatment with phenylalanine were unsuccessful. This seems to indicate that inhibiting excess of phenylalanine, phenylpyruvate or of some other metabolite is so tightly bound with the enzyme that it does not dissociate during dialysis. It can be also supposed that the conformation of the enzyme becomes altered or that the formation of the enzyme is suppressed.

The transamination activity in phenylketonuria, which has not been so far studied, was examined in rats with induced phenylketonuria. Contrary to expectations, the phenylalanine : α-oxoglutarate aminotransferase remained unaltered, whereas the transamination of tyrosine and tryptophan was increased several-fold. The administration of tyrosine caused only a twofold increase in the activity of phenylalanine aminotransferase, but it enhanced several-fold the transamination of tryptophan and tyrosine. Boggs & Waisman (1964) observed a similar lack of influence of phenylalanine on the activity of phenylalanine : pyruvate aminotransferase in rats with phenylketonuria induced by a diet containing 7% of phenylalanine. Lin & Knox (1957) found a two- and threefold increase in the activity of tyrosine : α-oxoglutarate aminotransferase after intraperitoneal administration to the rat of 1 m-mole of phenylalanine per 300 g. body weight, and a sevenfold increase after the injection of the same amount of tyrosine.

Administration of tryptophan caused in our experiments a threefold increase in the activity of phenylalanine aminotransferase, whereas the transamination of tryptophan and tyrosine rose to an even higher degree. Rosen & Milholland (1963) observed a several-fold increase in the activity toward tyrosine after intraperitoneal administration of tryptophan (3 m-moles per 1 kg. body weight) to rats.

Differences in the transamination activity toward particular aromatic amino acids seem to indicate that their transamination is catalysed by separate enzymes or by one enzyme exhibiting group specificity, with a preference toward tyrosine.

Nothing is known about changes in decarboxylase activity which may occur in phenylketonuria. Weil-Malherbe (1955) demonstrated a deficiency of adrenaline and noradrenaline in blood serum of phenylketonuric children, and Pare, Sandler & Stacey (1957) found also a lowered serotonin content. In our experiments, 4 hr. after phenylalanine administration the DOPA decarboxylase activity was lowered in liver, kidney and brain to a half of its initial values; this may indicate that the decrease of adrenaline and serotonin in phenylketonuria could be attributed to the reduction in decarboxylase activity. This supposition is in agreement with the report of Lovenberg, Weissbach & Udenfriend (1962) who demonstrated that formation of the three physiologically important amines: DOPAmine, serotonin and histamine, is catalysed by a single enzyme, the decarboxylase of aromatic L-aminoacids.

The inhibition of DOPA decarboxylase activity observed in our experiments was not due to the excess of phenylalanine, as from the experiments of Fellman (1956) it appears that *in vitro* phenylalanine has no effect on DOPA decarboxylase in extract from ox adrenal. Similarly, Boylen & Quastel (1961) reported that phenylalanine did not influence the biosynthesis of [^{14}C]adrenaline from [^{14}C]tyrosine in slices of guinea pig adrenal. Both Fellman (1956) and Boylen & Quastel (1961) found that phenylpyruvate inhibited DOPA decarboxylase activity as well as adrenaline formation. In our experiments *in vitro*, DOPAmine was a much stronger inhibitor of decarboxylase activity than phenylpyruvate and phenylacetate.

Albeit the physiological role of DOPAmine and serotonin has not been yet fully elucidated, it seems, nevertheless, that these two amines are of importance for the functioning of the central nervous system. Their decreased concentration in the brain due to inhibition of DOPA decarboxylase, could be among the causes of mental retardation observed in phenylketonuria.

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WPŁYW OBCIĄŻENIA FENYLOALANINĄ NA AKTYWNOŚĆ HYDROKSYLAZY FENYLOALANINY, NIEKTÓRYCH AMINOTRANSFERAZ I DEKARBOKSYLAZ U DOROSŁEGO SZCZURA

Streszczenie

1. Po dootrzewnowym podaniu dorosłym szczurom L-fenyloalaniny (0,5 m-mola/100 g wagi ciała) obserwowano przejściowy wzrost fenyloalaniny we krwi (maksimum po 1 godz.) i w wątrobie (maksimum po 4 godz.); aktywność hydroksylazy fenyloalaniny w wyciągu z wątroby obniżała się równocześnie do 30% wartości normalnej.

2. Aktywność aminotransferazy fenyloalaniny w wyciągu z wątroby nie ulegała zmianie po 4 godz. od obciążenia fenyloalaniną, podczas gdy podanie tyrozyny lub tryptofanu powodowało 2-3-krotny wzrost aktywności. Aktywność aminotransferazy tryptofanu i tyrozyny wzrastała kilkakrotnie po obciążeniu badanymi aminokwasami aromatycznymi.

3. Aktywność dekarboksylazy DOPA w nerce i wątrobie wzrastała 2-krotnie po 2 godz. od podania fenyloalaniny lub tyrozyny, natomiast po 4 godz. obniżała się w nerce, wątrobie i mózgu do połowy wartości wyjściowej.

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THE INTERACTION OF AZLACTONE WITH ALDOLASE

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1. The conditions of inactivation of rabbit muscle aldolase by azlactone of *p*-nitrobenzoylvaline were studied. Temperature, concentration of aldolase and azlactone, and especially the pH of the medium, had a marked effect. p*K* of the groups involved in enzyme activity was in carbonate buffer pH 10 and in borate buffer about pH 8.5. 2. A protective effect against inactivation was exhibited by thioglycolate > lysine > glycine > proline > phosphate. 3. K_m and V with fructose-1,6-diphosphate as substrate were unaltered for aldolase modified at pH 7.4, whereas after modification at pH 10 both these values were changed. 4. Some other properties of the modified aldolase, such as isoionic point, solubility, ability to reassociate, were also studied.

Formation of azlactones (2,4-substituted oxazolones-5) as intermediates in the course of chemical synthesis of peptides has been observed by many workers (Siemion, Nowak & Kaczorowski, 1962). Azlactones can arise from *N*-acylaminoacid chlorides (Carter & Hinman, 1949) from acylamino acids treated with ethyl chloroformate or dicyclohexylcarbodiimide (Nowak, Siemion & Siemieniewski, 1962; Siemion *et al.*, 1962), in the anhydride methods of Wieland, of Taschner, and other methods commonly used for peptide synthesis (Siemion & Nowak, 1960; Siemion *et al.*, 1962). Some azlactones are very labile, e.g. those arising from *N*-formylamino acids; others, like the azlactone of *p*-nitrobenzoylvaline, are relatively stable and in aqueous solution hydrolyse slowly (Baranowski, Kochman, Nowak & Siemion, (1963).

So far, azlactones were little used for protein modification. Lettre & Haas (1940) by treating blood serum protein with azlactone of benzoylvaline in the presence of 0.1 N-NaOH obtained a protein possessing new immunological properties, thus proving indirectly the modification of the protein structure. Baranowski *et al.* (1963) demonstrated the dependence of the degree of modification of gelatine on the pH value, and they described mild conditions under which the azlactone of *p*-nitrobenzoylvaline reacted with the amino groups of the protein. In preliminary communications the binding of azlactone to aldolase (Kochman & Baranowski, 1964) and to α-chymotrypsin (Siemieniewski, 1964) have been reported.

The aim of this work was a closer examination of the process of aldolase modification by azlactone, and an attempt was made to elucidate the nature of the bonds formed.

MATERIALS

Azlactone of *p*-nitrobenzoylvaline was obtained by treating the acyloamino acid with dicyclohexylcarbodiimide, as described previously (Baranowski *et al.*, 1963). *p*-Nitrobenzoylvaline was obtained after Karrer & Christoffel (1944).

Aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was prepared from rabbit muscle after Taylor, Green & Cori (1948). The spectrum of the six times crystallized aldolase was close to that given by Baranowski (1949) and the specific activity determined using glyceraldehyde-3-phosphate dehydrogenase was in agreement with that given by Taylor *et al.* (1948).

Glyceraldehyde-3-phosphate dehydrogenase was obtained after Beisenherz *et al.* (1953). The activity of the crystalline enzyme toward synthetic glyceraldehyde-3--phosphate (Szewczuk, Wolny, Wolny & Baranowski, 1961) was in agreement with published values (Beisenherz *et al.*, 1953).

NAD was a commercial preparation (Boehringer, Mannheim, Germany).

Fructose-1,6-diphosphate (F-1,6-P), dibarium salt, was obtained by fermentation of saccharose after Orłowski (1959) and purified as described by Szewczuk, Wolny, Wolny & Baranowski (1961). The preparation of 80% purity was free from other phosphate esters of fructose, as checked by chromatography according to Baranowski, Długajczyk & Kochman (1959) and Kochman (1962). To convert the barium salt into sodium salt, about 0.2 M-frucose diphosphate was suspended in 0.2 N-HCl and dissolved by adding 2 N-HCl. To the solution norite was added and the clear filtrate applied onto a column of Dowex 50 (Na^+ form). The eluate was adjusted to pH 7.4 with NaOH. The amount of fructose diphosphate was determined enzymically using aldolase and glyceraldehyde-3-phosphate dehydrogenase.

Buffers: 0.1 M-natrium phosphate, 0.1 M-natrium carbonate and 0.1 M-natrium borate of appropriate pH.

METHODS

Procedure of protein modification. A suspension of crystalline aldolase was dialysed against 0.1 mM-natrium versenate solution at 0 - 4° until free of sulphates. The undissolved residue was centrifuged off and the supernatant, after determination of protein concentration, diluted with 0.1 M appropriate buffer. The reaction was started by adding azlactone solution in ethanol. The final concentration of the buffer used was always 40 mM and that of ethanol 10%.

The activity of aldolase was assayed at room temp. according to Taylor, Green & Cori (1948) using glyceraldehyde-3-phosphate dehydrogenase. For the assay, the enzyme (6 - 15 μg.) was appropriately diluted with cold water and 0.1 ml. samples were taken. The change in extinction at 340 mμ between the 15th and the 75th second of the assay, was taken as the measure of activity. When the activity was very low,

the readings were done over a few minutes and calculated in relation to 1 minute. The activity of aldolase modified by incubation with azlactone was compared with the activity of aldolase incubated under the same conditions but without azlactone.

In a preliminary experiment, the effect of azlactone on the measurement of aldolase activity was checked. The addition of 0.1 mg. of azlactone to the incubation mixture containing 6 μg. of aldolase did not alter the activity.

Analytical. Protein was determined using the Folin-Ciocalteu reagent (Layne, 1955), six times recrystallized aldolase being used as standard.

Aldolase concentration was determined by measuring the extinction at 280 mμ and applying the specific extinction coefficient given by Baranowski & Niederland (1949), E_{280}/0.91 mg. of aldolase. The molecular weight of aldolase was taken after Taylor & Lowry (1956) to be 149 000.

p-Nitrobenzoylvaline incorporated into the protein was estimated by measuring the difference in extinction at 270 mμ between modified and non-modified aldolase. A solution of 1 μmole of *p*-nitrobenzoylvaline in 1 ml., pH 7, had the E_{270}^{1cm} value of 11.3.

Thiol groups were determined by the method of Swenson & Boyer (1957) in 5 M-urea at pH 7.

ε-Amino groups of lysine were determined spectrophotometrically by the method of Dellacha & Fontanive (1965) after dissolving DNP-aldolase in formic acid (1 vol.) and then diluting with glacial acetic acid (9 vol.).

The isoionic point was determined using ion-exchanger resins according to Eastoe & Courts (1963).

RESULTS

The effect of azlactone concentration on the activity of aldolase. To study the effect of various concentrations of azlactone, 0.1 ml. of azlactone solution in ethanol (0.05 - 1.0 μmole) was incubated with 3 mμmoles (0.45 mg.) of aldolase in 0.9 ml. of carbonate buffer, pH 10, or phosphate buffer, pH 7.4, at 0° for 15 min. Then samples were taken for enzyme assay (Fig. 1). The activity of aldolase incubated with azlactone at pH 10 decreased rapidly with the increase in inhibitor concentration, to about 10% of the initial activity at 1 mM azlactone concentration. At pH 7.4 the loss of aldolase activity was but slight.

The effect of time, aldolase concentration and pH. The effect of time on the activity of aldolase incubated at 0° with 4 mM-azlactone at pH 10 in carbonate buffer, is shown in Fig. 2. The experiment was carried out at two enzyme concentrations, 40 μM and 0.67 μM. In either case the inactivation had a similar course: first a rapid decrease in activity observed already after 5 min. of incubation, then a further slow decrease. However, the extent of inhibition was dependent on aldolase concentration. With 40 μM-aldolase, that is under the conditions when the concentration of azlactone was equimolar with respect to the amino groups of lysine, the activity of the enzyme after 2.5 hr. incubation decreased by about 70% in comparison with the control incubated without azlactone. With 0.67 μM-aldolase, a practically total inhibition was obtained.

The effect of pH on the inactivation of aldolase by azlactone was studied in a system containing carbonate or borate buffer, 40 mM; aldolase, 10 μM; azlactone, 4 mM; and ethanol, 10%. The incubation was carried out for 15 min. at 0°, then

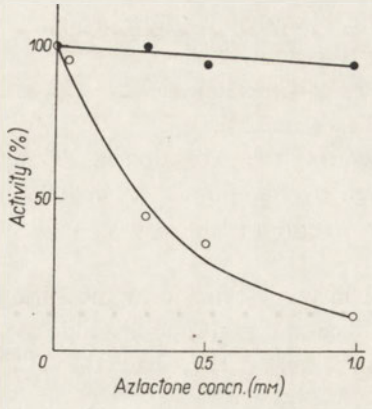

Fig. 1

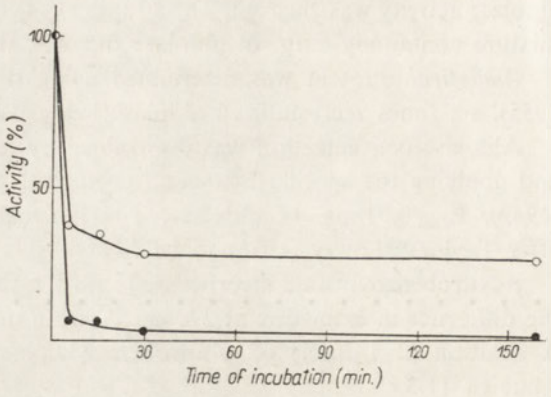

Fig. 2

Fig. 1. The effect of pH and azlactone concentration on the activity of aldolase. 3 μM-Aldolase was incubated for 15 min. at 0° with various amounts of azlactone in (●), 40 mM-phosphate buffer, pH 7.4, or (○), 40 mM-carbonate buffer, pH 10.

Fig. 2. The effect of time of incubation with 4 mM-azlactone, pH 10, at two aldolase concentrations on the activity of aldolase. The incubation was carried out at 0° in 40 mM-carbonate buffer. Aldolase concentration: (○), 40 μM; (●), 0.67 μM.

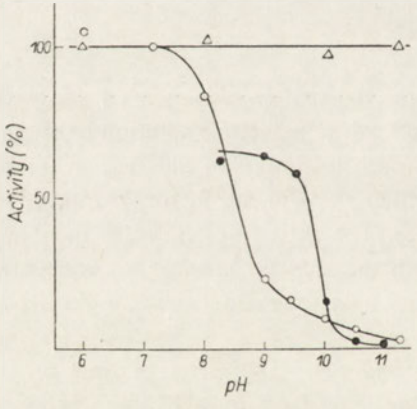

Fig. 3

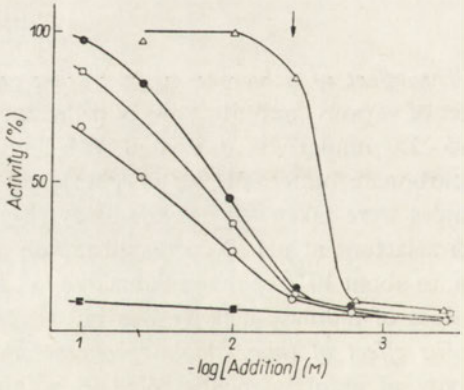

Fig. 4

Fig. 3. The effect of pH on inactivation of aldolase by azlactone. 10 μM-Aldolase was incubated at 0° for 15 min. with 4 mM-azlactone in: (●), 40 mM-carbonate buffer, pH 8.1 - 11.0, or (○), in 40 mM-borate buffer, pH 6.0 - 11.5. Then the samples were diluted 15-fold with cold water and the activity was determined in 0.1 ml. portions. The activity is expressed as percentages in relation to a control (△) incubated under the same conditions but without azlactone.

Fig. 4. The effect of compounds containing free thiol, amino, and carboxyl groups on the activity of aldolase incubated with azlactone. 10 μM-Aldolase was incubated at 0° for 1 hr. in 40 mM-carbonate buffer, pH 10, 4 mM-azlactone and various concentrations of: (△), thioglycolate; (●), lysine; (□), glycine; (○), proline, and (■), acetate. The arrow indicates equimolar concentration of azlactone and the particular compound added.

the activity was determined (Fig. 3). In control samples, no decrease of activity was observed over the pH range 6 - 11.5, whereas the effect of pH on the inactivation of aldolase by azlactone was dependent on the buffer used. For the carbonate buffer the point of inflection of the curve was at about pH 10, and for borate buffer it was at pH 8.2 - 8.4.

The protective effect of phosphate. As not only the pH value but also the kind of ion present was found to affect the degree of inactivation, in an analogous experiment the effect of phosphate ion was studied. When the incubation mixture containing carbonate or borate buffer at pH 10 was supplemented with 40 mM--sodium phosphate of pH 10, the inactivation of aldolase by azlactone was two- or threefold smaller.

Rowley, Tchola & Horecker (1964) demonstrated the binding of phosphate by aldolase and suggested that the receptors of the anion-binding groups are situated near the active centre of the enzyme. The partial protection of aldolase against inactivation by azlactone seems to indicate that the same receptors in the aldolase molecule are involved in binding of azlactone and phosphate.

The effect of compounds containing free thiol, amino and carboxyl groups. From the studies of Velick & Ronzoni (1948), Undenfriend & Velick (1951), Swenson & Boyer (1957) and Lai, Hoffee & Horecker (1965) it is known that aldolase possesses 29 thiol groups (10 - 12 of which react with *p*-chloromercuribenzoate in the absence of urea without loss of activity), two active sites with ε-amino groups of lysine, and three *N*-terminal proline residues, per one molecule of protein. It seemed worthwhile to study the effect of some compounds possessing similar groups, on the reaction of aldolase with azlactone.

The effect of glycine, lysine, proline, thioglycolate and also of acetate, on the inhibition of aldolase by azlactone was examined. Aldolase (final concn. 10 μM) was incubated at 0° in 10% ethanol and 40 mM-carbonate buffer, pH 10, with 4 mM--azlactone for 1 hr.; the sample contained also a solution of the appropriate amino acid, acetic acid or thioglycolic acid adjusted with 2 N-NaOH to pH 10, at the concentration shown in Fig. 4. The control samples contained the appropriate added compounds at the highest concentration applied in the proper experiment, but no azlactone. Thioglycolate was found to prevent most efficiently the inactivation of aldolase by azlactone. Already at an equimolar concentration of thioglycolate and azlactone, the activity of aldolase was retained in 80 - 90%, which seems to indicate a strong affinity of this compound toward azlactone. After 2 hr. of incubation in the presence of equimolar amounts of azlactone and thioglycolate, the aldolase contained about 0.6 mole of bound azlactone per 1 mole of the enzyme. When, however, the thioglycolate was added after 1 hr. of incubation of aldolase with azlactone, the enzyme contained as much as 17 moles of azlactone, and the activity was reduced by 50 - 60%. Aldolase activity was also protected, in decreasing order, by lysine, glycine and then proline, all of them being less effective than thioglycolate, whereas acetate had almost no effect.

The binding of thiol group by azlactone has been demonstrated by Lettre & Fernholz (1940) in the reaction of azlactone of benzoylalanine with thiophenol,

with the formation of thioester. In the present work it was found that during 1.5 hr. incubation of 4 mM-thioglycolate at pH 10 with an equimolar amount of azlactone, a decrease in thiol groups by about 60% occurred in relation to a control incubated under the same conditions but without azlactone.

The low protective effect of proline indicates that probably the *N*-terminal proline residues in aldolase form with azlactone much weaker bonds than do any other amino or thiol groups.

The type of inhibition. From the presented data it appeared that inhibition of aldolase depended not only on the concentration of azlactone and composition and pH of the medium, but on the concentration of the enzyme as well. In the experiment described below, an attempt was made to determine the type of the inhibition at an equimolar concentration of the amino groups of the enzyme and acyl groups of azlactone, and at a fourfold lower concentration of the enzyme. Aldolase, 40 μM or 10 μM, was incubated for 2.5 hr. at 0° in 10% ethanol and 40 mM-carbonate buffer at pH 10 with 4 mM-azlactone (1 mg. in 1 ml.). Then the incubation mixture was diluted with cold water containing 0.1 mM-versenate and 0.1 ml. samples were taken for activity determinations at various concentrations of fructose-1,6-diphosphate. The reciprocals of velocity plotted against the reciprocals of substrate concentration are shown in Fig. 5. It appeared that azlactone affected both the maximum velocity and the Michaelis constant, indicating a mixed type of inhibition. The

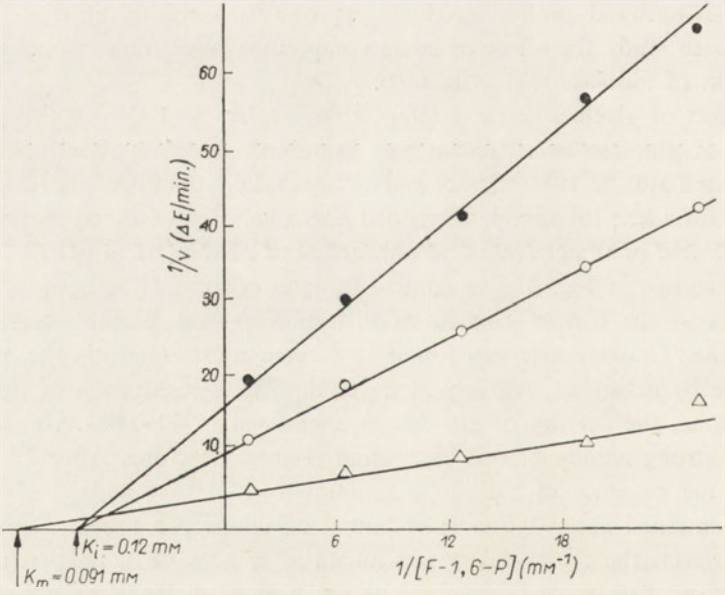

Fig. 5. Lineweaver-Burk plots on the effect of substrate concentration on the activity of modified and non-modified aldolase. 40 μM or 10 μM-aldolase was incubated for 2.5 hr. at 0° in 40 mM-carbonate buffer, pH 10, and 4 mM-azlactone. For activity determinations, the medium was diluted as indicated below, and 0.1 ml. samples were taken. Concentration of the incubated aldolase: (○), 10 μM-aldolase, for activity determination diluted×3; (●), 40 μM-aldolase (diluted×40); (△), control: 10 μM-aldolase incubated without azlactone (diluted×15).

value of K_m was 0.09 mM and that of K_i 0.12 mM. The K_i was independent of the concentration of the enzyme used for the modification in contrast to V, which was dependent on enzyme concentration. V expressed as moles of the substrate decomposed per 1 mole of the enzyme per 1 min. at room temperature, after introducing a correction for the dilution, was as follows: for control aldolase, 1610; for 40 μM-aldolase incubated with azlactone, 145; for 10 μM-aldolase incubated with azlactone, 29.5.

The effect of temperature. When the solution of azlactone was rapidly added to the aldolase, the temperature rose by a few degrees. Therefore the effect of temperature on the inactivation was studied. Azlactone dissolved in ethanol was slowly added, with stirring, to a solution of aldolase in carbonate buffer, pH 10, at 0°. The cool mixture was transferred to a water bath of given temperature for 1 hr., then the samples were diluted with cold water and the activity was determined.

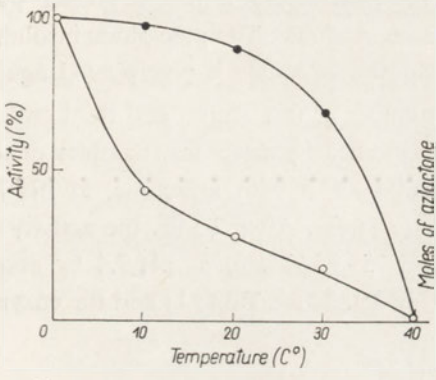

Fig. 6

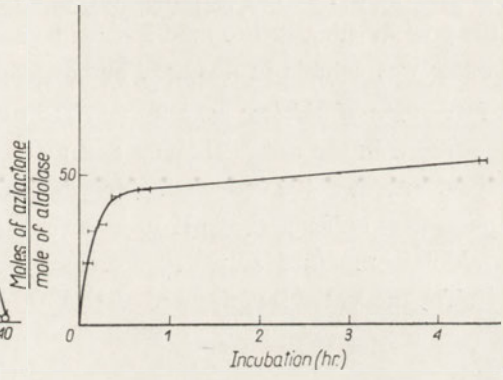

Fig. 7

Fig. 6. The effect of temperature on the inactivation of aldolase by azlactone. 10 μM-Aldolase was incubated at the indicated temperature (●), without and (○), with 4 mM-azlactone in 40 mM-carbonate buffer, pH 10. The activity is expressed as percentage of the activity of an analogous aldolase mixture incubated at 0°.

Fig. 7. The time-course of binding of azlactone to aldolase. 10 μM-Aldolase was incubated with 4 mM-azlactone at 20° in 40 mM-carbonate buffer, pH 10. At given time intervals, the reaction was stopped by transferring 1 ml. sample to a Sephadex G-25 column (1 × 10 cm.), equilibrated with 40 mM-carbonate buffer, pH 10. The time of application and penetration of the sample into the gel is indicated in the diagram. In the first 1 ml. of the effluent, protein was determined after Layne (1955), and the amount of azlactone calculated as described in Methods.

The effect of temperature on aldolase incubated for 1 hr. without azlactone was studied as well. The results presented in Fig. 6 showed that changes in temperature even by a few degrees, affected markedly the aldolase in the presence of azlactone, whereas in its absence their effect was rather insignificant over the temperature range 0 - 20°. A rise in temperature from 0 to 10° in the presence of azlactone resulted in inactivation by 50% in relation to the activity of aldolase incubated with azlactone at 0°, whereas without azlactone the activity decreased only by a few percent.

The time-course of combination of azlactone with aldolase at 20°. Samples of the incubation mixture containing 10 μM-aldolase, 4 mM-azlactone, 10% ethanol, and 40 mM-carbonate buffer, pH 10, were applied to a Sephadex G-25 column. The first 1 ml. fraction containing the protein was collected and the amount of azlactone bound to aldolase was determined. The results shown in Fig. 7 indicate that of the whole amount of bound azlactone determined after 4.5 hr., about 80% was bound within the first 30 min. of incubation. Simultaneous determinations of aldolase activity showed a decrease by 90% already after 2 min. of the reaction, and after 40 min. by 97%.

The solubility of the aldolase after 4 hr. of incubation with azlactone was smaller and the protein precipitated already at low ammonium sulphate concentration. The precipitate at 0.5 saturation could not be completely redissolved in water or carbonate buffer. If the precipitation was repeated several times, a greater part of the protein submitted to modification became insoluble. The precipitate insoluble in water was soluble in 4 M-urea but on addition of water it precipitated again.

The nature of aldolase modification. The number of free amino and thiol groups was assayed in the native aldolase and that modified by azlactone. Samples of the dialysed enzyme, 400 - 500 mg., were incubated at 0° with azlactone, at pH 7.4 in phosphate buffer and at pH 10 in carbonate buffer. After 2.5 hr. the activity of the aldolase modified at pH 10 decreased by 75 - 85%, and at pH 7.4 by about 10%. The incubated samples were dialysed (for details see Table 1) and the enzyme

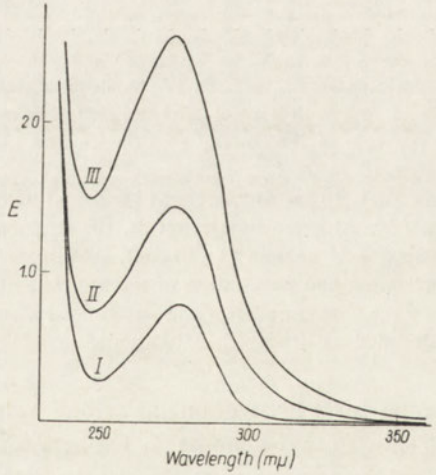

Fig. 8. Absorption spectra of aldolase. Samples of dialysed aldolase were freeze-dried and then dissolved in 5 M-urea containing 6.7 mM-phosphate buffer, pH 7.0. Conditions of modification of aldolase as described in Table 1. *I*, Native aldolase; *II*, aldolase modified at pH 7.4 (0.4 mg./1 ml.); *III*, aldolase modified at pH 10 (0.833 mg./1 ml.).

activity, number of free thiol and ε-amino groups of lysine, as well as the amount of azlactone, were determined. The absorption spectrum of the freeze-dried dialysed preparation dissolved in 5 M-urea, is presented in Fig. 8. The enzymic activity of the preparations modified at pH 7.4 and 10 was not altered by dialysis.

The results of analysis presented in Table 1 indicate that the incorporation of *p*-nitrobenzoylvaline groups into the protein was accompanied by binding of ε--amino groups of lysine and thiol groups. The modification carried out at pH 7.4 caused a loss of about three thiol groups and seven amino groups, which corresponds to the amount of bound azlactone. At pH 10 the decrease amounted to 11 thiol groups and 21 amino groups, whereas the amount of bound azlactone corresponded to 29 molecules per one molecule of protein. An attempt to confirm the disappearence of free amino groups in the modified aldolase by the ninhydrin method (Moore & Stein, 1954) using DL-leucine as standard, was unsuccessful; 70 amino groups were found per one protein molecule in the modified aldolase and 75 in the native preparation. This discrepancy may be explained by low accuracy of the method applied.

Table 1

Analysis of aldolase modified by azlactone at pH 7.4 *and* 10

Aldolase, 40 μM, was incubated for 2.5 hr. with 4 mM-azlactone, 40 mM-phosphate buffer, pH 7.4, or carbonate buffer, pH 10, and 10% ethanol. The scanty precipitate of denatured protein was centrifuged off. To the supernatant, 10% glycine solution was added to a final concentration of 1%. The turbid solutions were dialysed at 0° against frequently changed water containing 0.1 mM--versenate. After 12 hr. the turbidity disappeared almost completely, then the samples were centrifuged again for 20 min. at 18 000 *g* and the supernatants dialysed for 60 hr. at 0°. In the dialysed preparations ε-DNP-lysine was determined after Dellacha & Fontanive (1965), thiol groups by the method of Swenson & Boyer (1957) and azlactone as described in Methods. The results are expressed in moles per mole of aldolase.

Aldolase	Thiol		ε-DNP-lysine		Azlactone	Beginning of precipitation of 0.5% aldolase solution at pH 7.4, at ammonium sulphate sat.
	found	decrease	found	decrease	found	
Native	28.7		115			Crystallizes at 0.5 sat.
Control, incubated at pH 10	26	2.7	114			Crystallizes at 0.5 sat.
Modified at pH 7.4 (phosphate buffer)	26	2.7	108	7	10.6	Heavy submicrocrystalline sediment at 0.25 sat.
Modified at pH 10 (carbonate buffer)	17.5	11.2	94	21	28.7	Amorphous sediment, not fully soluble in water, at 0.066 sat.

Some properties of aldolase modified at pH 7.4. The aldolase modified by azlactone at pH 7.4 and 20° retained about 90% of the enzymic activity and did not lose the solubility in water even after several-fold precipitation with ammonium sulphate. The modified aldolase precipitated much earlier than the native enzyme,

already at 0.25 - 0.35 ammonium sulphate saturation. However, the attempt at crystallization was unsuccessful. In contrast to native aldolase, which after dissociation by 4 M-urea can be completely reactivated by dilution (Swenson & Boyer, 1957), the activity of the modified aldolase under these conditions was altered. This was observed in the following experiment: to a sample of modified aldolase dissolved in water was added at 0° an equal volume of cooled 8 M-urea in 5 mM--phosphate buffer, pH 7. After 20 min. at 0°, the mixture was 100-fold diluted with water and after a further 20 min. the enzyme activity was determined. The activity was recovered in about 70 - 80%, whereas native aldolase under the same conditions regained the activity in 95 - 100%.

The isoionic point of the modified aldolase was 7.6, whereas for the native enzyme it was 8.4. The K_m and V of the enzyme were almost unaltered.

DISCUSSION

Many attempts have been made recently to establish the possibly mildest conditions of modification of protein structure so as to permit controlling of the process of modification.

In the studies on the structure of aldolase, Rowley *et al.* (1964) applied chlorodinitrobenzene instead of the fluoro derivative, and Riordan, Vacker & Vallee (1965) instead of acetic anhydride used acetylimidazole which acetylates specifically the tyrosine residues, without acetylating, as it seems, the thiol groups. Even fluorodinitrobenzene, when applied in appropriate amounts and under suitable conditions, appeared to be useful for modification of the thiol groups of aldolase, without causing dinitrophenylation of the ε-amino groups (Cremona, Kowal & Horecker, 1965).

Azlactone, applied by Baranowski *et al.* (1963), belongs to the mild agents modifying the structure of protein, and its action can be controlled by changing the pH of the reaction medium. The rate of the reaction at room temperature is high, as about 80% of the total amount of bound azlactone becomes bound during the first 20 - 30 min. of the reaction. The number of introduced azlactone residues depends on the concentration of the components and the conditions of the reaction.

The majority of the performed modifications were carried out at an equimolar concentration of azlactone and amino groups of aldolase, although in the course of the experiments it appeared that the thiol groups are also reactive. Only a few experiments were carried out with a small excess of azlactone, and therefore complete substitution has not been achieved.

The protective effect of amino acids and thioglycolate against the inhibition of aldolase by azlactone, as well as the almost complete lack of binding of azlactone by aldolase in the presence of thioglycolate, indicate that amino and thiol groups participate in the process of binding of azlactone by aldolase. Quantitative data, although incomplete, seem to support this supposition. There is no direct evidence concerning the nature of the bond formed. Thioglycolate,

which is able to protect aldolase almost completely against modification, exhibited no such effect when it was added after the aldolase had become bound to the modifying agent, indicating the stability of the binding. The simplest conclusion based on the properties of azlactones would be the assumption that they form a peptide bond with the amino groups and an ester bond with thiol groups of the enzyme (Lettre & Fernholz, 1940). However, other possibilities should be also envisaged, as for instance that the reaction may proceed by steps and that one of the stages may give relatively stable linkages which would gradually be regrouped to form the bonds discussed above.

The modification of aldolase by azlactone at pH 7.4 in phosphate buffer was found to cause a decrease of three thiol groups and seven amino groups; simultaneously 10 - 11 azlactone groups were introduced into the enzyme molecule, and the concomitant loss of enzyme activity was insignificant. Riordan *et al.* (1965) using acetic anhydride at pH 7.5 in phosphate buffer also found a decrease of 3 - 4 thiol groups per protein molecule. However, in their aldolase preparation they found only 22.5 thiol groups instead of 29. It seems probable that silver titration in the presence of dodecyl sulphate, applied by these authors, does not permit to determine all thiol groups. The experiments of Kowal, Cremona & Horecker (1965) indicated the presence of three highly reactive thiol groups in aldolase; they demonstrated that the reaction between chlorodinitrobenzene and aldolase at pH 9.6 proceeds in two steps: in the first one, lasting about 2 min., three thiol groups are bound but the enzyme activity is unaffected; during the second step, further nine groups become substituted with a concomitant loss of activity.

The results obtained in the present work seem to indicate that none of the three groups substituted at pH 7.4 by azlactone are situated in the vicinity of the active site of aldolase. The experiments on the association of subunits of the modified aldolase showed that the introduced residues may act as a steric hindrance to recombination of the peptide chains into the active molecule.

At pH 10, azlactone caused a marked decrease in enzyme activity with a simultaneous loss of 11 thiol groups and 21 ε-amino groups of lysine. If it is assumed that the decrease in thiol groups is due to binding with azlactone and not to auto-oxidation, then the number of the introduced azlactone residues corresponds closely to the decrease in thiol and amino groups of aldolase. The total number of ε-amino groups of lysine, determined by the method of Dellacha & Fontanive (1965) in native aldolase amounted to 115 and was in agreement with 112 groups reported by Christen, Göschke, Leuthardt & Schmid (1965). The activity was partly protected by phosphate which indicates that azlactone interacts probably with thiol groups. Kowal *et al.* (1965) observed a similar protective effect of phosphate on the activity of aldolase treated with chlorodinitrobenzene. Under the experimental conditions described by Horecker and his collaborators (Cremona *et al.*, 1965; Kowal *et al.*, 1965), fluoro- and chloro- derivatives of dinitrobenzene reacted only with thiol groups. From preliminary experiments on the protective effect of substrate on aldolase activity (Kochman, unpublished data) it has not been possible so far to establish the nature of the groups which undergo modification by azlactone

and are involved in activity. Lai *et al.* (1965) found the same sequence of 28 amino acids, including one lysine, in the two active sites of the aldolase from rabbit muscle. It seems highly probable that ε-amino groups located at these sites are also susceptible to azlactone.

The observation that the aldolase modified at pH 10 easily became insoluble, can be explained by a considerable accumulation of hydrophobic residues of benzoylvaline on the surface of the molecule.

The aldolase modified at pH 7.4 had an almost unaltered K_m value and the maximum velocity with fructose-1,6-diphosphate as substrate. On the other hand, the aldolase modified at pH 10 exhibited a distinct change in the maximum velocity whereas the Michaelis constant was only slightly changed.

The course of the changes in activity due to the effect of pH on the modification of aldolase by azlactone, seems to indicate that the groups having the pK close to pH 10 for the carbonate buffer and to pH 8.5 for the borate buffer, are of importance for enzyme activity.

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WPŁYW AZLAKTONU NA ALDOLAZĘ

Streszczenie

1. Badano wpływ azlaktonu *p*-nitrobenzoilowaliny na aktywność aldolazy z mięśni królika. Stwierdzono, że wśród badanych czynników jak temperatura, stężenie aldolazy i azlaktonu, znaczny wpływ na aktywność aldolazy wywiera pH środowiska. p*K* grup odpowiedzialnych za aktywność leży przy pH około 10 w buforze węglanowym oraz około pH 8.5 w buforze boranowym.

2. Ochronne działanie przed inaktywacją aldolazy azlaktonem w pH 10 wywierają kolejno: tioglikolan > lizyna > glicyna > prolina > fosforany.

3. K_m i V mierzone wobec fruktozo-1,6-dwufosforanu nie ulegają zmianie dla aldolazy modyfikowanej w pH 7,4, podczas gdy po modyfikacji w pH 10 obie te wartości ulegają zmianie.

4. Zbadano również inne własności aldolazy zmodyfikowanej, jak punkt izojonowy, rozpuszczalność, zdolność do reasocjacji.

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PYRIMIDINE AND PURINE BASES AND NUCLEOSIDES IN WHEAT PLANTS

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1. Uracil, cytosine, adenine, uridine, cytidine, adenosine and guanosine were isolated from wheat seedlings and quantitatively determined; the presence of orotate was established by the isotope dilution method. 2. Rapid changes in the content of major bases and nucleosides in wheat were observed during germination and subsequent growth.

In studies on pyrimidine nucleotide biosynthesis in wheat seedlings, in addition to 5′-UMP, 5′-CMP and uridine, we have constantly observed the presence of uracil (Buchowicz, Reifer & Makowski, 1961; Buchowicz & Reifer, 1961, 1962). However, in leguminous plants Brown (1963, 1965) was unable to detect uracil or any other free base by anion-exchange chromatography and he assumed that reports on their presence in higher plants published in the first half of the XX-th century probably describe artifacts arising from the enzymic or chemical degradation of nucleotides during isolation procedures. Therefore it seemed worthwhile to reinvestigate the composition of the acid-soluble fraction from wheat seedlings with special attention to free bases. Simultaneously, quantitative changes of pyrimidine and purine bases and their ribosides during germination and growth of wheat seedlings were studied.

MATERIALS AND METHODS

Winter wheat, variety Dańkowska 40, was used throughout. Germination and subsequent growth was carried out at 25° in the light. For analysis dry grain, germinated grains together with whole seedlings and roots, as well as excised above-ground green parts of the plant were used.

Extraction procedure. Dry grains were ground in a laboratory mill, germinated grains were ground in a mortar and green parts were homogenized with 85% aqueous ethanol. After the disruption, 10 g. sample of the material was extracted four times with 30 ml. portions of 85% ethanol. The combined extracts were eva-

porated under reduced pressure at room temperature. The dry residue was treated with 50 ml. of ice-cold 0.2 N-$HClO_4$ and the crude extract immediately used for isolation of pyrimidine and purine derivatives.

Separation of pyrimidine and purine bases and nucleosides. Acid-soluble pyrimidine and purine derivatives were adsorbed from the crude extract on activated charcoal (800 mg. per extract from a 10 g. plant sample) and eluted with a warm mixture of acetone and 0.1 N-NH_4OH (4 : 1, v/v) as described by Buchowicz & Reifer (1962). The eluate was freed of ammonia and acetone by heating on a boiling water bath, and after cooling to room temperature, put on Dowex 1×1 (Cl^-) column (1×12 cm.). The column was washed with 300 ml. of water and the effluent, containing free bases and nucleosides, was evaporated under reduced pressure at room temperature to dryness. The dry material was redissolved and submitted to paper chromatography in butan-1-ol saturated with water (Buchanan, 1951). Ultraviolet light absorbing spots were eluted from the chromatogram with 5 - 10 ml. of water and rechromatographed in the propan-2-ol - HCl (d = 1.18) - water (170:41:39, by vol.) solvent (Wyatt, 1955). The purine and pyrimidine spots, corresponding to standard samples, were eluted with 5 ml. of 0.1 N-HCl and used for identification and quantitative determinations with the spectrophotometer SF 4 (U.S.S.R.). Criteria for identity were the same as previously (Buchowicz & Reifer, 1961). For calculations the millimolar extinction coefficients as given by Beaven, Holiday & Johnson (1955) were applied.

The efficiency of the isolation procedure was checked on commercial samples of free bases and ribosides, as well as on mixtures of these substances added to crude plant extracts. Practically no losses were observed on adsorption and desorption from charcoal. However, further purification, in particular paper chromatography and rechromatography, resulted in substantial losses. Finally, from 1 μmole of uracil, cytosine, adenine, uridine, cytidine, adenosine and guanosine, 0.69, 0.63, 0.87, 0.72, 0.72, 0.60 and 0.60 μmole, respectively, were recovered. Data given in the result section for plant material represent actually found, non-corrected, values.

In order to establish whether or not nucleotides and nucleosides can be degraded to corresponding free bases, 2′(3′)-UMP, 5′-UMP, 2′(3′)-AMP, 5′-AMP, uridine, and adenosine (1 μmole of each) were passed through the procedure leading to isolation of uracil and adenine. However, no measurable quantities of these bases could be isolated.

Isolation of orotate. The isotope dilution method was used for determination of orotate in wheat plants; 0.1 μmole of [6-^{14}C]orotate of high specific activity (24 000 counts/sec./μmole) was added to $HClO_4$-extract from a 100 g. sample of plant material. The procedure worked out earlier for isolation of orotate from carbamoylaspartate-fed plants (Buchowicz, Reifer & Makowski, 1961) was followed with additional rechromatography in the propan-1-ol - formate - water (7:1:2, by vol.) solvent system (Leone & Scale, 1950). The isolated orotate was checked for identity and purity by ultraviolet light absorption measurements and its specific activity was determined. The content of endogenous orotate in the plant extract

was calculated from the difference between specific activities of the added and recovered product, according to the equation of Rittenberg & Foster (1940).

Chemicals. [6-^{14}C]Orotic acid, The Radiochemical Centre, Amersham, England; uracil, cytosine, adenine, uridine, cytidine, adenosine, guanosine, 5′-UMP, and 2′(3′)-AMP, L. Light Co., Colnbrook, England; 5′-AMP and Dowex 1×1, 50--100 mesh, Fluka AG, Buchs, Switzerland; orotic acid, Nutritional Biochemicals Corp., Cleveland, U.S.A.; 2′(3′)-UMP, Calbiochem., Los Angeles, U.S.A.; activated charcoal, Zakłady Suchej Destylacji Drewna, Hajnówka, Poland.

RESULTS

Uracil, cytosine, adenine, uridine, cytidine, adenosine and guanosine were isolated from 6-day-old wheat plants and determined by the spectrophotometric method (Table 1). Uridine and adenine were predominant among nucleosides and free bases. Uracil was found in a quantity as high as 5′-UMP. Cytosine and cytidine were present in relatively low amounts and guanine could not be detected. The material contained, however, several non-identified, ultraviolet-light-absorbing compounds, free of phosphate and carboxyl groups. Orotate could be detected and quantitatively determined only by the isotope dilution method. Its quantity was considerably lower than that of any other isolated acid-soluble pyrimidine derivative.

Table 1

Amounts of pyrimidine and purine derivatives in 6-day-old wheat plants

Mean values ± standard deviation, with the number of determinations in parentheses, are given. No correction was made for losses during the isolation procedure.

Compound	μmoles/10 g. of fresh weight
Uracil	0.55 ± 0.22 (6)
Cytosine	0.13 ± 0.04 (3)
Adenine	0.90 ± 0.12 (6)
Uridine	1.63 ± 0.16 (6)
Cytidine	0.13 ± 0.05 (3)
Adenosine	0.17 ± 0.06 (5)
Guanosine	0.25 ± 0.04 (3)
Orotate	0.013 ± 0.003 (3)
5′-UMP	0.45*
5′-CMP	0.25*

* Data taken from the report of Buchowicz & Reifer (1962).

The content of the free bases and their ribosides in wheat was found to undergo rapid changes during germination and subsequent growth (Table 2). After the first day of germination a considerable increase in the amounts of adenine, uracil,

Table 2

Changes in the amounts of uracil, adenine and their ribosides in wheat during germination and growth

For all experiments wheat grains were sown on the same day and the material was always harvested at the same time of day. The results are expressed in μmoles per 10 g. of fresh weight and, parallelly, in μmoles per 100 plants. Results are from duplicate experiments, differing by not more than 10%. No correction was made for losses during the isolation procedure.

Age (days)	Part of the plant	Dry weight (%)	Uracil		Adenine		Uridine		Adenosine	
			μmoles per		μmoles per		μmoles per		μmoles per	
			10 g.	100 plants	10 g.	100 plants	10 g.	100 plants	10 g.	100 plants
0	Whole grain	87.5	0.14	0.04	0.10	0.03	0.27	0.08	0.06	0.02
1	Whole material	61.0	0.25	0.11	0.28	0.13	0.41	0.18	0.07	0.03
3	Whole material	31.0	0.25	0.21	0.12	0.10	0.62	0.52	0.04	0.03
6	Green parts	9.3	0.84	0.24	0.93	0.26	1.94	0.55	0.14	0.05
8	Green parts	10.5	0.26	0.08	0.98	0.29	1.83	0.55	0.19	0.06
10	Green parts	11.5	0.83	0.26	0.57	0.18	5.27	1.67	3.26	1.03
14	Green parts	13.0	0.71	0.35	0.29	0.15	1.85	0.92	1.17	0.59

uridine, and to a lesser degree, adenosine was observed both when the results were expressed in μmoles per 100 plants and per units of fresh weight. In the former case the same tendency was maintained for further two days. However, when the results were expressed per unit of fresh weight, a further rise was observed only for uridine, whereas the amounts of adenine and adenosine dropped markedly. The quantities of all substances tested were considerably higher in the green parts of 6-day-old wheat plants than in the 1 - 3-day-old material. No regular tendency of the changes in the amount of uracil, whether it was expressed per weight unit or per 100 plants, could be found during further growth (from 6th to 14th day). A constant drop in the amount of adenine was observed after the 8th day of growth. The amounts of both nucleosides, uridine and adenosine, reached a maximum on the 10th day of growth.

DISCUSSION

Previous observations on the presence of uracil in wheat seedlings (Buchowicz, Reifer & Makowski, 1961; Buchowicz & Reifer 1961, 1962) have been confirmed. In addition, two other free bases, cytosine and adenine, were found. It is difficult to assume that these compounds could arise during the isolation procedure, since neither nucleotides nor nucleosides were degraded to free bases under the conditions employed. The apparent discrepancy between our results and the data reported by Brown (1963, 1965) may be due to differences between wheat seedlings and leguminous plants. Uridine, adenosine and guanosine were isolated from wheat plants in quantities comparable to those reported by Brown for peas.

Occurrence of acid-soluble cytosine derivatives in wheat plants is a matter of controversy. Cytidine nucleotides were found by Bergkvist (1956) and later in our laboratory (Buchowicz & Reifer, 1961, 1962). However, more recently Keys (1963) and Elnaghy & Nordin (1966) were unable to detect acid-soluble cytosine derivatives in wheat seedlings. The data presented here showed that this material, in addition to 5′-CMP, contains cytidine and cytosine, although in relatively low quantities.

Orotate has been previously found only in plants fed with appropriate precursors, carbamoylaspartate or aspartate (Reifer, Buchowicz & Toczko, 1960; Buchowicz, Reifer & Makowski, 1961; King, Wang & Waygood, 1965; Ross, 1965). By the isotope dilution method it was possible to detect this compound in normal, non-fed plants, too.

The substantial increase in the amounts of adenine and uracil during the early phase of germination, followed by the decrease of the adenine content, may suggest that the free bases are involved in metabolic changes leading to the transition from dormant to germinating seeds.

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ZASADY I NUKLEOZYDY PIRYMIDYNOWE I PURYNOWE W SIEWKACH PSZENICY

Streszczenie

1. Z siewek pszenicy wyizolowano i oznaczono ilościowo uracyl, cytozynę, adeninę, urydynę, cytydynę, adenozynę i guanozynę. Zastosowanie metody rozcieńczenia izotopowego pozwoliło stwierdzić także obecność kwasu orotowego w tym materiale.

2. Obserwowano szybkie zmiany zawartości zasad i nukleozydów w pszenicy podczas jej kiełkowania i wzrostu.

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THE REDUCTION OF UBIQUINONE BY SARCOSINE DEHYDROGENASE SYSTEM

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1. Sarcosine in the presence of cyanide reduces ubiquinone in rat liver mitochondria washed with phosphate buffer as well as in NAD-depleted mitochondria. 2. Mitochondria, the sonically disintegrated preparation and the 100 000 *g* supernatant solution reduce ubiquinone in the presence of sarcosine. 3. The fractions obtained by ammonium sulphate precipitation are separately inactive. When fractions 0.4-0.6 and 0.6-0.8 sat. are combined, the activity is restored.

The studies of Mackenzie (1955) on sarcosine metabolism have established that sarcosine is oxidized in mitochondria to glycine and an "active one-carbon compound" which possesses the oxidation level of formaldehyde. This active one-carbon entity either may condense with glycine to yield serine or, alternatively, it may be converted irreversibly to formaldehyde. Sarcosine dehydrogenase system was isolated from mitochondria in soluble form, and characterized as the enzyme containing non-haeme iron and tightly bound FAD (Hoskins & Mackenzie, 1961; Frisell & Mackenzie, 1962). Our earlier investigations (Szarkowska & Heller, 1961; Szarkowska & Drabikowska, 1963; Drabikowska & Szarkowska, 1965) suggested ubiquinone to be an electron acceptor for flavoprotein enzymes containing iron. Therefore a study was undertaken on the behaviour of sarcosine oxidizing system towards this acceptor. It was established that during oxidation of sarcosine in rat liver mitochondria both endogenous and added ubiquinone (UQ_6) became reduced. A preparation of the soluble sarcosine dehydrogenase system was also studied.

MATERIAL AND METHODS

Preparation of mitochondria. Adult Wistar rats weighing 200 - 250 g. were used. The animals were killed by decapitation, the liver excised, chilled in cold isotonic sucrose solution, and homogenized in a glass homogenizer with 9 vol. of 0.25 M-sucrose. The homogenate was centrifuged at 500 *g* for 10 min., and the supernatant solution was centrifuged at 4500 *g* for a further 10 min. The sedimented mitochondria

were washed once with 0.25 M-sucrose, then with 7.5 mM-phosphate buffer, pH 7.5, and suspended in 0.25 M-sucrose to contain about 20 mg. of protein per millilitre.

Mitochondria depleted of NAD were prepared according to Ernster & Navazio (1956).

Preparation of soluble sarcosine oxidizing system. The washed mitochondria were suspended in 7.5 mM-potassium-phosphate buffer, pH 7.5, in a volume equivalent to the original weight of liver, placed in an ice-bath and subjected to sonic oscillation for 1 hr. in MSE 20-kc oscillator at a current output of 1.5 amperes. From the sonically treated mitochondria, the soluble sarcosine oxidizing system was prepared and fractionated according to Hoskins & Mackenzie (1961). The sonically treated mitochondria were centrifuged for 30 min. in MSE ultracentrifuge at 100 000 *g* to remove mitochondrial debris, and the supernatant solution was centrifuged again at 100 000 *g* for 3.5 hr. to remove the electron transfer particles. The obtained supernatant was separated by ammonium sulphate precipitation into three fractions: 0-0.4, 0.4-0.6 and 0.6-0.8 ammonium sulphate saturation. Each fraction was dissolved in 7.5 mM-potassium-phosphate buffer, pH 7.5, and dialysed against the same buffer for 1 hr. in the cold. Primary sarcosine dehydrogenase was present in fraction 0.4-0.6, while fraction 0.6-0.8 contained the so-called "electron transferring flavoprotein".

Analytical methods. The content of endogenous ubiquinone in mitochondria was determined as described previously (Drabikowska & Szarkowska, 1965). Reduction of exogenous ubiquinone (UQ_6) was determined by the method of Redfearn & Pumphrey (1960), and reduction of 2,6-dichlorophenolindophenol was measured in the recording Eppendorf photometer at 578 mμ.

Protein in mitochondria was determined by the biuret procedure after Szarkowska & Klingenberg (1963), and in the soluble enzyme preparation by the method of Lowry, Rosebrough, Farr & Randall (1951).

Reagents. Sarcosine chlorohydride and choline chloride (Light Co, Ltd, Colnbrook, Bucks, England); phenazine methosulphate (Sigma Chem. Comp., St. Louis, U.S.A.); FAD (Fluka A. G., Buchs, Switzerland); ubiquinone, UQ_6 (Farmochimica Cutolo-Calosi S.P.A., Napoli, Italy); semicarbazide and potassium acetate were Polish commercial products. Triton X-100 was kindly given by Rohm & Haas (Philadelphia, Pa., U.S.A.).

RESULTS

Reduction of endogenous ubiquinone in mitochondria. In rat liver mitochondria washed with phosphate buffer, ubiquinone was present mainly in the oxidized state. The addition of sarcosine in the presence of cyanide induced an appreciable reduction of ubiquinone (Table 1). Semicarbazide had no effect on the reduction by sarcosine; this indicates that formaldehyde as the product of sarcosine oxidation cannot participate in this reaction. Acetate at 40 mM concentration inhibited completely the reduction of ubiquinone by sarcosine. Formaldehyde added as substrate reduced mitochondrial ubiquinone but its effect was somewhat smaller than that

Table 1

The redox state of ubiquinone in rat liver mitochondria

Composition of the medium: 10 mM-TRA (triethanolamine hydrochloride), pH 7.5, 0.22 M-sucrose, 1 mM-potassium cyanide (adjusted to pH 8). Sarcosine and formaldehyde were added at concentration of 5 mM, and where indicated 60 mM-semicarbazide or 40 mM-acetate. Mitochondrial protein, 5.2 mg. per ml. Total volume 2.5 ml. The reaction mixture was placed in a water bath at 37°. After 3 min. the reaction was initiated by the addition of substrate. The reaction was stopped after 5 min. of incubation by addition of 7.5 ml. of methanol at -20°.

Additions	Oxidized ubiquinone (mμmoles/mg. protein)	Reduction (%)
None	1.89	—
None + semicarbazide	1.86	—
Sarcosine	0.98	47
Sarcosine + semicarbazide	0.98	47
Sarcosine + acetate	1.86	1
Formaldehyde	1.17	38
Formaldehyde + semicarbazide	1.81	2

Table 2

The reduction of ubiquinone in rat liver mitochondria by different substrates

Conditions of reaction as described in the legend of Table 1. The mitochondrial protein 5.7 mg./ml.

Exp. no.	Substrate	Time of incubation (min.)	Oxidized ubiquinone (mμmoles/mg. protein)	Reduction (%)
1	None	5	2.32	
	Succinate	5	0.53	77
	Choline	5	1.30	44
	Sarcosine	5	1.35	42
	Sarcosine + choline	5	1.30	44
	None	15	2.40	
	Sarcosine	15	1.42	41
2	None	5	2.44	
	Succinate	5	0.34	86
	Choline	5	0.86	65
	Formaldehyde	5	1.47	40
	Sarcosine + choline	5	0.83	66
	None	15	2.54	
	Sarcosine	15	1.17	54

of sarcosine, and the reduction was inhibited by semicarbazide. Choline as substrate gave the same degree of reduction as did sarcosine (Table 2) but the addition of choline to the mitochondria together with sarcosine did not increase the degree of ubiquinone reduction above that given separately by each of these substrates. Succinate induced much higher reduction of the endogenous ubiquinone. Pro-

Table 3

Reduction of ubiquinone in NAD-depleted mitochondria

Reaction mixture and conditions of reaction as described in the legend of Table 1. Mitochondrial protein 5.05 mg./ml.

Exp. no.	Substrate	Time of incubation (min.)	Oxidized ubiquinone (mμmoles/mg. protein)	Reduction (%)
1	None	5	3.82	—
	Sarcosine	5	1.87	51
	Formaldehyde	5	3.78	0
	Succinate	5	0.39	90
2	None	15	3.85	—
	Sarcosine	5	1.87	51
	Sarcosine	10	1.87	51
	Sarcosine	15	1.87	51

Table 4

Reduction of ubiquinone (UQ_6) *by successive preparations of sarcosine oxidizing system*

The medium contained: 7.5 μmoles of phosphate buffer, pH 7.5, 1 μmole of potassium cyanide (adjusted to pH 8), 198 mμmoles of ubiquinone (UQ_6); 2 mg. of Triton X-100; 10 μmoles of sarcosine and 0.2 ml. of each enzyme fraction. Total volume 1 ml. Incubation: 30 min. at 37°.

Preparation	Protein (mg./ml. of the preparation)	UQ_6 red. (μmoles/sample)	Activity (UQ_6 red.)
Whole mitochondria	24.5	181	37
Sonically treated mitochondria	24.5	190	39
100 000 *g*, 30 min. supernatant	14.0	167	60
100 000 *g*, 3.5 hr. supernatant	11.0	165	75

longation of the time of incubation with sarcosine did not change the degree of reduction.

In NAD-depleted mitochondria (Table 3), the reduction of ubiquinone by sarcosine was of the same degree as in the phosphate-buffer-washed mitochondria, and also independent of the time of incubation. In these mitochondria, formaldehyde did not reduce ubiquinone at all while succinate reduced 90% of its amount.

Soluble sarcosine oxidizing system. Phosphate-buffer-washed mitochondria as well as the preparations of sonically disintegrated mitochondria reduced added ubiquinone (UQ_6) in the presence of sarcosine (Table 4). The activity per mg. protein of the 3.5 hr. supernatant solution (free from electron transfer particles) towards UQ_6 was twice as high as in the mitochondria.

Table 5

Reduction of ubiquinone (UQ_6) *by the preparations of sarcosine oxidizing system fractionated by ammonium sulphate*

The experimental conditions are the same as in Table 4. Where indicated 0.05% phenazine-methosulphate or 40 μM-FAD were added.

Ammonium sulphate saturation fraction	Protein (mg./sample)	UQ_6 red. (mμmoles)
0-0.4	0.8	0
0.4-0.6	3.2	0
0.6-0.8	1.1	0
0.4-0.6 + 0.6-0.8	3.2 + 0.55	101
0-0.4 + 0.4-0.6 + 0.6-0.8	0.8 + 3.2 + 0.55	108
0.4-0.6 + phenazine	3.2	65
0.4-0.6 + 0.6-0.8 + FAD	3.2 + 0.55	98

Neither of the three fractions obtained by ammonium sulphate precipitation, was able alone to reduce UQ_6 with sarcosine as substrate (Table 5). However, the recombination of fraction 0.4-0.6 and 0.6-0.8 restored the activity, whereas the addition of fraction 0-0.4 had no effect. Fraction 0.6-0.8 could be replaced by phenazine metosulphate but the reaction was not so efficient, whereas FAD had no effect.

The results presented in Fig. 1 show that ammonium sulphate 0.4-0.6 sat. fraction, reduced indophenol only insignificantly. The addition of fraction 0.6-0.8 brought

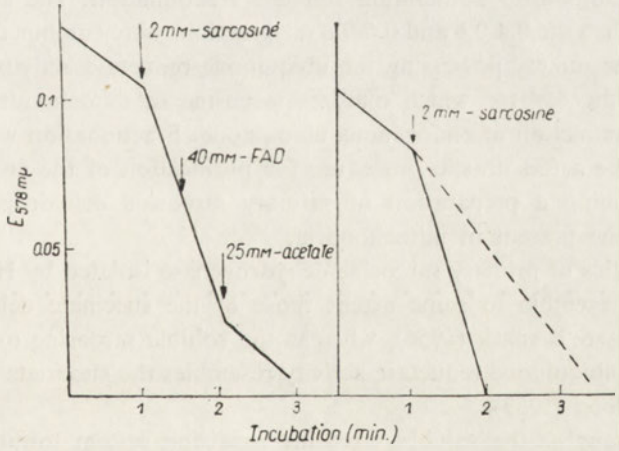

Fig. 1. Reduction of 2,6-dichlorophenolindophenol by ammonium sulphate-precipitated fractions of sarcosine oxidizing system. Conditions: 504 μM-indophenol, 10 mM-cyanide (adjusted to pH 8), 0.1 ml. of each enzyme fraction and 7.5 mM-potassium-phosphate buffer, pH 7.5, to 1 ml. Sarcosine, FAD, and acetate were added successively as indicated by arrows. (— — —), Fraction 0.4-0.6 sat. (1.2 mg. of protein/sample); (———), fraction 0.4-0.6 + 0.6-0.8 sat. (1.2 + 0.63 mg. of protein/sample).

about an evident increase in the reduction rate. FAD did not stimulate this reaction but addition of 25 mM-acetate inhibited completely the reduction. Phenazine methosulphate was as effective as fraction 0.6-0.8.

DISCUSSION

The experiments presented in this paper indicate that in rat liver mitochondria, endogenous ubiquinone becomes reduced after addition of sarcosine. This reduction can be due only to the oxidation of sarcosine to glycine and "active formaldehyde" but not to oxidation of "active formaldehyde" to formate, because after adding semicarbazide as the trapping agent for carbonyl group the reduction of ubiquinone was unaltered. Moreover, the reduction was completely inhibited by acetate which is a competitive inhibitor for sarcosine oxidase (Mackenzie, 1955). The direct reduction of ubiquinone by sarcosine is also substantiated by the experiments with NAD-depleted mitochondria in which exogenous formaldehyde did not reduce ubiquinone at all as it did in normal mitochondria. Thus the participation of formaldehyde in reduction of ubiquinone by sarcosine is excluded.

The extent of reduction was never higher than 50% of total ubiquinone present in NAD-depleted as well as in NAD-containing mitochondria. Under the same conditions succinate reduced ubiquinone up to 90%.

It was also shown that exogenous ubiquinone acted as an electron acceptor for hydrogen transfer from sarcosine by sarcosine oxidizing system, both when the enzyme was bound to the mitochondrial structure and when it was released by sonic disintegration and free from the electron transfer particles. The activity of the soluble sarcosine oxidizing system toward ubiquinone was not found in the particular fractions after ammonium sulphate fractionation. The activity was recovered only when the 0.4-0.6 and 0.6-0.8 sat. fractions were combined. This suggests that the enzyme moiety possessing the ubiquinone reductase activity was split off. It seems that the enzyme which oxidizes sarcosine in mitochondria, is involved as well in the reduction of endogenous ubiquinone. Fractionation with ammonium sulphate may be a too drastic procedure for purification of the enzyme and may result in obtaining a preparation of primary sarcosine dehydrogenase differing from the enzyme present in mitochondria.

The properties of primary sarcosine dehydrogenase isolated by Hoskins & Mackenzie (1961) resemble to some extent those of the succinate dehydrogenase of Singer, Kearney & Bernath (1956), whereas the soluble sarcosine oxidizing system possessing the ubiquinone reductase activity resembles the succinate dehydrogenase of Ziegler & Doeg (1959).

The behaviour of the soluble sarcosine oxidizing system towards indophenol before and after fractionation by ammonium sulphate, was studied by Hoskins & Mackenzie (1961). The same behaviour of the soluble preparation toward exogenous ubiquinone was found in our experiments except that, contrary to the findings of the above authors, we were unable to demonstrate any stimulation of the enzyme activity after addition of FAD, either with indophenol or ubiquinone as acceptor.

It is worth noting that in our earlier investigations on the role of ubiquinone as acceptor for flavoprotein enzymes containing iron, we have always observed that soluble dehydrogenases (glycerophosphate dehydrogenase and choline dehydrogenase), similarly as the sarcosine oxidizing system studied in this work, if they were able to reduce 2,6-dichlorophenolindophenol, they reduced external ubiquinone as well (Szarkowska & Drabikowska, 1963; Drabikowska & Szarkowska, 1965). This may indicate that indophenol accepts electrons from the respiratory chain at the level of ubiquinone or below it.

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REDUKCJA UBICHINONU PRZEZ SYSTEM DEHYDROGENAZY SARKOZYNY

Streszczenie

1. Sarkozyna w obecności cyjanku powoduje redukcję ubichinonu w mitochondriach wątroby szczura przemytych buforem fosforanowym, jak również w mitochondriach, z których usunięto NAD.

2. Mitochondria, preparaty mitochondrialne rozbite ultradźwiękami i frakcje supernatantu po wirowaniu przy 100 000 *g* katalizują redukcję egzogennego ubichinonu w obecności sarkozyny.

3. Poszczególne frakcje otrzymane przez wysalanie siarczanem amonu nie wykazują aktywności enzymatycznej. Po połączeniu frakcji wytrąconych przy 0.4-0.6 i 0.6-0.8 nasycenia, aktywność jest przywrócona.

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[*CARBAMOYL*-^{14}C]CITRULLINE IN PYRIMIDINE SYNTHESIS IN WHEAT SEEDLINGS

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1. Incorporation of [^{14}C]bicarbonate, [^{14}C]carbamoylphosphate and [*carbamoyl*-^{14}C]citrulline into pyrimidine and purine derivatives was studied in excised wheat seedlings. Citrulline was most efficiently utilized in pyrimidine synthesis and rates of its incorporation were not influenced by non-radioactive carbamoylphosphate. 2. Degradation of uracil showed that ^{14}C of these precursors labels only the C-2 position of the ring.

It has been established (see reviews by Reichard, 1959, and Wang & Waygood, 1964) that [^{14}C]bicarbonate and [*carbamoyl*-^{14}C]citrulline are incorporated into pyrimidine derivatives in animal tissues and bacterial cells. The radioactive carbon of these precursors has been found to be located in the C-2 position of the pyrimidine ring. Studies *in vitro* have shown that the ureide fragment of the pyrimidine ring is derived directly from carbamoylphosphate, formed either *de novo* from CO_2, NH_3 and ATP or on the path of citrulline phosphorolysis. This implies carbamoylphosphate as the intermediate on the route of incorporation irrespectively whether the bicarbonate or citrulline was used as precursor.

To establish whether similar pathways occur also in higher plants, wheat seedlings were fed with bicarbonate, carbamoylphosphate or citrulline and incorporation of these compounds into pyrimidine derivatives was compared. Preliminary accounts of some of this work have been presented (Rybicka, Buchowicz & Reifer, 1966; Rybicka & Buchowicz, 1966).

MATERIAL AND METHODS

Reagents. Radioactive carbamoylphosphate, dilithium salt, was prepared from K^{14}CNO according to the method of Jones, Spector & Lipmann (1955). K^{14}CNO and L-citrulline labelled with ^{14}C in the carbamoyl carbon were purchased from The Radiochemical Centre, Amersham, England. NaH$^{14}CO_3$ was obtained from Instytut Badań Jądrowych, Świerk, Poland. Non-labelled L-citrulline and all pyri-

midine and purine derivatives were purchased from L. Light Co., Colnbrook, England.

Plants. Green, five-day-old wheat plants, variety Dańkowska 40, were used throughout.

Feeding. Preparation of plant material and general conditions of feeding experiments were described previously (Reifer, Buchowicz & Toczko, 1960). All the details are given in Table 1.

Extraction, separation and determination of pyrimidine and purine derivatives. The acid-soluble pyrimidine and purine derivatives were extracted from the plant material with ice-cold 0.3 N-$HClO_4$ and then freed from most of the acid-soluble contaminants by charcoal treatment (Buchowicz & Reifer, 1962). The obtained mixture of partially purified pyrimidine and purine derivatives was either used for the separation and determination of the individual components according to the previously described procedure (Buchowicz, Reifer & Makowski, 1961) or hydrolysed to the corresponding bases with 9 N-$HClO_4$ at 100° for 2 hr. The resulting bases were then further purified on the Dowex-1 column and separated by paper chromatography as described in detail by Grzelczak & Buchowicz (1967). Products corresponding to the reference samples of uracil, cytosine, adenine and guanine were eluted, checked for purity and identity by spectrophotometric measurements and quantitatively determined, using the SF-4 spectrophotometer (U.S.S.R.). The radioactivities were determined with the mica window GM counter, AAH 55, with the counting time long enough to reduce the error to less than 5%. No corrections were made for self-absorption, which was negligible under the conditions described. Chromatographic procedures were repeated until specific activities of the isolated products were constant and the absorption spectra identical with those of the authentic commercial samples. In the preliminary report (Rybicka & Buchowicz, 1966) the possibility of contamination of cytosine and guanine (isolated from plants fed with citrulline) by spectrophotometrically non-interfering products was unfortunately overlooked.

The acid-insoluble fraction was hydrolysed with 1 N-HCl at 100° for 1 hr. The resulting pyrimidine mononucleotides were isolated and determined as described for the acid-soluble fraction.

Degradation of uracil. Radioactive uracil isolated from the plant material was diluted with commercial non-labelled uracil to 0.7 mg. and degraded according to the method of Heinrich & Wilson (1950). The resulting urea and oxalic acid were then assayed for radioactivity. The carbon atom of urea corresponds to C-2 position, whereas the two carbon atoms of oxalic acid correspond to C-5 and C-4 (or C-6) positions of the pyrimidine ring. Urea was estimated quantitatively by the method of Reifer & Toczko (1959).

RESULTS

To compare the incorporation of [^{14}C]bicarbonate, [^{14}C]carbamoylphosphate and [*carbamoyl*-^{14}C]L-citrulline into acid-soluble pyrimidine and purine derivatives, wheat plants were fed with these substrates under comparable experimental condi-

Table 1

Amounts and specific activities of acid-soluble pyrimidine and purine derivatives isolated from wheat seedlings fed with different substrates

Samples of excised wheat seedlings (10 g.) were fed with 2 ml. portions of the indicated substrates at 20° for 2 hr. under exclusion of the atmospheric CO_2. Each solution, in addition to the specified substrates, contained 100 μmoles of potassium phosphate, pH 7.2, and L-aspartate in quantity equimolar to the radioactive precursor. The amounts are expressed in μmoles per 10 g. of fresh weight, specific activities in counts/sec./μmole. Results are from duplicate experiments.

Expt. no.	Substrates			Uptake* (counts per sec. $\times 10^{-3}$)	Acid-soluble derivatives of							
					Uracil		Cytosine		Adenine		Guanine	
	radioactive	spec. act. $\times 10^{-3}$	non-radioactive		amount	spec. act.	amount	spec. act.	amount	spec. act.	amount	spec. act.
I	43 mM-bicarbonate	1.2	—	81.8	0.84	0.8	0.50	0.9	1.15	0.6	0.35	0.2
	25 mM-carbamoylphosphate	1.1	—	37.8	1.07	6.3	0.58	1.0	1.40	4.2	0.30	1.0
	25 mM-citrulline	1.1	—	7.9	0.80	10.5	0.60	3.2	0.80	< 0.1	0.20	< 0.1
II	0.6 mM-citrulline	60.3	—	28.8	1.75	9.9	0.40	5.5	1.10	< 0.1	0.37	< 0.1
	0.6 mM-citrulline	60.3	6.0 mM-carbamoylphosphate	24.0	1.75	9.7	0.30	7.5	1.00	< 0.1	0.28	< 0.1
III	0.3 mM-citrulline	87.5	—	41.0	2.00	9.0	—	—	0.93	< 0.1	0.25	< 0.1
	0.3 mM-citrulline	87.5	3.0 mM-carbamoylphosphate	37.0	1.80	6.0	—	—	1.15	< 0.1	0.22	< 0.1
IV	12 mM-carbamoylphosphate	1.1	—	20.2	1.80	8.0	0.32	1.4	1.34	2.5	0.32	1.0
	12 mM-carbamoylphosphate	1.1	60 mM-citrulline	21.7	2.00	6.6	0.30	1.9	1.27	2.8	0.44	1.2

* Difference between total radioactivities of the feeding solution before and after incubation.

tions (Table 1, expt. I). It was found that each of the three substrates was incorporated into pyrimidines, substantial differences in the specificity and efficiency were, however, observed. When bicarbonate or carbamoylphosphate were used, both pyrimidine and purine derivatives were radioactive. On the other hand, citrulline was incorporated only into the pyrimidine derivatives. Moreover, despite the low uptake of citrulline, specific activities of both uracil and cytosine derivatives were the highest when this compound was used as precursor. As regards carbamoylphosphate incorporation, the results obtained were probably affected by spontaneous decomposition of this compound (approximately 10% was degraded to orthophosphate during the incubation period). Nevertheless, the intensity of its incorporation was several times higher than that of bicarbonate.

Table 2

Specific activities of individual uracil derivatives isolated from plants fed with [carbamoyl-^{14}C]*citrulline in the presence or absence of non-radioactive carbamoylphosphate*

Feeding conditions were the same as given in Table 1, expt. I. The results are expressed as counts/sec./μmole.

Product	Carbamoylphosphate	
	not added	added
Uracil	5.0	5.8
Uridine	7.5	6.0
5′-UMP	5.0	4.0
2′(3′)-UMP	0.13	0.15

To establish whether or not carbamoylphosphate is an obligatory intermediate on the path of citrulline incorporation into pyrimidine derivatives, dilution experiments were carried out. It was found that a tenfold excess of non-labelled carbamoylphosphate in the medium did not decrease the rate of [*carbamoyl*-^{14}C]citrulline incorporation into acid-soluble pyrimidine derivatives (Table 1, expts. II

Table 3

Radioactivity of uracil and its degradation products

From plants fed with labelled precursors (under conditions given in Table 1), uracil was isolated and degraded. Radioactivity is expressed in counts/sec./μmole.

Radioactive precursor	Uracil	Degradation products	
		Urea	Oxalate
Bicarbonate	0.23	0.14	< 0.1
Carbamoylphosphate	0.74	0.72	< 0.1
Citrulline	1.53	1.09	< 0.1
Citrulline	1.02	0.70	< 0.1

and III). Similarly, non-labelled citrulline did not show dilution effects on [^{14}C]carbamoylphosphate incorporation (Table 1, expt. IV).

The apparently independent ways of carbamoylphosphate and citrulline incorporation into acid-soluble pyrimidine derivatives, as indicated by data given in Table 1, brought up the question whether some of them might be synthesized with carbamoylphosphate, whereas the others with citrulline as respective donors of the carbamoyl group. Therefore dilution effects of carbamoylphosphate on the citrulline incorporation into individual uracil derivatives were investigated. It was found that all compounds tested, uracil, uridine, 5′-UMP and 2′(3′)-UMP were labelled when [^{14}C]citrulline was used as substrate. The presence of an excess of non-radioactive carbamoylphosphate in the medium caused no measurable differences (Table 2).

It was of interest to determine whether the radioactive carbon atom of each of the three precursors was incorporated into the same position of the pyrimidine ring. Therefore the degradation of radioactive uracil isolated from plants fed with labelled bicarbonate, carbamoylphosphate or citrulline was carried out and radioactivities of the resulting products determined (Table 3). It was found that independently of the precursor used only urea (derived from C-2 of the ring) was radioactive. The specific activity of this product was comparable to that of the degraded uracil.

DISCUSSION

The general similarity of the pathways of pyrimidine synthesis in higher plants and other organisms has been further substantiated. It has been found that CO_2 and the carbamoyl group of L-citrulline are incorporated into the C-2 position of the pyrimidine ring in wheat seedlings, similarly as it was earlier observed for animal tissues and micro-organisms (Kusama & Roberts, 1963; Hager & Jones, 1965; and the older reports as reviewed by Reichard, 1959, and Wang & Waygood, 1964). Furthermore, as it could be expected, it was shown that carbamoylphosphate is incorporated with much higher intensity than bicarbonate. The more efficient incorporation of carbamoylphosphate than of bicarbonate also into purine derivatives may be due to formation of "active" CO_2, that is known as an intermediate in carbamoylphosphate metabolism (Anderson & Meister, 1965).

Some observations concerning utilization of the carbamoyl group of citrulline for pyrimidine synthesis in wheat plants are, however, hardly compatible with the current views. As it was originally demonstrated by Smith & Reichard (1956) on animal enzyme systems, citrulline takes part in this synthesis indirectly, after its phosphorolysis to ornithine and carbamoylphosphate, which then serves as the direct donor of the carbamoyl group. Should citrulline utilization in wheat plants follow the same route, the quantitative comparison of carbamoylphosphate and citrulline incorporation should reveal preferential utilization of the former. Moreover, the presence of an excess of non-labelled carbamoylphosphate in the medium should show a dilution effect on citrulline incorporation into pyrimidines. Neither

of these effects was observed in our experiments. On the contrary, the carbamoyl group of citrulline was found to be more efficiently and more specifically incorporated into pyrimidines than carbamoylphosphate, and the presence of a tenfold excess of non-labelled carbamoylphosphate did not decrease the rate of this incorporation.

According to these observations it is difficult to accept the view that carbamoylphosphate is an obligatory intermediate in utilization of citrulline for pyrimidine biosynthesis. Instead, it appears more likely that these two compounds are alternative donors of the carbamoyl group in reactions leading to pyrimidine ring formation in wheat seedlings. Whereas the mechanism of carbamoylphosphate utilization in these reactions is well known, the part played by citrulline so far has not been elucidated.

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[*KARBAMOIL*-^{14}C]CYTRULINA W SYNTEZIE POCHODNYCH PIRYMIDYNOWYCH W SIEWKACH PSZENICY

Streszczenie

1. Badano wbudowywanie [^{14}C]dwuwęglanu, [^{14}C]karbamoilofosforanu i [*karbamoil*-^{14}C]cytruliny do pochodnych pirymidynowych i purynowych w odciętych siewkach pszenicy. Cytrulina wcielała się z największą intensywnością do pochodnych pirymidynowych i szybkość jej wbudowywania nie ulegała zmianom pod wpływem nieradioaktywnego karbamoilofosforanu.

2. Degradacja uracylu wykazała, że ^{14}C tych prekursorów znakuje tylko pozycję C-2 pierścienia pirymidynowego.

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PURIFICATION AND SOME PROPERTIES OF THE NEURAMINIDASE FROM RABBIT KIDNEY

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1. A preparation of partially purified mucopolysaccharide *N*-acetylneuraminyl hydrolase was obtained from rabbit kidney. 2. The optimum pH was 5.5 and the enzyme was not activated by calcium ions.

Neuraminidase (mucopolysaccharide *N*-acetylneuraminyl hydrolase, EC 3.2.1.18) has been isolated from many species of bacteria. In spite of the widespread distribution of neuraminic acid-containing substances in mammalian tissues, only a few papers have been published so far concerning the presence of neuraminidase activity in mammals. Warren & Spearing (1960) found neuraminidase activity in commercial preparations of Cohn's fraction IV from bovine and human plasma, and they succeeded in 30 - 50 fold purification of the enzyme. Carubelli, Trucco & Caputto (1962) reported neuraminidase activity in lactating mammary gland, liver and brain of the rat, and traces of activity in kidney, small intestine, spleen and testes. The activity was present in the 105 000 *g* soluble fraction. Cook & Ada (1963) found neuraminidase activity in the soluble fraction of the chorioallantois, skin, and spinal cord of chick and guinea pig embryos. They emphasized the much higher content of neuraminidase in the embryonic than in the mature tissues.

Recently a new method of neuraminidase purification from *Clostridium perfringens* has been described by Cassidy, Jourdian & Roseman (1965). In the present work this method was adopted for isolation of neuraminidase from rabbit kidney. Some properties of the enzyme are also presented.

MATERIAL AND METHODS

Reagents. N-Acetylneuraminic acid (*N*-AN), cryst., stored at 0° and dried under P_2O_5, and neuraminidase from *Vibrio cholerae* (activity 100 units/ml., 1600 units/mg. of protein), were products of Light & Co. (Colnbrook, Bucks, England); *N*-acetylneuraminlactose from bovine colostrum and thiobarbituric acid (anal. grade) were from Sigma Corp. (St. Louis, USA); Sephadex G-75, medium size, was from

Pharmacia (Uppsala, Sweden); DEAE-cellulose from Serva (Heidelberg, German Federal Republic). Seromucoid was prepared from bovine plasma according to the method of Weimer, Mehl & Winzler (1950) and contained 10% of sialic acid. All other chemicals used were commercial products of analytical grade.

Analytical methods. Free sialic acid was estimated by the thiobarbituric acid method of Aminoff (1961). Total sialic acid was determined by the same method after 1 hr. of hydrolysis in 0.2 N-H_2SO_4 at 80° or by the method of Svennerholm (1958), *N*-acetylneuraminic acid being used as standard. Protein was determined by the method of Ellman (1962) using human albumin as standard. Pyruvic acid was determined by the method of α-oxoacids estimation of Friedemann (1957).

Purification of the enzyme. The method described by Cassidy *et al.* (1965) was applied for the purification of neuraminidase from animal tissue with minor modifications: final concentration of ammonium sulphate was 0.75 instead of 0.85; Sephadex G-75 column was smaller and 10 ml. instead of 40 ml. portions were collected.

Kidneys from two normal adult male rabbits were quickly removed under light ether anaesthesia and carefully washed from blood in ice-cold 0.85% natrium chloride solution. All further steps were done at 4°. The renal capsula, pelvis and connective

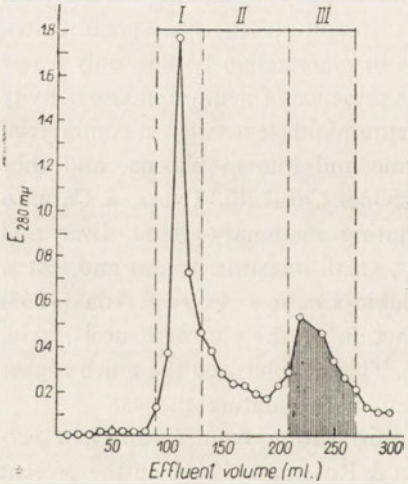

Fig. 1. Gel-filtration on Sephadex G-75 of the 0.5 - - 0.75 ammonium sulphate sat. fraction of the 105 000 *g* kidney supernatant. The shadowed areas indicate the presence of neuraminidase activity.

tissue were cut off. The kidneys were minced, then homogenized with 4 volumes of buffer solution (0.154 M-KCl containing 0.25 vol. of 0.5 M-sodium acetate, pH 7.3) in the Elvehjem-Potter glass homogenizer with teflon pestle. The homogenate was centrifuged at 10 000 *g* for 20 min. The precipitate was discarded and the supernatant was centrifuged at 105 000 *g* for 60 min. in MSE Spinco high-speed centrifuge.

To the 105 000 *g* soluble fraction, ammonium sulphate was added to 0.5 saturation and after 5 hr. at 4° the precipitate was centrifuged off. To the supernatant, $(NH_4)_2SO_4$ was added to 0.75 sat. After the next 18 hr. the centrifuged precipitate

was dissolved in 20 ml. of bidistilled water and dialysed against 4 l. of water for 48 hr., water being changed 10 - 12 times during this period. The dialysis residue was freeze-dried, dissolved in 5 ml. of 1 mM-potassium-phosphate buffer, pH 6.0, applied onto Sephadex G-75 column (45×2.5 cm.) and eluted with the same buffer. The effluənt collected in 10 ml. samples was assayed for protein content at 280 mμ. A typical elution pattern is presented in Fig. 1. The effluent was pooled into three fractions (*I, II, III*), each fraction was dialysed overnight against bidistilled water, freeze-dried, and neuraminidase activity was determined. A sample of the 105 000 *g* supernatant was also dialysed and freeze-dried.

The assay of enzyme activity. Incubation mixture contained in 2 ml.: 5 - 10 mg. of seromucoid or 0.2 mg. of *N*-acetylneuraminlactose; enzyme preparation (4 mg. dry wt.); and 0.05 M-potassium-phosphate buffer, pH 6.0. After appropriate time of incubation at 37°, the enzyme was inactivated by heating for 4 min. at 60°, and in the clear supernatant the amount of free sialic acid was determined. Blanks did not contain either the enzyme preparation or the substrate.

In some of the experiments the properties of the studied enzyme were compared with commercial *Vibrio cholerae* neuraminidase of known activity. Enzyme activity was expressed as μg. of *N*-AN liberated from the substrate per 1 mg. enzyme protein during 30 min. at 37°.

RESULTS

The course of purification of the enzyme is shown in Table 1. The low value of the neuraminidase activity in the 105 000 *g* soluble kidney fraction could be due to the presence of *N*-AN-aldolase (*N*-acetylneuraminate pyruvate-lyase, EC 4.1.3.3.). Therefore pyruvate was determined in the incubated samples. A high increase in pyruvate content was observed in the starting material, but at further stages of purification no increase was found.

Calcium ion had no influence on enzyme activity over the range 0.45 - 450 μM--$CaCl_2$. The dependence of the activity on pH in the presence of seromucoid as

Table 1

Purification of neuraminidase from rabbit kidney

The reaction mixture (2 ml.) contained: *N*-acetylneuraminlactose, 0.2 mg., enzyme preparation, 4 mg. dry wt., and 0.05 M-potassium-phosphate buffer, pH 6.0. Incubation: 30 min. at 37°.

Stage	Activity (μg. *N*-AN/mg. protein/30 min.)
105 000 *g* supernatant	0.041
Ppt. at 0.5 - 0.75 $(NH_4)_2SO_4$ sat.	0.7
Sephadex fraction *I*	0.0
Sephadex fraction *II*	2.8
Sephadex fraction *III*	152.5

substrate, is shown in Fig. 2. The optimum pH value was 5.5; the same optimum was found with *N*-acetylneuraminlactose. The reaction rate was proportional to enzyme concentration (Fig. 3).

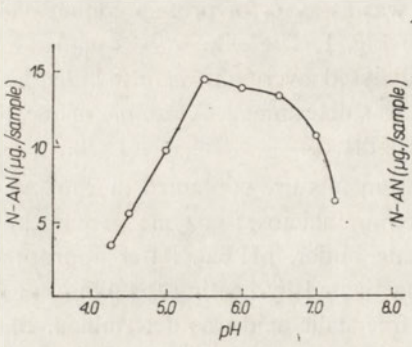

Fig. 2

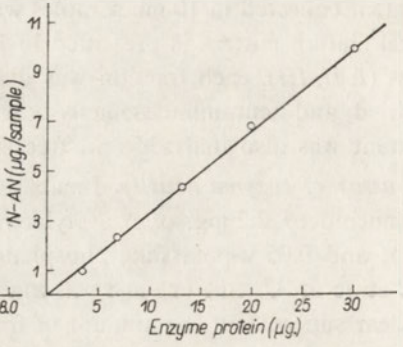

Fig. 3

Fig. 2. Effect of pH on enzyme activity with seromucoid as substrate. Incubation, 30 min.
Fig. 3. Effect of enzyme concentration on the amount of *N*-AN released from seromucoid (5 mg./ /sample). Incubation, 30 min.

Under the conditions used, neuraminidase was more active toward *N*-acetylneuraminlactose than toward seromucoid. After 30 min. of incubation, 70% of *N*-AN was released from acetylneuraminlactose, and 94% after 3 hr., whereas the respective values for seromucoid amounted to 43% and 75%.

DISCUSSION

The partially purified renal neuraminidase was not activated by calcium ion. In this the enzyme differs from the *Vibrio cholerae* (Ada, French & Lind, 1961) and bovine plasma (Warren & Spearing, 1960) neuraminidases, but is similar to the enzyme obtained from *Cl. perfringens* (Cassidy *et al.*, 1965) and from viruses (Wilson & Rafelson, 1963; Laver, Pye & Ada, 1964) as well as that from lactating mammary glands of rats (Carubelli *et al.*, 1962).

In kidney, Comb & Roseman (1960) and Brunetti, Jourdian & Roseman (1962) found *N*-AN-aldolase which can be the reason of unreliable results in neuraminidase activity determinations. In the present work, the neuraminidase preparation obtained by ammonium sulphate fractionation was free from *N*-AN-aldolase activity, and about 200-fold purification of neuraminidase was achieved by Sephadex gel-filtration.

The role of the kidney in the metabolism of aminosugars and sialoglycoproteins has not been elucidated so far. It is known from the data of Comb & Roseman (1960), Warren & Felsenfeld (1962) and Spiro (1965) that the kidney is an organ rich in aminosugars-containing compounds, which seem to play a biological role in renal function as components of tubular membranes and glomerular vascular

epithelium. Many enzymes participating in the synthesis, catabolism and interconversions of glycoprotein have been found in the kidney. In addition to the above mentioned *N*-AN-aldolase, enzymes catalysing the main steps of *N*-AN synthesis: cytidinemonophospho-*N*-AN-synthetase and *N*-acyl-D-glycosamine 6-phosphate epimerase (Ghosh & Roseman, 1962) were also reported to be present in kidney tissue.

The results described in this paper indicate the presence of significant neuraminidase activity in the kidney. Probably neuraminidase plays a role in the catabolism of glycoproteins in the kidney. As an enzyme present in the soluble fraction it could be an element of bigger multienzymic complex of other cytoplasmic enzymes participating in the catabolism of aminosugar compounds.

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OCZYSZCZANIE I NIEKTÓRE WŁASNOŚCI NEURAMINIDAZY NERKI KRÓLICZEJ

Streszczenie

1. Otrzymano częściowo oczyszczony preparat neuraminidazy z nerki królika.
2. Optimum pH wynosi 5,5. Aktywność enzymatyczna nie zależy od obecności jonów Ca.

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BASIC EXTRANUCLEAR PROTEINS OF PORCINE RENAL CORTEX

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1. A fraction of basic extranuclear proteins was isolated from the porcine renal cortex by gel-filtration of a formic acid extract from the tissue. 2. Chromatographic properties of the proteins on Sephadex G-75 and CM-cellulose are different from those of histones from cell nuclei. 3. α-Amino nitrogen was determined in basic, neutral and acidic amino acids of these proteins.

In recent years, basic proteins of animal tissues, and particularly nuclear histones, have attracted considerable attention. It has been stated that the proteins of this type are present not only inside the cell nucleus (Burston, Tombs & Maclagan, 1961, 1962; Mejbaum-Katzenellenbogen & Wieczorek, 1966). They were found in microsomes (Crampton & Petermann, 1959; Butler, Cohn & Simson, 1960), in mitochondria (Rzeczycki, Grudzińska & Hillar, 1962; Rzeczycki, 1964) and in sarcoplasma (Hartshorne & Perry, 1962), but except for lysozyme, ribonuclease and cytochrome *c*, little is known about their nature and biological significance.

In the present paper the isolation and partial characterization of a fraction of basic extranuclear proteins from porcine renal cortex are described. Preliminary results of this work have been presented (Toczko & Kanabus, 1966).

MATERIALS AND METHODS

The cortex layer of the porcine kidneys removed immediately after killing the animals, was used for all experiments.

Extract from renal cortex. The tissue previously cooled to 0° was homogenized for 3 min. in a Pragomix mixer with an equal volume (w/v) of cold N-formic acid containing 15 mM-thioglycolic acid. The homogenate was then transferred to a beaker immersed in ice, two volumes of the same solvent being added. The suspension was stirred for 30 min. and centrifuged in the cold for 15 min. at 2 500 *g*. The supernatant fluid was freeze-dried, yielding about 16 g. of dry material from 100 g. of the tissue.

Histones from cell nuclei. The cell nuclei from porcine renal cortex were isolated by the citric acid method (Mirsky & Pollister, 1946; Daly & Mirsky, 1955). Histones were prepared by extraction of nuclei with 0.25 N-hydrochloric acid according

to Davison, James, Shooter & Butler (1954) and with N-formic acid as follows: nuclei obtained from 200 g. of the tissue were suspended in water in a final volume of 100 ml. and 100 ml. of 2 N-formic acid containing 30 mM-thioglycolic acid were added. The suspension was stirred for 40 min. at 0°, centrifuged for 10 min. at 1 000 *g* and the clear supernatant fluid freeze-dried.

Fractionation on Sephadex G-75. Two grams of the freeze-dried preparation were extracted for 40 min. at 0° with 25 ml. of N-formic acid and then centrifuged for 10 min. at 8 000 *g*. The clear supernatant fluid (15 ml.) was applied to a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column (24 mm. × 560 mm.) prepared by the method of Flodin (1961) and equilibrated with N-formic acid. The effluent was tested by measuring extinction at 280 mμ and by the biuret method of Ellman (1962).

Chromatography on CM-cellulose was carried out on a carboxymethylcellulose (Serva, Entwicklungslabor, Heidelberg) column (10 mm. × 140 mm.) using the method of Phillips & Johns (1959).

Assay methods. Hydrolysis was performed with 5.7 N-hydrochloric acid in sealed tubes at 105 - 110° for 20 - 24 hr.

α-Amino nitrogen was determined in the acid hydrolysates by the manometric ninhydrin method of Van Slyke, MacFadyen & Hamilton (1943). Amide nitrogen was determined by the method of Raacke (1957). Basic, neutral and acidic amino acid contents were determined by the copper-ninhydrin method as described by Blackburn (1965) in fractions of the acid hydrolysates separated by means of high-voltage electrophoresis on Whatman no. 3 paper at pH 5.6. Cysteine was determined as cysteic acid in effluents from Amberlite IR-120 (form H^+) columns (10 mm. × × 100 mm.) by the colorimetric method of Yemm & Cocking (1955).

RESULTS AND DISCUSSION

Elution diagrams for the Sephadex G-75 column of the N-formic acid extracts from renal cortex and from isolated cell nuclei are shown in Figs. 1a and 1b respectively. The curves for tissue extract, obtained by reading the extinction at 280 mμ and by the biuret method of Ellman (1962), both possess three peaks with maxima at 70 - 90 ml., 195 - 205 ml. and 255 - 260 ml. The elution pattern of the extract from cell nuclei obtained by reading the extinction at 280 mμ shows two peaks with maxima at 70 - 80 ml. and 115 - 120 ml.

These results indicate that the components of the whole tissue extract can be separated on Sephadex G-75 column into three fractions: T_1 65 - 150 ml., T_2 150 - - 225 ml. and T_3 225 - 300 ml. Fraction T_1 contains proteins which were not examined in the present work. Fraction T_3 includes low-molecular weight compounds such as free amino acids and oligopeptides which were also observed in the water extracts from this tissue (Toczko, Kanabus & Pisarek, 1966). Fraction T_2 was found to contain low-molecular weight proteins and it was subjected to further analysis. Although these proteins are well soluble in water after isolation, they were not

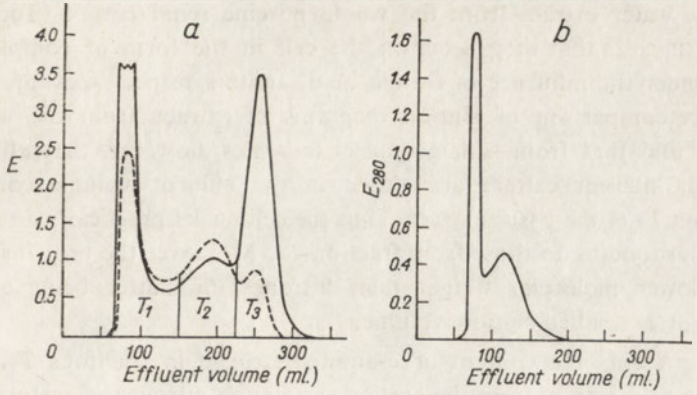

Fig. 1. Gel-filtration of N-formic acid extracts from: (*a*), 7 g. of renal cortex; (*b*), cell nuclei isolated from 7 g. of renal cortex. Column: Sephadex G-75 (560 mm. × 24 mm.); eluent: N-formic acid; fraction volume: 6 ml.; flow-rate: 30 ml./hr.; (———), $E_{280\ m\mu}$; (- - -), $\Delta E_{263\ m\mu}$.

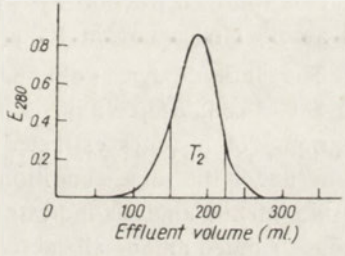

Fig. 2. Gel-filtration of crude fraction T_2. Same conditions as in Fig. 1.

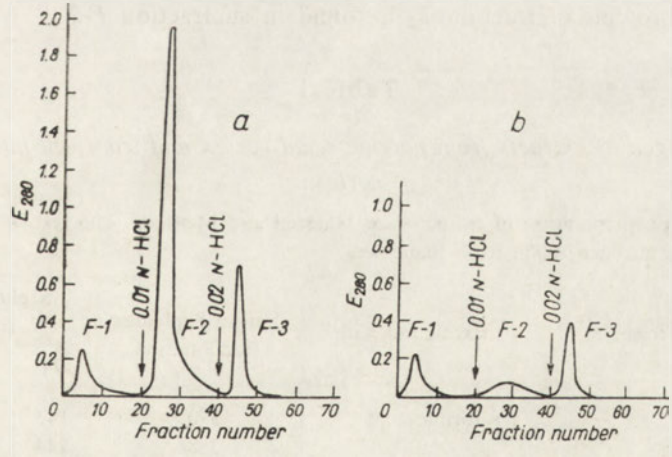

Fig. 3. Chromatography of: (*a*), fraction T_2, and (*b*), N-formic acid extract from cell nuclei. Column: CM-cellulose (140 mm. × 10 mm.); eluent: as indicated in the diagram; flow-rate: 40 ml./hr.; fraction volume: 5 ml.

present in a water extract from the whole porcine renal cortex (Toczko *et al.*, 1966). This suggests that they occur in the cell in the form of complexes which dissociate under the influence of formic acid. In this respect they are similar to histones. The comparison of elution diagrams of extract from the whole tissue homogenate and that from isolated nuclei indicates, however, that all the components of the nuclear extract are eluted at the effluent volume corresponding to the fraction T_1 of the tissue extract. Thus the cell nuclei practically do not contain proteins corresponding to those from fraction T_2. Moreover the proteins of fraction T_2 are of lower molecular weight than histones, the latter being eluted from the column at a smaller elution volume.

Table 1 presents the content of α-amino nitrogen in fractions T_1, T_2 and T_3 of the formic acid extract from the cortex, and amino nitrogen of histones obtained from isolated nuclei. About 390 mg. of amino nitrogen was extracted with formic acid from renal cortex and only 27 mg., i.e. about 14 times less, from cell nuclei.

Further study was confined to the components of fraction T_2 rechromatographed on a Sephadex G-75 column in order to remove the contaminations from fractions T_1 and T_3 (Fig. 2). The compounds eluted between 150 ml. and 225 ml. of the effluent volume (purified fraction T_2) were collected and freeze-dried yielding 115 - 130 mg. of amino nitrogen from 100 g. of wet tissue.

The elution diagram obtained during chromatography of proteins of fraction T_2 on a CM-cellulose column is presented in Fig. 3a. For comparison the chromatography of histones extracted with N-formic acid from isolated nuclei was also done under the same conditions, and a diagram of this is shown in Fig. 3b. The results of this analysis indicate that the proteins of fraction T_2 as well as histones are distributed among all subfractions *F-1*, *F-2* and *F-3* but in different proportions. The distribution of these proteins among individual subfractions is shown in Table 2. The nuclear proteins are located mainly in subfraction *F-3*, whereas as much as 87% of the proteins of fraction T_2 is found in subfraction *F-2*.

Table 1

α-Amino nitrogen of extracts from porcine renal cortex and from cell nuclei of renal cortex

For details on preparation of extracts, see Material and Methods. The results are expressed as mg. α-amino nitrogen/100 g. fresh tissue wt.

Material	Extraction with	Before Sephadex fractionation	Sephadex G-75 fraction		
			T_1	T_2	T_3
Renal cortex	N-formic acid	390	143	140	—
		388	144	158	93
		366	140	134	96
Renal cortex nuclei	N-formic acid	27.4	—	—	—
Renal cortex nuclei	0.25 N-HCl	22.7	—	—	—

Table 2

α-Amino nitrogen in hydrolysed subfractions from CM-cellulose column

The freeze-dried material was dissolved in 0.002 N-hydrochloric acid and applied to a CM -cellulose column. Fractions were eluted with: 0.005 N-HCl (fraction *F-1*), 0.01 N-HCl (*F-2*) and 0.02 N-HCl (*F-3*), 100 ml. of each. α-Amino nitrogen was determined in each fraction after hydrolysis. The results are expressed as % of the total.

Material	Subfraction from CM-cellulose		
	F-1	*F-2*	*F-3*
Fraction T_2 from Sephadex G-75	2	87	11
N-Formic acid extract from renal cortex nuclei	4	30	66
0.25 N-HCl extract from renal cortex nuclei	3	25	72

Table 3

Forms of nitrogen in freeze-dried hydrolysed fraction T_2

Fraction T_2 from Sephadex G-75 column was freeze-dried and rechromatographed. After hydrolysis and high-voltage paper electrophoresis the basic, neutral and acidic amino acids were determined. α-Amino nitrogen was calculated from the amount of respective amino acids. Cystine was determined as cysteic acid in effluents from Amberlite IR-120 (form H^+) by the colorimetric method of Yemm & Cocking (1955). Amide nitrogen was determined by the method of Raacke (1957).

Form of nitrogen	% of preparation weight	% of total α-amino N
Total α-amino	11.2	100
α-Amino of basic amino acids	2.8	25
α-Amino of neutral amino acids	6.2	55
α-Amino of acidic amino acids	2.2	20
α-Amino of 1/2 cystine	0.17	1.5
Amide	1.0	9

Table 3 presents characteristics of nitrogen forms in the freeze-dried fraction T_2. The preparation does not contain great amounts of non-amino acid compounds, since the total α-amino nitrogen represents as much as 11.2% of its dry weight. The content of basic and dicarboxylic amino acids was 25 and 20 moles per 100 moles, respectively. From the quantity of the amide nitrogen it follows that about 45% of dicarboxylic acids is in the amide form. Thus the ratio of basic to acidic amino acids (amides deducted) amounts to 2.3, indicating the basic character of the proteins of the fraction T_2, which is compatible with the results of chromatography on CM-cellulose.

The proteins of the fraction T_2 are very well soluble in water at pH below 4 and cannot be thermally coagulated in this solution. This is most probably due

to their low molecular weight and a low content of sulphur amino acids. The increase of pH value over 4 causes their partial precipitation in the cold. Ethanol added in the cold to a final concentration of 80% precipitates about 27% of the proteins from a 2.5% aqueous solution and as much as 76% is precipitated with 5% trichloroacetic acid.

The results presented above indicate that a considerable amount of extranuclear basic protein does occur in porcine renal cortex. It seems that they are specific for this tissue, since no similar proteins were found in bovine heart muscle, when the same method of isolation was used. This may indicate that these proteins are taking part in some processes characteristic for renal cortex.

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POZAJĄDROWE ZASADOWE BIAŁKA KORY NEREK WIEPRZA

Streszczenie

1. Metodą sączenia molekularnego wydzielono z wyciągu kwasem mrówkowym z kory nerek wieprza frakcję zasadowych białek pozajądrowych.

2. Badane białka różnią się od histonów kory nerek wieprza, na co wskazują wyniki chromatografii na kolumnach z żelu Sephadex G-75 i z karboksymetylocelulozy.

3. Oznaczono azot α-aminowy aminokwasów zasadowych, obojętnych i kwaśnych.

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CARBOHYDRATES IN THE WAX MOTH DURING DEVELOPMENT

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1. The distribution of glycogen, trehalose and some other carbohydrates in tissues of fully grown wax moth larvae was estimated. The main carbohydrate in the wax moth, trehalose, is present in 80% in the haemolymph. The amount of glycogen is lower than in most other insects. 2. The content of glucose is very low, less than 0.1 mg./g. of fresh tissue. Much higher but unreliable values were obtained when deproteinization was carried out not rapidly enough to inhibit the enzymic hydrolysis of trehalose. 3. During the period of spinning of cocoons and during pupation an increase in the amount of glycogen and simultaneous disappearance of trehalose occur. The possibility of conversion of trehalose into glycogen is discussed.

Glycogen and trehalose are the main carbohydrates in most insects (cf. Chefurka, 1965). Glycogen is accumulated mainly in the fat body, while trehalose is present mostly in the haemolymph. Small amounts of other carbohydrates have also been found in insect tissues, namely glucose, some aminosugars (Wyatt, 1961), fructose (Levenbook, 1950), cellobiose, maltose (Hansen, 1964).

Wax moth, *Galleria mellonella*, is known to be rich in lipids, but it was assumed by some earlier authors that glycogen is almost completely absent in this insect at least at certain developmental stages (Taylor & Steinbach, 1931). This view persisted for 20 years (Rockstein, 1950). However, the presence of glycogen in this insect was demonstrated in this laboratory (Niemierko & Cepelewicz, 1950; Załuska & Niemierko, 1961; Niemierko & Niemierko, 1964); moreover, Wyatt & Kalf (1957) found a high concentration of trehalose (1.7%) in the haemolymph.

The aim of the present work was to examine more closely the distribution of particular carbohydrates in various organs of the wax moth and to study the changes in the amount of carbohydrates during development. Special attention was paid to determination of glucose because it seemed that the discrepancies between the data reported in the literature, even for the same insect species (Ralph & McCarthy, 1964), could depend, at least in part, on specific difficulties in analysing this compound in the insect body.

MATERIAL

The wax moth was bred on honeycomb as described by Niemierko & Cepelewicz (1950). The experiments were performed on larvae from the last instar just after ecdysis, weighing about 50 mg. (later on called small larvae); on fully grown larvae weighing 150 - 200 mg.; on spinning larvae; on one-, two-, and three-day-old pupae; and on adults, both male and female. The whole body of the insect and the following organs of fully grown larvae were analysed: haemolymph, fat body, gut with its content, and the remaining organs, i.e. mainly integument and muscles taken together. The organs were isolated as described earlier (Lenartowicz, Rudzisz & Niemierko, 1964). Ten to twenty specimens were taken for each analysis made in duplicate.

METHODS

The whole bodies of larvae and pupae were homogenized in 10% trichloroacetic acid (TCA), about 10 ml. of the acid per 1 g., in glass at 0°. The homogenate was heated for 3 min. at 100° to extract the whole glycogen present in the tissues. In the supernatant, the total amount of carbohydrates was determined by the anthrone method according to Scott & Melvine (1953). Glycogen was estimated by the anthrone method after being precipitated from the supernatant with 2 vol. of ethanol at pH 7.0 and dissolved in 1% TCA. Trehalose was determined according to Wyatt & Kalf (1957).

For analyses of the particular organs of the larvae and of the whole body of adults a slightly modified procedure was used. The material was homogenized in 70% ethanol, then heated for 15 min. at 60° and centrifuged. Glycogen present in the sediment was dissolved in water and determined after Good, Kramer & Somogyi (1953), while trehalose present in the supernatant was separated from other sugars by ion exchange chromatography on Dowex 1 (borate form) according to Khym & Zill (1952) and determined by the anthrone method.

Aminosugars, fructose and pentoses were analysed in the supernatant of the TCA extract prepared at 0°. Acetyloglucosamine and glucosamine were determined according to Elson & Morgan (1933). The amount of acetyloglucosamine was calculated from the difference between the total amount of glucosamine (found after hydrolysis in N-HCl during 1 hr.) and the amount of free glucosamine found directly in the chilled extract.

Fructose was estimated by resorcinol Roe reaction according to Niemierko & Janasik (1952). Pentoses were determined by the method of Mejbaum (1939) in the TCA extract after removal of nucleotides and other phosphorus compounds by Dowex 1 (formate form).

Glucose was determined after homogenization of the material with following protein precipitants: (1), with 10% TCA at 0°; (2), with $Zn(OH)_2$ (equal volumes of 5% $ZnSO_4$ and 1% NaOH) at 0° or at room temp.; (3), with 70% ethanol at room temp.

The estimation of glucose was performed in the supernatant either with hexokinase and glucose-6-phosphate dehydrogenase, or with glucose oxidase. In the assay of glucose by the glucose oxidase method, difficulties were encountered because the commercial enzyme preparations (Boeringer, Mannheim, Germany; Worthington Corp., Freehold, U.S.A.) were sometimes contaminated with trehalase. This does nor interfere with determinations in a material free from trehalose, but in insects, which are known to contain trehalose, the assay is unreliable giving too high values for glucose. It was necessary therefore to separate glucose from trehalose before glucose determination. The procedure was as follows: after deproteinization with TCA, the supernatant was shaken with ethyl ether to remove TCA and then treated with Dowex 50 (H^+). Samples of the purified extract were concentrated on a water bath to a small volume (50 - 100 µl.), applied on Whatman no. 1 paper and ascending chromatograms in ethylacetate - acetic acid - water (3:3:1, by vol.) were developed for 16 hr. at room temperature. The sugars were located with $AgNO_3$ according to Trevelyan, Procter & Harrison (1950); the R_F values of trehalose and glucose were found to be 0.15 and 0.33, respectively. The areas of paper corresponding to the spots of glucose were cut out and incubated directly with the glucose oxidase solution and the amount of glucose was determined in the incubation mixture according to Hugett & Nixon (1957).

The estimation of glucose by means of hexokinase and glucose-6-phosphate dehydrogenase ("true glucose test", Calbiochem, Lucerne, Switzerland) was performed in the supernatant after deproteinization with TCA and removal of the acid with ether, or after deproteinization with $Zn(OH)_2$. The procedure was adapted for the very small quantities of glucose: the content of the capsule with the mixture of enzymes was dissolved in 2.4 ml. of water and 0.4 ml. of the solution was added to 0.1 ml. of the investigated supernatant (10 - 20 mg. of fresh weight). The measurements of extinction were performed in microcells (0.5 ml., 10 mm. light-path) in Unicam SP 500 spectrophotometer at 340 mµ. It was proved that the various substances present in the analysed material did not interfere with the enzymic reaction and the recovery of added glucose was quantitative.

RESULTS

The amount of various carbohydrates in the whole body of larvae

The fully grown larvae contain about 1% of carbohydrates (Table 1). This is distinctly less than is generally found in other insects, and may be related to the exceptionally low amount of glycogen (about 2 mg./g.) which forms only 18% of the total quantity of carbohydrates, while trehalose forms 65% (about 6 mg./g.). The remaining carbohydrates comprise small quantities of fructose, pentoses, glucosamine and acetylglucosamine and a still smaller quantity of glucose (see below).

Determination of glucose required special attention. Table 2 shows clearly that the amount of this sugar found in *Galleria mellonella* depended greatly on the pro-

Table 1

Carbohydrates in fully grown larvae of Galleria mellonella

The amount of glycogen was calculated as glucose. Mean values ± S. D. are given.

Carbohydrate	No. of expts.	Content (mg./g. of fresh wt.)	Percentage of total carbohydrates
Total amount	9	9.80 ± 0.65	100
Trehalose	9	6.33 ± 0.49	65
Glycogen	9	1.80 ± 0.26	18
Acetyloglucosamine	4	0.47 ± 0.05	5
Glucosamine	4	0.32 ± 0.05	3
Fructose	3	0.16 ± 0.02	2
Pentoses	3	0.22 ± 0.13	2

Table 2

The influence of deproteinization on the amount of estimated glucose in fully grown larvae of Galleria mellonella

Mean values ± S. D. are given.

Procedure of deproteinization	Method of determination	No. of expts.	Glucose (mg./g. of fresh wt.)
5% $ZnSO_4$ and 1% NaOH at room temp. and at 0°	Reaction with hexokinase and glucose-6-phosphate dehydrogenase	2	0.77; 0.81
70% ethanol at room temp.	Anthrone reaction after separation by column chromatography	9	1.08 ± 0.10
10% trichloroacetic acid at 0°	Reaction with hexokinase and glucose-6-phosphate dehydrogenase	5	0.05 ± 0.007
10% trichloroacetic acid at 0°	Reaction with glucose oxidase after separation by paper chromatography	8	0.10 ± 0.012
5% $ZnSO_4$ and 1% NaOH at room temp.	Data of Niemierko & Janasik (1952)	18	0.99 ± 0.10

cedure used for deproteinization. Irrespective of the method of determination the content of glucose after deproteinization by TCA appeared to be extremely low (0.1 mg./g. or even less) but it was about 10 times higher after deproteinization either by $Zn(OH)_2$ (even at 0°) or by 70% ethanol. It could be suspected that during some of the deproteinization procedures the activity of trehalase was inhibited not rapidly enough and as result a part of trehalose was split enzymically. This view

was supported by the fact that when trehalose was added during deproteinization of the wax moth larvae by $Zn(OH)_2$ a still higher content of glucose was found in the supernatant fluid. A minute increase of glucose was found, in analogous experiment, also after deproteinization by TCA at 0°. It cannot be excluded, therefore, that even this very small amount of glucose found in chilled TCA extracts of *Galleria mellonella* may partly be due to the action of trehalase during homogenization of the tissue.

Distribution of trehalose and glycogen in the tissues of wax moth larvae

The concentration of the two main carbohydrates, in different organs of larvae is presented in Table 3. As in other insects, the highest concentration of trehalose occurs in the haemolymph (1.5%) and that of glycogen in the fat body, but it accounts only to 0.5%, much less than in most other insects. It is noteworthy that in all tissues the concentration of glucose is extremely small; in the haemolymph and in the fat body it is less than 0.01% and in other organs only traces of glucose could be detected.

Table 3

The content of carbohydrates in different tissues of fully grown larvae of Galleria mellonella

Mean values ± S. D. are given; in parentheses the number of experiments.

Organ	Trehalose (mg./g. of fresh wt.)	Glycogen (mg./g. of fresh wt.)	Glucose (mg./g. of fresh wt.)
Haemolymph	15.1 ± 0.33 (9)	0.36 ± 0.09 (9)	0.08 ± 0.005 (4)
Fat body	2.36 ± 0.30 (9)	5.38 ± 0.60 (9)	0.09 ± 0.005 (3)
Intestinal tract	1.32 ± 0.39 (9)	0.56 ± 0.06 (9)	0.008*
Remaining tissues	1.50 ± 0.24 (9)	1.05 ± 0.18 (9)	

* Calculated from the amount of glucose found in the whole body and that in the haemolymph and the fat body.

Table 4

Distribution of trehalose and glycogen in the organs of larvae as percentage of their total amount in the body

Material	Percentage of the body weight*	Trehalose (%)	Glycogen (%)
The total body	100	100	100
Haemolymph	28.3	77.4	5.6
Fat body	21.5	9.2	68.3
Intestinal tract	10.0	2.4	3.0
Remaining tissues	40.2	11.0	23.1

* According to Zielińska & Wroniszewska (1957).

From the total amount of trehalose found in the larval body, about 80% is present in the haemolymph and about 70% of glycogen is found in the fat body (Table 4).

Changes in the amount of carbohydrates during development of the wax moth

During the last instar the total amount of carbohydrates in the larval body decreases from 1.6 to 1.0%, calculated per fresh weight. From previous studies from this laboratory (Niemierko & Cepelewicz, 1950) it is known that in this period the content of water diminishes, from 70% to about 60%, and simultaneously the amount of lipids highly increases, from 10% to about 25%.

Table 5

Trehalose and glycogen at different developmental stages of Galleria mellonella

Mean values ± S. D. are given.

Stage	No. of expts.	Trehalose (mg./g. of fresh wt.)	Glycogen (mg./g. of fresh wt.)
Larvae			
Small	4	10.80 ± 1.50	1.63 ± 0.18
Fully grown	9	6.33 ± 0.49	1.80 ± 0.26
Pupae, 2-day-old			
Whole body	3	3.11 ± 0.51	7.80 ± 0.98
Haemolymph	5	7.15 ± 0.51	0.33 ± 0.07
Adults			
Females	5	2.99 ± 0.41	3.27 ± 0.07
Males	5	1.98 ± 0.12	0.02 ± 0.01

In Table 5 changes in the quantity of trehalose and glycogen during development of the wax moth are shown. The percentage of trehalose during the last instar significantly decreases, whereas that of glycogen remains approximately on the same level. In two-day-old pupae a further decrease of the amount of trehalose occurs and in contrast the amount of glycogen rises. In adult females the concentration of trehalose is as high as in pupae and similar to that of glycogen. In males, however, only traces of glycogen are present and the quantity of trehalose is also lower than that found in females. The differences in the amount of carbohydrates in males and females resemble those found in *Bombyx mori* (Załuska, unpublished results); these differences depend chiefly on accumulation of glycogen in the eggs. Simultaneously, adult males of the wax moth contain distinctly more lipids than females, namely about 11 and 8%, respectively (S. Niemierko, unpublished results).

Figure 1 shows the differences in the behaviour of trehalose and glycogen during spinning and pupation. During spinning of cocoons and during the first two days of metamorphosis the quantity of trehalose decreases sharply to about one third

of the amount found in the fully grown larvae. On the other hand the quantity of glycogen increases: during spinning the increase is small but it is very sharp during the first day of pupation; thus the amount of glycogen becomes greater than that of trehalose. On the third and fourth day of metamorphosis the quantity of glycogen slightly decreases but the level of trehalose still remains lower than that of glycogen. It should be pointed out that the quantities of carbohydrates were calculated in relation to the weight of the fully grown larvae when they stopped feeding; consequently, the data presented in Fig. 1 show the absolute changes

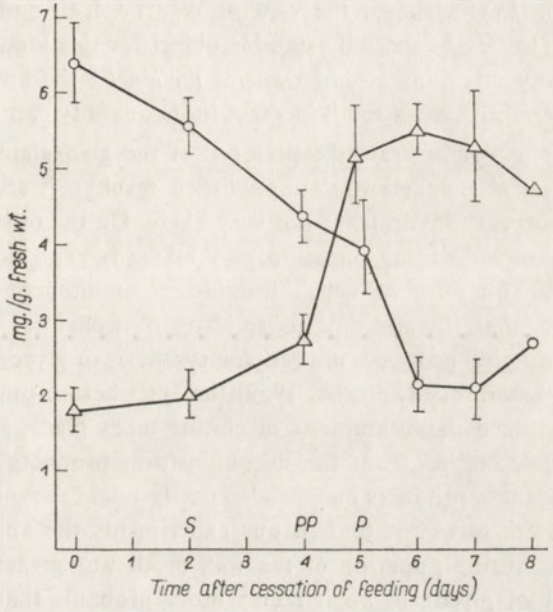

Fig. 1. The changes in the content of (○), trehalose; and (△), glycogen during metamorphosis of the wax moth calculated as mg./g. of fresh weight of the larvae just before the cessation of feeding; *S*, larvae spinning the cocoons; *PP*, prepupae; *P*, pupae.

of the amount of glycogen and trehalose. It is possible to calculate from these results that during the investigated period of metamorphosis the sum of trehalose and of glycogen decreases. But it may be supposed that in the wax moth, similarly as in many other insects, the total quantity of carbohydrates utilized during metamorphosis is much smaller than that of lipids.

DISCUSSION

Changes in the content of trehalose and glycogen in the insect body during pupation have been observed by many workers. A decrease in the amount of glycogen with a simultaneous increase in trehalose was found in the fat body and haemolymph of *Bombyx mori* (Duchateau-Bosson, Jeuniaux & Florkin, 1963). Similar changes were observed in the whole body of *Antherea pernyi* by Egorova (1965). On the

other hand, it has been known for a long time that after cessation of feeding, just before pupation, in larvae of some insects the amount of glycogen increases (Needham, 1929; Załuska, 1959; Karlson & Sekeris, 1964). It was assumed by some authors that a conversion of lipids into carbohydrates takes place, and this view, although poorly substantiated, is still rather popular. On the other hand, Wyatt & Kalf (1957) suggested that the increase in glycogen found at the beginning of metamorphosis may be due to splitting of trehalose. The possibility of formation of glycogen from trehalose, after injection of glucose into the body of *Antherea pernyi*, was also envisaged by Egorova (1965). It seems that the results presented in this paper give additional evidence for the view of Wyatt & Kalf and of Egorova. The wax moth appears to be an especially suitable object for this kind of investigations. In contrast to many other insects, in *Galleria mellonella* the amount of glycogen is very low and therefore even a small increase in its quantity can be easily detected.

As the attempt to demonstrate the presence of the glyoxalate cycle in the wax moth was unsuccessful (Lenartowicz, unpublished results), it seems that the conversion of lipids into carbohydrates is not very likely. On the other hand, the sharp rise in glycogen observed during pupation may be easily explained by its synthesis from trehalose, the quantity of which diminishes simultaneously. It cannot be excluded, however, that, in addition to splitting of trehalose, another catabolic process as well may give products utilized for synthesis of glycogen. It was shown previously in this laboratory (Załuska, 1959) that just before pupation of the silkworm the degradation of large amounts of chitine takes place, and the possibility of formation of new chitine from the decomposition products was discussed. It seems possible that these products may be also partly used for synthesis of glycogen. It should be stressed, however, that in our experiments the amount of trehalose which disappeared during pupation of the wax moth was greater than the simultaneous increment of glycogen. So it seems more probable that during pupation glycogen is formed from trehalose and not from chitine.

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WĘGLOWODANY U MOLA WOSKOWEGO PODCZAS ROZWOJU

Streszczenie

1. Gąsienice mola woskowego w ostatnich dniach żerowania zawierają 1% węglowodanów, w tym 65% trehalozy i 20% glikogenu; ponadto wykryto w nich małe ilości aminocukrów, pentoz i fruktozy. Zawartość glukozy nie przekracza 0.1 mg/g. Wyższe wartości otrzymuje się przy niewystarczająco szybkim zahamowaniu enzymatycznego rozkładu trehalozy.

2. Trehaloza występuje w 80% w hemolimfie, natomiast glikogen w 70% w ciele tłuszczowym.

3. W okresie snucia kokonu i przepoczwarczania ilość glikogenu wzrasta trzykrotnie, prawdopodobnie kosztem znikającej równocześnie trehalozy. W ciele dorosłych owadów ilość trehalozy jest niska; glikogen występuje tylko u samic.

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INFLUENCE OF DNA ON GROWTH AND VIABILITY OF TRANSFORMABLE GROUP H STREPTOCOCCI*

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1. The addition of transforming DNA to a highly competent culture of group H *Streptococci* leads to death of a part of the population, and probably an increase in generation time of the viable cells. This effect of DNA manifests itself at saturation concentrations for transformation (about 5 μg./ml.), and is accentuated by increasing DNA concentrations up to 50 μg./ml. The maximum observed lethal effect led to the death of *at least* 65% of the recipient cells during the early logarithmic growth phase. Several hours incubation of the recipient population with transforming DNA under optimal culture conditions leads to a ten- to fifteen-fold decrease in the number of colony-forming units, relative to that of a non-treated control. Deoxyribonuclease--treated DNA is completely without effect on cell growth. 2. The influence of DNA on cell growth is not due to its transforming activity, since the effect is accentuated by partially inactivated (by ultraviolet irradiation) DNA; and since both homologous and heterologous DNA are almost equally effective. However, the influence of DNA is exerted only on competent cells, i.e. when DNA uptake occurs. 3. The dependence of the lethal effect of DNA on the competence of recipient cells made it possible to establish that, in a highly competent *S. Challis* recipient population, at least 71% of the cells were capable of taking up exogenous DNA. 4. Related observations on the effect of DNA in other transforming systems, and on mammalian cells, have been culled from the literature, and the overall findings compared and discussed.

During the course of an investigation on the group H streptococcal transforming system, the principal objective of which was to obtain a high yield of transformants, it was observed that saturation (and higher) concentrations of transforming DNA affected the growth characteristics of the recipient strain. A more detailed study of this phenomenon appeared of interest since the yield of transformants attained with the above system was in excess of 50 - 60%, in terms of viable units (Piechowska & Shugar, 1967); consequently the ability of a large fraction of the cell popu-

* These results were presented in preliminary form at the Symposium on "*Isolierung und Struktur von DNS aus Mikroorganismen*", Jena, German Democratic Republic, July, 1966. We are indebted to Mme. Galina Zizina of the Institute of Physical Chemistry, Moscow, for assistance with part of this investigation.

lation to incorporate DNA made it possible to estimate the effects of exogenous DNA by a comparison of the survival and growth of the DNA-treated entire bacterial population with that of a non-treated control.

A number of investigators have previously observed that the uptake of DNA by competent bacterial cells may lead not only to the expression of new genetic traits, but also to some influence on the growth of some of the cells (Nester & Stocker, 1963; Gimlin, Farquharson & Leach, 1963; Lambina & Mansurova, 1964; Kammen, Beloff & Canellakis, 1966; Bodmer, 1966). During the preparation of this manuscript a rather interesting report appeared by Gimlin, Hardman, Kelley, Butler & Leach (1966) on the influence of DNA containing incorporated 5-bromouracil on the growth of transformable *Bacillus subtilis*. Related effects of DNA on animal cells have also been reported by Zahn & Tiesler (1963), Glick & Goldberg (1965), and Halpern, Halpern, Ciliv & Smith (1966).

MATERIALS AND METHODS

The *Streptococcus Challis*[1] group H transforming system, originally developed by Pakuła, Fluder, Hulanicka & Walczak (1958), is described in detail elsewhere (Pakuła, Piechowska, Bańkowska & Walczak, 1962). The recipient strain was sensitive to dihydrostreptomycin (25 μg./ml.), cathomycin (12.5 μg./ml.) and erythromycin (0.05 μg./ml.). We are indebted to Dr. H. Osowiecki for the *Streptococcus Wicky* group H strain, which was likewise sensitive to all of the above antibiotics.

The *Streptococcus Challis* group H donor strain was resistant to dihydrostreptomycin (2000 μg./ml.), cathomycin (30 μg./ml.) and erythromycin (4 μg./ml.). The three markers are non-linked.

The thymine-dependent *Escherichia coli* CR-34 strain was kindly made available by Dr. M. Meselson of Harvard University.

Transforming DNA from *Streptococcus Challis* was isolated both by the method of Marmur (1961) as well as by a modification of the phenol method based in part on the results of Saito & Miura (1963) and Berns & Thomas (1965). A detailed description of these preparations is presented elsewhere (Piechowska & Shugar, 1967).

DNA from *E. coli* CR-34T$^-$ was isolated essentially as described by Berns & Tomas (1965), supplemented by RNase digestion, additional phenol deproteinization, and final precipitation of DNA with ethanol. The DNA was then dissolved and dialysed against the required solvent to free it from phenol.

DNA from bacteriophage T2 was isolated by the phenol method (Mandell & Hershey, 1960).

Poly-d(AT), isolated from the crab *Cancer magister*, but contaminated with 10% of the main DNA fraction, was kindly supplied by Dr. H. Sierakowska and Dr. M. Laskowski Sr.

[1] For purposes of simplicity, the terms *S. Challis* and *S. Wicky* will be used to denote the Challis and Wicky strains, respectively, of group H haemolytic streptococci.

The DNA preparations all exhibited characteristic ratios of maximum to minimum absorption in the ultraviolet testifying to the presence of only traces of protein. Quantitative determinations by the method of Lowry (see Layne, 1957) showed that the protein content of the various samples ranged from 0.8 to 1.6% protein. DNA concentrations were determined spectrophotometrically, as well as by the method of Burton (1955); the difference between these two was regarded as a measure of the content of RNA which, in all instances, was below 5%. The biological activity of samples of transforming DNA was evaluated from the minimal concentration required to give a single transformant, and was 5×10^{-6} μg./ml. (with 1×10^6 cells/ml.) for DNA isolated by the method of Marmur (1961) and the modified phenol method.

E. coli sRNA was a gift of Dr. M. Bagdasarian.

DNase was a crystalline Worthington (U.S.A.) preparation and was employed at a concentration of 2 μg./ml.

Competent cultures of *Streptococcus* were prepared as described elsewhere, together with the procedure and conditions used for transformation (Piechowska & Shugar, 1967).

The growth medium for *S. Challis* and *S. Wicky* was identical to that employed for transformation and contained beef extract, neopeptone, yeast extract and swine serum; full details will be found elsewhere (Piechowska & Shugar, 1967). *E. coli* B was cultivated on the synthetic medium *C* of Roberts, Cowie, Abelson, Bolton & Britten (1955).

The number of viable cells was determined by plating on agar, using agar in broth supplemented with 3% lamb's blood for *Streptococci*, and agar in broth for *E. coli*. In addition optical densities of cultures were measured at 650 mμ. Counts were also made under the microscope of the number of cells per cluster, as well as the distribution of the number of cells in clusters on the basis of counts carried out on 500 clusters.

In all figures, colony counts are for 0.5 ml. culture samples.

RESULTS

Multiplication of S. Challis *transformants*. The starting point for our studies on the influence of DNA on growth of streptococci was our finding with regard to the multiplication of transformants, carried out as described by Hotchkiss (1957) and Ephrussi-Taylor (1959) for transformation of pneumococci and Voll & Goodgal (1961) for *Hemophilus influenzae*. To a thawed, low-competent culture of *S. Challis*, diluted with fresh medium, was added transforming DNA to a concentration of 1 μg./ml. After 10 min. contact at 37°, DNase was added and, at given time intervals, samples were withdrawn, diluted with agar and plated. Following 3 hr. incubation at 37° the agar plates were covered with a second layer of agar containing the necessary concentration of dihydrostreptomycin for selection of transformants (250 μg./ml. which, following diffusion through the first layer, gave a mean con-

centration of 125 μg./ml.). Simultaneously a series of controls was plated with a second antibiotics-free layer.

Growth of the entire recipient population commenced after a lag phase lasting about 30 min. During the logarithmic growth phase the generation time was about 36 min. The same samples showed no increase in the number of viable units of transformants for a period corresponding to three normal generations of the recipient population (Fig. 1), similar to that previously reported for *H. influenzae*

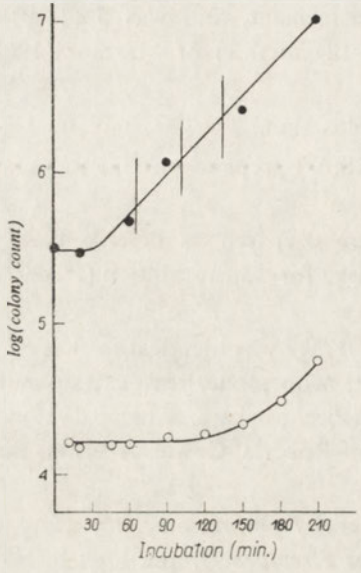

Fig. 1

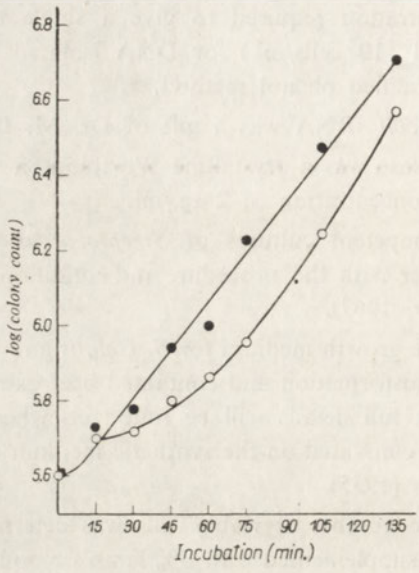

Fig. 2

Fig. 1. Growth curves for (●), *S. Challis* group H competent recipient population (the vertical lines represent growth corresponding to one generation), and (○), dihydrostreptomycin-resistant transformants from the same population.

Fig. 2. Influence of short treatment with transforming DNA on the growth of *S. Challis* recipient cells: (●), control population treated with DNase, and (○), culture exposed to transforming DNA, 10 μg./ml., at time 0, for 15 min., then DNase added to 2 μg./ml., and incubation continued.

(Alexander & Leidy, 1953) and *Diplococcus pneumoniae* (Hotchkiss, 1957; Ephrussi-Taylor, 1959). The question then arises as to whether the observed lag in growth of the number of transformants was due simply to transient inhibition of cell division as a result of DNA uptake. The foregoing results by themselves do not make it possible to answer this question. One of the difficulties associated with the interpretation of the curves in Fig. 1 is the tendency of *S. Challis* cells to form clusters (in the form of chains), so that one transformant cell could be present in a cluster consisting of two or more non-transformants (the number distribution of cells in *S. Challis* culture clusters is presented elsewhere, Piechowska & Shugar, 1967). In such instances the increase in the number of clusters containing transformants would lag behind the rate of increase of the overall number of clusters in the culture.

A lag in the doubling of the number of transformants could also be due to incorporation of the new marker in only one of the DNA strands of the recipient, so that a doubling of the number of transformed cells will occur only following the second replication of the chromosome (Fox & Allen, 1964; Ephrussi-Taylor, 1966). A third possibility which may be envisaged is the formation of impure lines of progeny in the transformants, resulting from cell division without transfer of the new genetic traits; such a phenomenon has been observed by Stocker (1963) in transformation of *Bacillus subtilis*.

In view of the foregoing, it became necessary to examine the influence of DNA on the growth of recipient cells by additional methods.

Influence of transforming DNA on highly-competent cultures of S. Challis *recipients*. Having obtained highly competent *S. Challis* cultures with a transformation yield of about 50% (Piechowska & Shugar, 1967), it was to be anticipated that an increase in generation time of the transformants would be reflected in the growth curve of the entire population, of which the transformants formed an important fraction. The growth of control and DNA-treated cultures were compared in the following manner.

To a thawed culture of recipients, diluted with fresh growth medium to a density of 8×10^5 cells/ml., was added 10 μg./ml. DNA (the plateau in the calibration curve begins at 2 to 5 μg./ml.). The culture was left for 15 min. at 37°, DNase added, and incubation continued at 37°. To the control suspension was added only DNase. Samples were then withdrawn from both cultures for plating on agar. The resulting growth curves, shown in Fig. 2, demonstrate a net decrease in the number of viable units in the DNA-treated culture. A difference in number of viable units is visible after 30 min. and this difference reaches a maximum after 75 min. incubation, at which time the number of viable units from the DNA-treated culture is only one-half that of the control. These results confirm the existence of some effect of transforming DNA on the growth of recipients, while the concave course of the growth curve of the DNA-treated culture suggests a transient inhibition of cell division in part of the cell population, followed by its subsequent participation in the normal cycle of cell division.

Death of recipient cells provoked by transforming DNA. An increase in DNA concentration to 50 μg./ml., as well as prolongation of the time of contact of DNA with the recipient cells by elimination of the addition of DNase, led to a gradual decrease in the number of viable units to a level of 46% in 45 min., following which, up to 90 min., the number of viable units remained stationary (Fig. 3). During the same period the non-treated control multiplied normally so that, after 90 min., it exhibited a ninefold higher colony count that the DNA-treated culture.

Effect of DNA concentration on growth of S. Challis *recipients*. An examination was made of the effect of various concentrations of transforming DNA on the increase in the number of viable units during 75 min. incubation. From the results, shown in Fig. 4, it is apparent that at 5 μg./ml. there is a clearly-defined effect of DNA on the growth characteristics, which increases progressively up to 50 μg./ml. While this experiment adequately illustrates the effect of increasing DNA con-

centrations, it differed in one important respect from other experiments in that, although there was no increase in the number of viable units during the 75 min. incubation, there was no decrease below the initial value. Since the competence of the recipient culture was of the same order as in other experiments (50% yield of transformants), the reason for the diminished effect observed here is not clear.

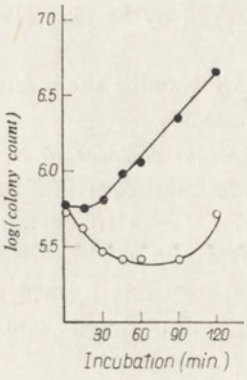

Fig. 3

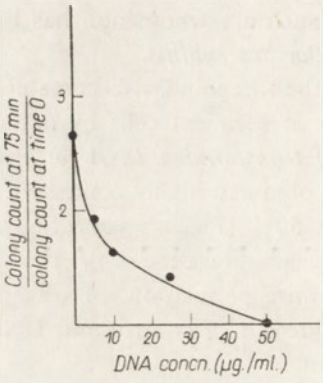

Fig. 4

Fig. 3. Influence of constant exposure to transforming DNA on the growth of *S. Challis* recipient cells: (●), control population, and (○), culture to which, at time 0, was added transforming DNA to a concentration of 50 μg./ml.

Fig. 4. Effect of different concentrations of transforming DNA on the growth of *S. Challis* recipient cells; DNA added at time 0.

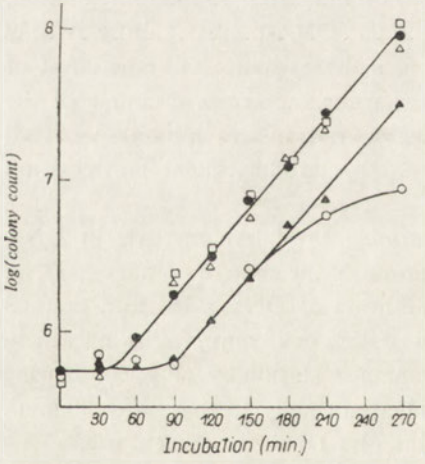

Fig. 5. Growth curves for DNA-treated *S. Challis* recipient cells and effect of DNase: (●), control culture; (○), culture to which at time 0 was added transforming DNA to concentration of 50 μg./ml.; (△), culture to which at time 0 was added DNase--hydrolysed transforming DNA to a concentration of 50 μg./ml.; (▲), culture to which at time 0 was added transforming DNA to a concentration of 50 μg./ml., followed by addition, after 60 min., of DNase to a concentration of 2 μg./ml.; (□), control, untreated, culture to which was added, after 60 min., DNase to a concentration of 2 μg./ml.

On the other hand, Fig. 4 shows that the effect of DNA on the growth characteristics is by no means maximal at 50 μg./ml.; but higher concentrations of transforming DNA were not employed in order to conserve material.

Effect of DNase. The observed influence of transforming DNA on the growth of *S. Challis* recipients is a property of the DNA itself, and is not due to some po-

tential impurity in the DNA preparation, since treatment with DNase completely abolished the effect (Fig. 5). It was also established that this effect is not due to clumping of the cells, or to the possible union of two cells by a single DNA molecule, since addition of DNase after 60 min. contact of the recipients with DNA did not eliminate the difference between the DNA-treated culture and the control (Fig. 5). Maintenance of the culture in continuous contact with DNA apparently leads to a "second wave" in the effect of DNA, due presumably to the appearance of a successive "competence wave" in the culture. In this experiment the number of viable units after 270 min. culture in the presence of DNA was one-tenth of that in the control; and in the sample where DNase was added after 60 min. contact with DNA, this figure was one-third. The differences in optical densities of the cultures were, however, considerably less, e.g. for the tenfold difference in colony counts, the difference in optical densities was only threefold. This difference can be due only to the presence of non-viable cells in the culture, since a microscopic count demonstrated no differences in the distribution of the number of cells per cluster between the two cultures, even after 270 min. incubation.

Relationship between competence and influence of DNA on bacterial growth. It may be inferred from the above that the effect of DNA on bacterial growth is related to the ability of the cells to take up DNA. This was in effect established by making use of the fact that *Streptococcus Wicky*, a non-transformable strain, can take up DNA and undergo transformation with *S. Challis* DNA only following

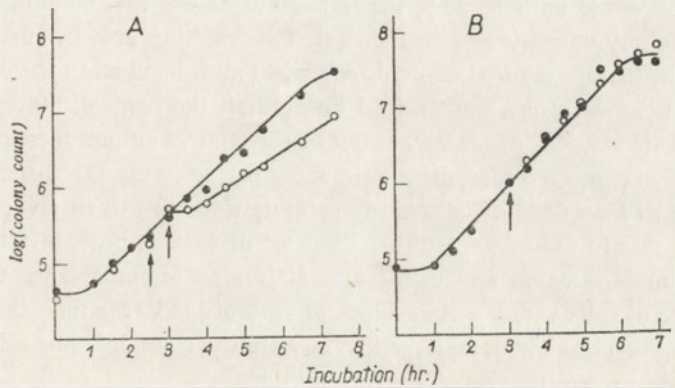

Fig. 6. Effect of transforming DNA on the growth of *S. Wicky*. *A*: (●), Control culture, to which after 2.5 hr. (indicated by arrow) was added "competence factor" from *S. Challis* supernatant; (○), culture to which, after 2.5 hr. (indicated by arrow), was added "competence factor" from *S. Challis* supernatant and, after 30 min. (indicated by 2nd arrow), transforming DNA to a concentration of 50 μg./ml. *B*: (●), Control culture; (○), control culture to which, after 3 hr., was added transforming DNA to a concentration of 50 μg./ml.

addition to the culture medium of a "competence factor" isolated from a culture of competent *S. Challis* (Pakuła & Walczak, 1963; Osowiecki & Łancow, 1965; Dobrzański & Osowiecki, 1966). Under these conditions *S. Challis* transforming DNA affects the growth of *S. Wicky*, whereas the same DNA is quite inert against a non-competent culture which is unable to take up DNA (Fig. 6A and 6B).

Influence of DNA on survival of transformants and entire population of S. Challis *recipients.* A competent *S. Challis* culture was incubated in transforming medium in the presence of 50 μg./ml. DNA. Colony counts demonstrated a decrease in

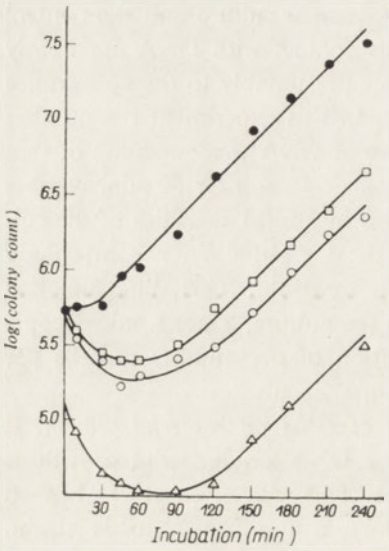

Fig. 7. Comparison of effect of transforming DNA on growth and viability of *S. Challis* recipient population and on the transformed fraction of this culture, and comparison of homologous and heterologous DNA. (●), Control culture; (○), culture to which at time 0 was added transforming DNA to a concentration of 50 μg./ml.; (△), dihydrostreptomycin-resistant transformants from culture to which at time 0 was added transforming DNA to a concentration of 50 μg./ml.; (□), culture to which at time 0 was added *E. coli* CR-34 DNA to a concentration of 50 μg./ml.

the number of viable units to 35% the initial during the first 60 min. (Fig. 7). At 90 min. the colony count began to increase, the resulting growth curve being parallel to that of the non-treated control. After 240 min. incubation the colony count of the DNA-treated culture was 14-fold lower than the control. Since, during the initial 60 min. incubation, the colony count of the control culture increased twofold, it may logically be inferred that, during the same period in the DNA-treated culture, not 65%, but *at least* 65% of the colony-forming units fail to survive; this follows from the fact that in the treated culture what we observe is probably the difference between cell multiplication and cell death. It is also of importance to note that the percentage of cells which undergo loss of viability is larger than the percentage of colony-forming units which undergo loss of viability, since only one cell in a cluster must be viable to produce a colony on plating.

Measurement of the number of viable units of dihydrostreptomycin-resistant transformants likewise demonstrated a loss in cell viability. Extrapolation to zero time indicated a loss in viable units of 73%. This may suggest that account must be taken of the lower probability of the appearance of clusters of transformed cells.

It follows from the foregoing that the number of transformants actually measured is less than the total number of cells which took up DNA. This may be seen as follows: the maximum number of colonies of dihydrostreptomycin-resistant transformants, determined by extrapolation to time 0, is 127×10^3. For *S. Challis* recipients the number of cathomycin-resistant transformants is always at least equal to that for dihydrostreptomycin-resistant transformants; hence the number of trans-

formants in a given experiment is at least 254×10^3 (the number of transformants to erythromycin-resistance is so low, about 1%, that it may be ignored). After 60 min. the number of transformants is reduced by 73%, i.e. by 186×10^3, which is only 35% of the initial, overall number of colony forming units in the recipient population (536×10^3). Since 65% of the total number of viable units undergo loss of viability, it may be concluded that the number of cells which lose their viability as a result of DNA uptake is larger than the number of killed transformants.

The number of cells which take up DNA in a recipient culture such as the above may be estimated in the following manner. During 60 min. incubation *at least* 65% of the cells lose their viability, due to their uptake of DNA. The surviving cells still contain some transformants, i.e. cells which took up DNA but did not lose their viability. The number of these latter, in terms of viable units, amounts to about 14% of the total number of viable units at zero time. The majority of clusters consist of three cells, so that, correcting for this, we find 5% transformed cells. The sum of (% cells killed) and (% cells transformed), i.e. 70%, is the number of cells in the recipient culture which took up DNA.

Influence of growth phase on action of DNA. In all of the foregoing experiments, DNA was added to a fresh *S. Challis* culture following thawing and fivefold dilution of the competent recipients with fresh medium. A different type of experiment was now carried out, in which transforming DNA was added both at the beginning and end of the logarithmic phase of the recipient culture; this resulted in a growth pattern corresponding to that for synchronous growth and an approximately fourfold increase in generation time (Fig. 8A). Further examination showed that the observed synchronization of culture growth was due to accentuation of the partial synchronization exhibited by the control culture (Fig. 8B) when growth measurements were performed at more frequent intervals (10 min.). It is, however, pertinent in relation to the foregoing, to note that in young cultures a microscopic count de-

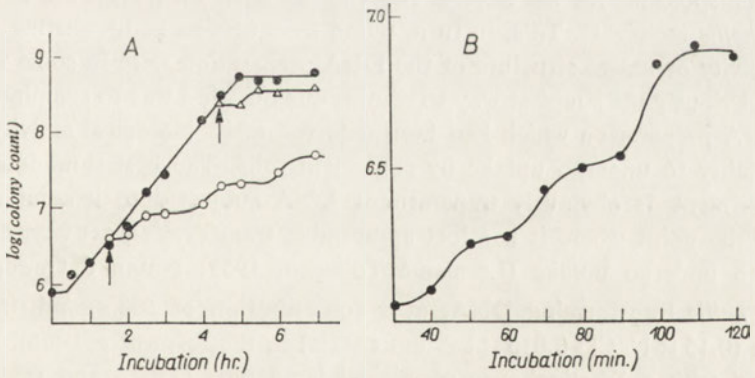

Fig. 8. Growth of *S. Challis* recipients in presence of transforming DNA (50 μg./ml.) added during the logarithmic phase. *A*: (●), Control culture; (○), culture to which DNA was added after 1.5 hr. (indicated by arrow); (△), culture to which DNA was added after 4.5 hr. (indicated by arrow). *B*: (●), Control culture; partial synchronization of growth, as revealed by measurements at 10 min. intervals.

monstrated that there is no difference in the number of cells per cluster as between a control population and one that has been subjected to treatment with DNA (see above). By contrast, at the end of the logarithmic phase the DNA-treated population exhibits about 50% more cells per cluster than the control population.

Influence of homologous and heterologous DNA on growth and viability. It was obviously of importance to determine whether the difference between the DNA's of donor and recipient cells could account for the adverse effect of donor DNA on recipient cells. An experiment analogous to the above was therefore carried out, in which donor *S. Challis* cells were treated with donor DNA. The growth curve for the latter was, in fact, similarly affected by the added DNA; after 9 hr. incubation the colony count in the treated culture being 17-fold less than in the control At the same time there was a fourfold difference in the optical densities of the two cultures, testifying to the presence in the treated culture of non-viable cells.

Attention was next directed to an examination of the effects of DNA differing markedly from that of the recipient cells. Analogous experiments were therefore performed with DNA preparations from *E. coli* CR-34T$^-$ and bacteriophage T2, and with the poly-d(AT) fraction of DNA from *Cancer magister*. All the foregoing preparations, at a concentration of 50 μg./ml., affected the growth of *S. Challis* recipient cells, the degree of activity of all three preparations being practically identical to that of *S. Challis* transforming DNA. The results for *E. coli* DNA are illustrated in Fig. 7. The addition of such *E. coli* DNA to a culture of *E. coli* B was without any visible effect on cell growth; this is in agreement with the finding of Lerman & Tolmach (1957) that *E. coli* B cells do not take up DNA. It was also found that *E. coli* B s-RNA had no effect on the growth of *S. Challis* recipient cells.

Role of biological activity of DNA in relation to influence of latter on growth of S. Challis. The results of the experiments described in the previous section demonstrate unequivocally that it is not the transforming activity of the DNA which is responsible for the adverse effect of the latter on the growth and viability of *S. Challis* recipients. This, in turn, posed the question as to whether the biological activity or native structure of the DNA preparation were in some way implicated. Obviously the simplest way to resolve this question was to examine the effect of a DNA preparation which had been deprived of its biological activity, but not of its ability to undergo uptake by competent cells. The best candidate for such an experiment is obviously transforming DNA subjected to irradiation by UV light to the point where it has lost appreciable transforming activity, but not its ability to undergo uptake (Lerman & Tolmach, 1959; Notani & Goodgal, 1965).

S. Challis transforming DNA, at a concentration of 500 μg./ml. in 0.5 SSC solution (0.15 M-NaCl - 0.015 M-sodium citrate) in the form of a 1-mm. layer, was irradiated with a Westinghouse germicidal lamp type G30T8 and samples withdrawn at various time intervals, and their transforming activity determined. The variously inactivated samples were also added to competent cultures of *S. Challis* recipient cells to a concentration of 50 μg./ml at time 0. The results, Fig. 9, show that progressive destruction of transforming activity to 10% of the initial

leads to an increase of 20 - 50% in the adverse effect of DNA on cell viability. With higher doses of UV radiation the effect of DNA on the cultures decreased.

Effect of DNA concentration on calibration curve for S. Challis *transformants.* One might expect the observed killing effect of DNA to be reflected in the calibration curve of frequency of transformants *versus* DNA concentration, i.e. the usual experimentally observed plateau (at so-called saturation concentrations of

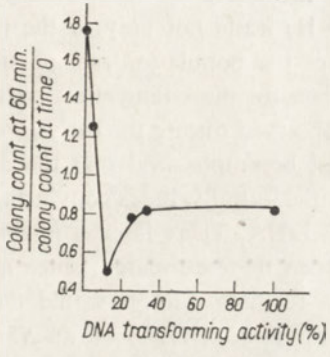

Fig. 9

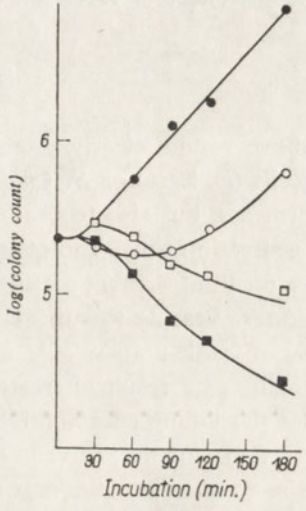

Fig. 10

Fig. 9. Influence of degree of ultraviolet inactivation of transforming DNA (50 μg./ml.) on growth and viability of *S. Challis* recipient cells (see text for details).

Fig. 10. Effect of transforming DNA (50 μg./ml.) on growth and viability of *S. Challis* recipients in presence of chloramphenicol (25 μg./ml.). (●), Control culture; (○), culture to which DNA was added at time 0; (□), culture to which chloramphenicol was added at time 0; (■), culture to which at time 0 were added both DNA and chloramphenicol.

DNA) would be the resultant of the number of cells transformed and the number which have lost their viability. If this is so, then, at sufficiently high concentrations, there should be a decrease in the yield of transformants, relative to the plateau value. In an earlier study (Pakuła *et al.*, 1962), no such effect is visible, the plateau level for transformation frequency remaining essentially constant up to a DNA concentration of 100 μg./ml. However, it should be recalled that account must be taken of both the culture density and the ratio of DNA concentration to population density. When a calibration curve was prepared under the conditions employed in this study (i.e. an eightfold lower culture density, 2×10^6 viable units/ml., with a consequent ratio of DNA/cell eight times higher), a pronounced decrease in frequency of transformants was, in fact, observed: at a DNA concentration of 50 μg./ml., the number of transformants decreased to 68% of the plateau level, and at 80 μg./ml. to 54%. This is in reasonable agreement with expectations, since the time of contact between the recipient cells and DNA in this instance was 15 min.

Effect of chloramphenicol on the growth of DNA-treated recipient cells. One series of experiments was carried out with a view to determining whether the lethal effect of DNA is in some way linked to protein synthesis. The growth of control and DNA-treated recipient cultures was examined in the presence of 7.5 μg. and 25 μg./ml. chloramphenicol, a known inhibitor of protein synthesis. The results, illustrated in Fig. 10, demonstrate unequivocally that protein synthesis is not involved in the effect of DNA on recipient cells.

DISCUSSION

The above results clearly establish that the effect of transforming DNA on recipient cells (at least for *S. Challis* group H) leads not only to the production of transformants, but also to death of part of the population and possibly an increase in generation time. The quantitative data for the killing effect possess added weight as a result of the fact that they were observed during the early growth phase of the cultures. Bearing this in mind, it must be emphasized that the decrease in the number of viable units to 50% or 35% of the initial value is convincing evidence of cell lethality as a result of treatment with DNA. There is even justification for stating that the killing effect of DNA has been underestimated, since no account was taken of the number of cells per colony-forming unit. It would therefore be more precise to state that the maximum lethal effect of DNA on an *S. Challis* recipient culture involves the death of *at least* 65% of the cells.

It is more difficult to evaluate the effect of DNA under conditions where the number of viable units does not fall below the initial value, or when DNA was added during the logarithmic growth phase. In the latter instance the growth curves obtained by plating a liquid culture suspension on agar do not suffice to determine whether a certain fraction of cells is killed in successive generations or whether the entire population exhibits an increased generation time. Optical density measurements and counts of viable units facilitate an evaluation of the results, but more accurate results will have to await the application of supplementary counting techniques to establish the proportion of viable cells in a culture.

Nonetheless it may be inferred from the present findings that the character of the growth curves of a highly competent *S. Challis* culture, following 15 min. contact with DNA, suggests the existence of a transitory inhibition of cell division of a portion of the cell population. The regularity observed in the synchronized curve of a DNA-treated culture in the early logarithmic growth phase is most reasonably interpreted as a fourfold increase in generation time.

The effect of DNA on growth and viability is clearly dependent on DNA uptake, and this is what made it possible to establish that *at least* 71% of the cells in a highly competent *S. Challis* culture take up DNA. It follows that the 50% yield of transformants with the two non-linked markers, dihydrostreptomycin and cathomycin obviously is by no means an upper limit. This, in turn, points to the advisability of attempts to estimate by autoradiographic methods the number of cells

which take up DNA, the more so in that the experiments of Perry & Slade (1962) showed that some non-transforming strains of *Streptococci* take up as much DNA as transformant strains. Furthermore it has recently been shown by Young (1967) that, in a transforming strain of *B. subtilis* with a yield of transformants for several traits of 1 - 4%, as much as 20% of the cells exhibited uptake of DNA, as estimated by autoradiography.

It will obviously be necessary to determine how much DNA is taken up by *S. Challis* cells as a function of the exogenous DNA concentration, since the present results do not permit of a conclusive decision as to whether the lethal effect is due to the uptake of a simple excess of DNA or of a requisite amount of a given fraction. It should be underlined here that relatively little is as yet known about the amount of DNA taken up by competent cells, particularly at higher DNA concentrations. Furthermore the practice of calculating the amount of DNA uptake by competent cells, on the assumption that all the DNA has been taken up by transformants, is a procedure which, while widely applied, is of doubtful validity.

On the other hand, the fact that heterologous DNA, including such an unusual sample as poly-d(AT), has almost the same effect on growth and viability as homologous DNA, argues against the involvement of a particular fraction. It also suggests that integration of the DNA into the bacterial genome is not the decisive factor in the lethal effect of DNA. This is in agreement with the findings of Pène & Romig (1964) who demonstrated the absence of any incorporation of heterologous DNA in the genome of *B. subtilis* recipients; and of Notani & Goodgal (1965) showing diminished incorporation of UV irradiated DNA in *H. influenzae*.

As already mentioned in the Introduction, the results reported here find their counterpart in several other publications on bacterial transformation. If we assume that similar processes accompany the transmission of new genetic traits by recipient cells, the interpretation of these processes is perhaps facilitated by our findings.

One example is the lag in growth of transformants with the following systems: *D. pneumoniae* (Hotchkiss, 1957; Ephrussi-Taylor, 1959), *H. influenzae* (Alexander & Leidy, 1953), *B. subtilis* (Nester & Stocker, 1963), and *S. Challis*. To date there has been no unequivocal interpretation of this phenomenon, partly because of reservations of various authors with regard to experimental techniques (see Results, above). However, the effect of DNA uptake on the growth curves of highly competent *S. Challis* recipient cells, as reported above, is highly suggestive of a transient inhibition of transformant cell division in all of the above-mentioned systems.

Another example is the metabolic latency of *B. subtilis* transformants described by Nester & Stocker (1963); we would suggest, in the light of our results, that this is due rather to DNA uptake than to a difference in properties of the competent cell fraction. The foregoing authors also observed a decrease in the number of transformants during the initial period following DNA uptake, a phenomenon subsequently studied in more detail by Kammen *et al.* (1966), and which our own findings would suggest as being due to cell death.

Our findings appear at first sight to parallel those of Gimlin *et al.* (1966) with the *B. subtilis* system. These authors found that high concentrations of bromo-

uracil-containing homologous DNA inhibited the growth of competent cultures, and noted as well that higher concentrations of normal DNA exhibited a similar, but feebler, effect. While there is most likely some similarity in the effects of DNA as between their system and ours, this will require further study. The concentrations of DNA required in the *B. subtilis* system are much higher than those used here; but this may be related to the fact that the transformation frequency (and hence percentage of competent cells) is much higher in our system. More significant is the fact that heterologous DNA was found to be practically inert in the *B. subtilis* system.

As regards the mechanism of the lethal effect of DNA on recipient cells, the experimental data are as yet insufficient to allow for more than speculation. It seems clear that protein synthesis is not involved, but it remains to be established whether RNA and/or DNA synthesis play a role here. It appears most likely that cellular DNA synthesis is inhibited by incorporated exogenous DNA, e.g. by a shift in the equilibrium point for cellular DNA synthesis, or by inhibition of DNA polymerase by the 3′-terminal oligonucleotides resulting from enzymic degradation of incorporated DNA. Such inhibition of DNA polymerase *in vitro* has been demonstrated by Smellie (1963).

The effect of exogenous DNA on growth and viability of cells may find application as a means of placing in evidence DNA uptake by mammalian cells. The difficulties which still exist in attempts to transform animal cells are enhanced by the absence of convincing biological evidence for DNA uptake by such cells. It is consequently necessary to recall, as mentioned above, that there is some evidence for an effect of exogenous DNA on growth of animal cells (Zahn & Tiesler, 1963; Glick & Goldberg, 1965; Halpern *et al.*, 1966) similar to that described here for the *S. Challis* transforming system.

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WPŁYW DNA NA WZROST I ŻYWOTNOŚĆ ZDOLNYCH DO TRANSFORMACJI PACIORKOWCÓW Z GRUPY H

Streszczenie

1. Transformujący DNA, dodany do wysoko kompetentnej hodowli biorcy *Streptococcus* z grupy H powoduje śmierć części populacji i prawdopodobnie przedłużenie czasu generacji przeżywających komórek. Działanie to występuje przy nasycającym dla transformacji stężeniu DNA, to jest około 5 μg/ml, i wzmaga się wraz ze wzrostem stężenia DNA do 50 μg/ml. Maksymalne letalne działanie DNA prowadzi do obumarcia conajmniej 65% komórek biorcy w czasie wczesnej logarytmicznej fazy wzrostu. Kilkugodzinna inkubacja szczepu biorcy z transformującym DNA, w warunkach optymalnych dla wzrostu hodowli, prowadzi do otrzymania kilkunastokrotnie mniejszej ilości jednostek tworzących kolonie niż w kontroli. DNA traktowany DNazą zupełnie nie wpływa na wzrost bakterii.

2. Działanie DNA na wzrost komórek nie wynika z jego aktywności transformującej, ponieważ wzmaga się w przypadku DNA częściowo inaktywowanego promieniami UV, a preparaty DNA heterologicznego i homologicznego mają prawie taki sam wpływ. Jednakże DNA działa tylko na wzrost komórek kompetentnych, t.j. takich, które mogą pobrać DNA.

3. Zależność letalnego działania DNA od kompetencji komórek biorcy umożliwiła obliczenie, że w przypadku wysoko kompetentnej hodowli biorcy szczepu *Challis* conajmniej 71% komórek posiada zdolność pobrania egzogennego DNA.

4. Zebrano i przedyskutowano doniesienia innych autorów o działaniu DNA na układy transformujące i komórki zwierzęce.

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REACTION OF AMINES WITH DIHYDROCYTOSINE ANALOGUES AND FORMATION OF AMINOACID AND PEPTIDYL DERIVATIVES OF DIHYDROPYRIMIDINES

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1. In the presence of an amino acid, in neutral or alkaline medium, the amino group of dihydrocytosine undergoes rapid exchange with the amino group of the intact amino acid, leading to formation of 4-amino acid derivatives of 2-keto-5,6-dihydropyrimidine. This reaction also occurs with dihydrocytidine and with the dihydrocytidine residues in oligo-dihydrocytidylic acid. The reaction proceeds with a variety of amino acids; as well as with di- and tri-peptides to give 4-peptidyl-2-keto-5,6-dihydropyrimidine derivatives. A completely analogous reaction occurs with a variety of other amines such as histamine, putrescine, cadaverine, semicarbazide.
2. The resulting 4-substituted dihydropyrimidines exhibit considerably enhanced stability as compared to the parent dihydrocytosine derivatives. This enhanced stability, also at physiological pH, points to the possibility of the formation of such compounds in living cells. The potential role of such compounds is discussed.

The metabolic role of 2,4-diketo-5,6-dihydropyrimidines, such as dihydrouracil, dihydrothymine and dihydro-orotic acid, is relatively well known. There is, on the other hand, practically no evidence that reduced cytosine derivatives possess any metabolic function. Dihydropyrimidine dehydrogenase (Grisolia & Cardoso, 1957), which reduces uracil and thymine, is inactive against cytosine. To our knowledge there is only one reference in the literature dealing with the possible existence of dihydrocytosine in living cells, viz. that of Grossman & Visser (1955), who reported the isolation of dihydrocytidine-5′-phosphate following incubation of cytidine in the presence of rat liver slices.

In previous studies on the characterization of some of the properties of a number of dihydropyrimidines, it was not found possible to obtain homogeneous preparations of dihydrocytosine derivatives by catalytic reduction (Janion & Shugar, 1960). During the course of further attempts to obtain a single product of reduction, it was noted that the presence of amino acids during catalytic reduction appeared to stabilize the resulting product. It will be shown in what follows that this apparent stabilization is, in fact, due to formation of an entirely new product, resulting from replacement of the exocyclic amino group by the amino group of the amino

acid, the resulting product being a 5,6-dihydropyrimidinyl amino acid. Furthermore, it was found that similar reactions occur in the presence of other amines, including, e.g. polyamines, semicarbazide, etc. The present communication describes the conditions under which the foregoing reactions occur, some of the properties of the resulting products, and the potential role of such compounds in living systems.

MATERIALS AND METHODS

Except where otherwise stated, compounds were commercial products. Where necessary, purities were checked by chromatography and spectral methods.

Spectral measurements were carried out with a Unicam SP-500 instrument; pH measurements were made with a Radiometer (Copenhagen) Type PHM 28 meter, using glass microelectrodes.

Reductions of the appropriate compounds were carried out in buffered medium in the presence of a 5% rhodium catalyst (Cohn & Doherty, 1956) at atmospheric pressure. Commercial hydrogen was used for this purpose, and was first bubbled through solutions of permanganate and pyrogallol. The concentration of the compound to be reduced was of the order of 0.02 - 0.03 M, in the presence of an equal amount of catalyst (by weight). It should be emphasized that, in reduction of cytosine analogues by the foregoing method, the concentration employed is an important factor. Higher concentrations than the foregoing are reduced very slowly and occasionally not at all, for reasons which are not clear, but undoubtedly involve some inhibitory effect of the amino groups on the catalyst. The course of reduction was controlled by paper chromatography, and by spectral methods on a suitably diluted sample.

RESULTS

In contrast to 2,4-diketopyrimidines, the catalytic reduction of cytosine does not normally lead to the formation of a single product. This is due to the facility with which 5,6-dihydrocytosine derivatives undergo deamination to the corresponding 5,6-dihydrouracils, as well as the tendency of the cytosine ring to take up more than one mole hydrogen (Green & Cohen, 1957). The latter effect has been shown to result in the formation of trimethyleneurea which, according to Iwashaki (cited in: Wempen, Brown, Ueda & Fox, 1965) is due to saturation of two double bonds on the pyrimidine ring (presumably the 5,6 and 3,4), with concurrent deamination.

In the present investigation, attempts were made to find conditions under which the deamination reaction was appreciably reduced. Lowering the temperature during reduction was without any marked effect. Somewhat interesting, however, was the chance observation that deamination was considerably diminished in alkaline medium. It was then observed that catalytic reduction of cytosine in alkaline medium gave different products when the reaction was carried out in borate or glycine buffers. When this was further examined, it was found that the difference was due to reaction of glycine with 5,6-dihydrocytosine; and, in fact, when cytosine

was first reduced in borate buffer and glycine then added to the reaction medium, the same product was obtained.

The reaction of 5,6-dihydrocytosine with glycine is easily observed by spectral methods. In Fig. 1, it will be noted that the ultraviolet absorption maximum for dihydrocytosine at 238 mμ is shifted to 243 mμ on addition of glycine, furthermore the pK, which is 6.5 for dihydrocytosine, is reduced to 5.7 for the complex with glycine (Figs. 2a, 2b). The stability of the complex with glycine is also appreciably enhanced. For example, when a preparation of dihydrocytosine at pH 5.0 was

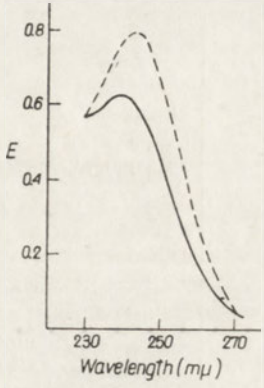


Fig. 1. Absorption spectra at pH 7.4 of dihydrocytosine (———), and dihydrocytosine-glycine complex (- - - -). Conditions: following complete reduction of cytosine in 0.2 M-borate buffer, pH 9.8, the solution was divided into two portions. To one was added an equal volume of water, to the other an equal volume of 0.2 M--glycine. After 20 min., each solution was diluted with buffer at pH 7.4, and spectra recorded.

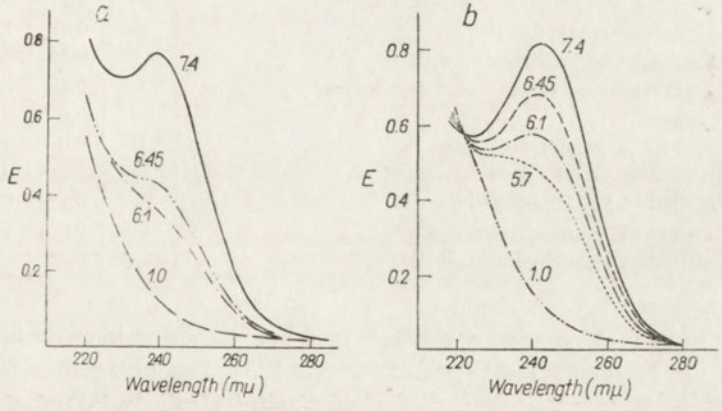


Fig. 2. Absorption spectra at various pH values of: (*a*), cytosine reduced in the presence of borate buffer, pH 9.8; estimated pK 6.5; (*b*), cytosine reduced in presence of glycine buffer, pH 9.8; estimated pK 5.7.

stored at -15° for 5 days, it was completely deaminated to dihydrouracil; when stored under the same conditions in borate buffer, pH 9.8, for 19 days, only 50% was transformed to dihydrouracil. At the same pH the product of reaction of dihydrocytosine with glycine underwent only 33% decomposition (to dihydrouracil) after 6 months storage at -15°.

The dihydrocytosine-glycine complex is also more stable than dihydrocytosine itself in strong alkali. In 0.1 N-NaOH at 21° the half-time for decomposition of dihydrocytosine is about 2 hr.; for dihydrocytosine-glycine it is 24 hr.

Table 1

Paper chromatography of various compounds

Ascending technique on Whatman no. 1, with the following solvent systems: *A*, water-saturated butanol; *B*, water-saturated butanol with ammonia in the gas phase; *C*, isopropanol - 28% NH_4OH - H_2O (7:1:2, by vol.).

Compound	R_F values in solvents		
	A	*B*	*C*
Uracil	0.23	0.23	0.71
Cytosine	0.22	0.23	0.71
5,6-Dihydrocytosine	0.08	0.08	0.50
	0.14	0.17	—
Cytidine	—	0.15	0.49
Dihydrocytidine	—	0.05	0.39
4-Glycyl-5,6-dihydrouridine	—	0.0	0.26
4-Ethoxyuracil	0.78	0.76	0.98
4-Semicarbazidouracil	0.0	0.0	0.52
4-Semicarbazido-5,6-dihydrouracil	—	0.20	0.63
1-Methyl-4-ethoxyuracil	—	0.85	1.0
1-Methyl-4-semicarbazidouracil	—	0.08	0.58
1-Methyl-4-semicarbazido-5,6-dihydrouracil	—	0.27	0.82
1,5-Dimethyl-4-ethoxyuracil	—	0.88	1.0
1,5-Dimethyl-4-semicarbazidouracil	0.25	0.20	0.56
1,5-Dimethyl-4-semicarbazido-5,6-dihydrouracil	—	0.48	0.77
4-Glycyluracil	0.0	0.0	0.33
4-Glycyl-5,6-dihydrouracil	0.0	0.0	0.38
4-Glycylphenylalanyl-5,6-dihydrouracil	—	streak	0.57
4-Glycylleucyl-5,6-dihydrouracil	—	0.15	0.56
4-Alanylglycyl-5,6-dihydrouracil	—	0.0	0.46
4-Methylamino-5,6-dihydrouracil	—	0.35	—

The addition of glycine to a solution of dihydrocytosine likewise modifies the mobility of the latter in several systems (see Table 1). This observation is, however, complicated by the fact that preparations of dihydrocytosine were found frequently to give two UV absorbing spots with some solvent systems (*A* and *B*, Table 1). Both of these spots, on elution, exhibited identical spectra at neutral pH. But whereas the one with the lower R_F (Table 1) showed no change in spectrum at alkaline pH (in agreement with the behaviour of the original solution), the eluate of the spot with higher R_F exhibited a marked increase in extinction at 235 mμ, which rapidly decreased with time to that of the spot with the lower R_F. Although no explanation for this behaviour can at present be offered, it appears to be due to some artifact during chromatography, for the following reasons: (a) The original solution of dihydrocytosine shows no increase in absorption in alkali; (b) the ratio of the in-

tensities of the spots in solvents *A* and *B* varies appreciably from one chromatogram to another; (c) both spots, following elution, give dihydrouracil on deamination; (d) treatment of each spot with glycine gives products with identical R_F values, spectra and stabilities.

A similar reaction was observed between dihydrocytidine and glycine, although the resulting modification in UV absorption spectrum was less pronounced than for the reaction product with cytosine. The formation of the glycine-dihydrocytidine complex was even more clearly demonstrated by the change in R_F value and by the remarkably enhanced stability of the product as compared to dihydrocytidine. In this case the borate buffer employed above in the reduction medium was replaced by phosphate in order to avoid modifications in R_F produced by complexing of borate with the ribose moiety of cytidine and its reduction products.

A similar reaction was found to occur with dihydrocytosine residues in reduced oligo-C. It was previously shown that cytosine residues in oligo-C [with chain lengths of 5 to 15 residues and a mixture of 2′,5′ and 3′,5′ diester linkages (Michelson, 1959)], were susceptible to reduction on a rhodium catalyst (Janion & Shugar, 1960), although at a much slower rate than for free cytosine or cytidine,

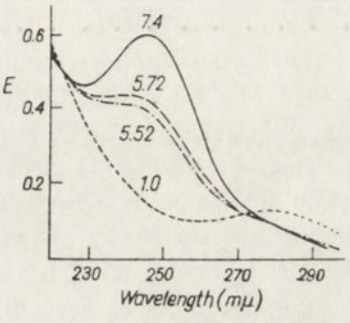


Fig. 3. Absorption spectra at various pH values of oligo-C which had been previously reduced in the presence of glycine buffer. Note from maximum at 280 mμ of pH 1 curve that a small proportion of cytosine residues still remained. Estimated pK_a of dihydrocytosine residues in oligo-C, about 5.5.

and was accompanied by extensive deamination. Reduction of oligo-C in alkaline medium in the presence of glycine was more rapid, and the reduced cytosine residues reacted smoothly with glycine (Fig. 3). On the other hand, high molecular weight poly-C, even under these conditions, was resistant to reduction.

The reaction of glycine with dihydrocytosine may be observed only within certain pH limits. At pH 5, for example, reduction of cytosine in the presence of glycine leads to a product with an absorption spectrum identical to that for reduction of cytosine in the absence of glycine. At pH 6.6 spectral observations show that the reaction with glycine proceeds to a small extent, while at pH 7.8 the reaction with glycine is as complete as at pH 9.8.

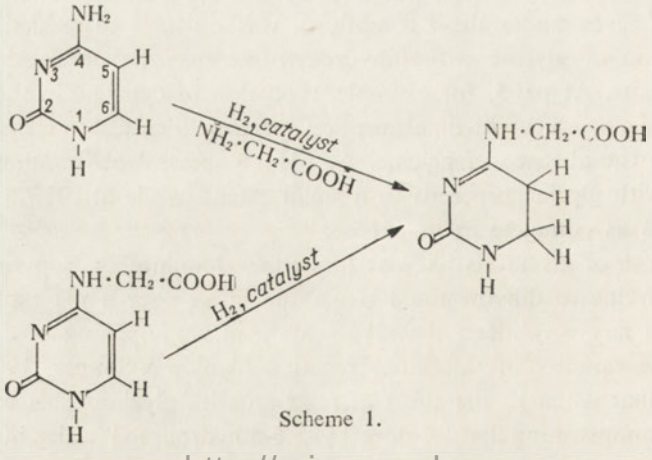
The influence of glycine is likewise limited to 4-aminodihydropyrimidines. The addition of glycine to dihydrouracil is without effect over a pH range up to 13, nor does it in any way affect the alkali-catalysed ring-opening of dihydrouracil. In view of the rapidity of this latter reaction (Janion & Shugar, 1960), it might be suspected that it masks the effect of reaction with glycine. This was, however, excluded by demonstrating that 1,6-dimethyl-5,6-dihydrouracil (which in 0.1 N-NaOH

has a half-life of more than 20 hr. at 25° as compared to about 4 min. for dihydrouracil) is unaffected in the presence of glycine.

All the spectral observations reported above lead logically to the inference that the reaction between the reduced cytosine ring and glycine involves in some manner the amino group of the former. Furthermore, the reaction with glycine is by no means a specific one. A precisely similar reaction was exhibited in the presence of other amino acids, including alanine, β-alanine, leucine, glutamine, glutamic acid, histidine, tryptophan, valine; as well as dipeptides such as glycylglycine, glycylleucine, glycylphenylalanine, alanylglycine; and the tripeptide valylglycylglycine. Further examination, with a view to determining whether the amino or carboxyl groups of the amino acids or peptides are involved, demonstrated unequivocally that the reaction involved only the amino group. A variety of other amines such as histamine (the product of decarboxylation of histidine), putrescine, cadaverine reacted equally effectively to give products with markedly enhanced stabilities. However, the influence of these amines could be revealed only by reacting them directly with dihydrocytosine. Attempts to carry out the reduction of cytosine in the presence of these amines were unsuccessful, due to poisoning of the catalyst. This latter fact was established by showing that in the presence of such amines the reduction of even uracil was completely inhibited.

By analogy with the reaction of hydroxylamine on cytosine derivatives (Janion & Shugar, 1965), it was inferred that the reaction between reduced cytosine analogues and amino acids and amines involves an exchange reaction, the amino group of the amines replacing the exocyclic cytosine amino group. This is, of course, rather difficult to demonstrate directly because of the impossibility of isolating the reaction products in sufficiently pure form. It proved feasible, however, to demonstrate the correctness of the above hypothesis in an indirect manner, as follows.

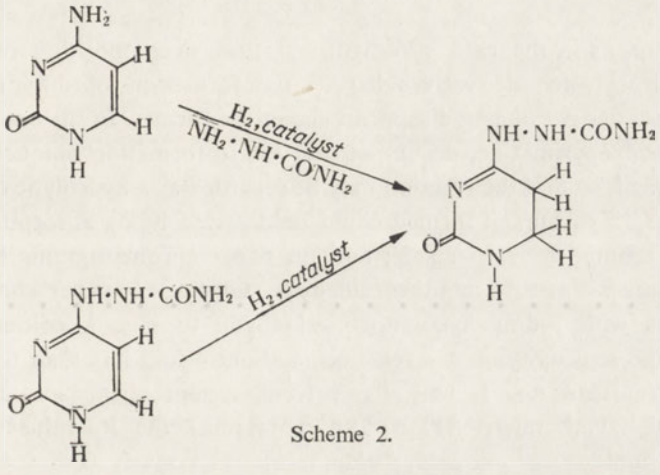
If the reaction between dihydrocytosine and glycine does involve the above exchange reaction, then it should be possible to obtain the same product by the catalytic reduction of 2-keto-4-glycylpyrimidine, as illustrated in Scheme 1.



Scheme 1.

As shown elsewhere (Janion & Shugar, in preparation), 2-keto-4-glycylpyrimidine can be readily prepared in crystalline form by reaction of 2-keto-4-ethoxypyrimidine with glycine. When this compound was reduced on a rhodium catalyst according to the usual procedure, it gave a product which was identical with that resulting from the reaction of dihydrocytosine with glycine, as judged by paper chromatography, stability, degradation rate in strongly alkaline medium, and absorption spectrum over a wide range of pH values.

A similar exchange reaction was found to occur between dihydrocytosine and semicarbazide, the resulting product being identical with that obtained by reduction of 4-semicarbazido-2-ketopyrimidine (Scheme 2).



Scheme 2.

The conditions used for reduction of cytosine in the presence of semicarbazide (relatively low concentration of the latter, 0.2 M, at pH 9.8) were such as to exclude reaction of cytosine with semicarbazide prior to reduction (cf. Hayatsu & Ukita, 1964).

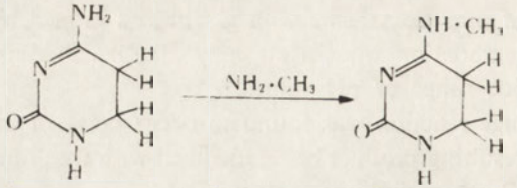
The 4-semicarbazido-2-ketopyrimidine used in the above experiment, which was originally prepared by Hayatsu & Ukita (1964) by reaction of cytosine with semicarbazide at pH 4.2, was more conveniently synthesized by treatment of 4--ethoxy-2-ketopyrimidine with semicarbazide, as described elsewhere (Janion & Shugar, in preparation). The 4-ethoxy is a much better "leaving" group than the 4-amino, as previously shown for the reaction of 4-amino and 4-ethoxy-2-ketopyrimidines with hydroxylamine (Janion & Shugar, 1965).

Additional evidence for the existence of the foregoing exchange reactions involving the amino group of dihydrocytosine analogues was forthcoming from a comparison of the reaction products of the reduction of 4-methylaminocytosine on the one hand, and the treatment of dihydrocytosine with methylamine on the other. The latter reaction was deduced to be as shown in Scheme 3.

The methylamino group in 4-methylamino-5,6-dihydrocytosine was in turn found to be susceptible to exchange with an added amino acid.

Like the 4-aminoacid derivatives of dihydrocytosine, 4-methylamino-5,6-dihydrocytosine is considerably more stable than dihydrocytosine itself. This is in

agreement with previous observations on the pronounced enhancement in stability of the photohydrates (i.e. 5-hydro-6-hydroxy derivatives) of 4-alkylaminocytosines as compared to the parent cytosine analogues (Fikus, Wierzchowski & Shugar, 1962).



Scheme 3.

Furthermore, as in the case of dihydrocytosine, decomposition of the 4-substituted dihydrocytosine derivatives led to the formation of dihydrouracil, the increase in the latter paralleling disappearance of the former. In the case of 4-glycyl--2-keto-5,6-dihydropyrimidine, decomposition led to formation uniquely of glycine and dihydrouracil, so that the reaction may be regarded as a hydrolytic deamination. In solution the dihydrouracil formed could be detected by its absorption spectrum and rate of decomposition in alkali; and on paper chromatograms by means of its reaction with *p*-dimethylaminobenzaldehyde. Glycine on paper chromatograms likewise reacts with *p*-dimethylaminobenzaldehyde to give a coloured (yellow) product, but the sensitivity of this reaction is about 4-fold less than that with ninhydrin. On Whatman no. 1, with the solvent system phenol - water - ammonia (80 ml. phenol, 20 ml. water, 500 µl. 28% ammonia), the R_F values of dihydrouracil, glycine, ureidopropionic acid, β-alanine are, respectively, 0.88, 0.43, 0.63, 0.63.

DISCUSSION

The question naturally poses itself as to whether compounds such as those described above possess any physiological significance. As mentioned in the introduction, there has been only one recorded instance of the isolation of a dihydrocytosine derivative from living cells, viz. dihydrocytidine-5′-phosphate (Grossman & Visser, 1955). What our findings do indicate is that, if dihydrocytosine analogues are involved in some metabolic processes, then, in the presence of amines, polyamines, amino acids or peptides, they would undergo *rapid* transformation to 4-substituted 5,6-dihydroderivatives which are more stable at physiological pH values than the corresponding parent dihydrocytosines.

The fact that this reaction can also occur in polymers points to the possibility of the existence, at least in transfer RNA, of such 4-substituted 5,6-dihydro analogues. In fact such compounds would be *much* more stable at alkaline pH than the known dihydrouracil residues in various transfer RNA's.

This point has an important bearing on the reported finding of Huang & Bonner (1965) with respect to histone-associated RNA of bud chromatin. The nucleotide composition of this RNA was determined by column chromatography, following hydrolysis to mononucleotides in 0.1 N-NaOH for 18 hr. at 37°, and was reported

to contain 27.5 mole% of dihydrouridylic acid. It is, however, clear that the hydrolysis conditions used should have led to ring opening of the $N_{(3)}$-$C_{(4)}$ bond in the pyrimidine rings of all the dihydrouridylic acid residues, since the half-time for decomposition of these, in 0.1 N-NaOH at 22°, is of the order of 6.5 min. (Janion & Shugar, 1960).

Our results suggest a plausible and *reasonable* explanation for the findings of Huang & Bonner (1965). If the presumed dihydrouridylic acid residues in their RNA preparation consisted instead of dihydrouridylic acid residues in which the 4-keto group is replaced by some other substituent such as an amine or peptide, then these residues would have been resistant to the alkali hydrolysis conditions used. Subsequently, during elution from the column, the acid eluants used would have led to "deamination" of these residues to give free dihydrouridylic acid in the eluate. It consequently appears likely that the histone-associated RNA contains substituted dihydropyrimidine residues such as those described above. It would obviously be highly desirable to re-examine the nucleotide composition of this histone-associated RNA, with the use of enzymic, instead of alkaline, hydrolysis conditions.

From a general point of view, the stability at physiological pH of such 4-substituted dihydropyrimidines points to the possibility, not only of their existence in nucleic acids and nucleoproteins, but also of their contributing to the stability of nucleoproteins.

Finally it should be recalled that plants have been found to contain a number of chemical complexes of amino acids with purines, pyridines and pyrimidines, e.g. villardine, $N_{(1)}$-alanine uracil (Gmelin, 1959); mimosine, $N_{(1)}$-alanine pyridine (Renz, 1936); 6-peptido uracil (Brown & Silver, 1966). Yeast transfer RNA has been shown to contain adenine linked to an amino acid *via* the purine amino group (Hall & Chheda, 1965). A variety of *N*-purinyl, *N*-purinoyl and pyrimidinyl amino acids have also been chemically synthesized (Shabarova, Sokolova, Boikova & Prokof'ev, 1959; Cohen, Thom & Bendich, 1962; Ueda & Fox, 1963; Springer, Haggerty & Cheng, 1965; Janion & Shugar, in preparation); but their physiological significance is as yet not clear.

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REAKCJA AMIN Z ANALOGAMI DWUHYDROCYTOZYNY I TWORZENIE AMINOKWASOWYCH I PEPTYDYLOWYCH POCHODNYCH DWUHYDROPIRYMIDYN

Streszczenie

1. Grupa aminowa dwuhydrocytozyny w obecności aminokwasu w środowisku obojętnym lub alkalicznym ulega szybkiej wymianie z grupą aminową aminokwasu z wytworzeniem 4-aminokwasowej pochodnej 2-keto-5,6-dwuhydropirymidyny. Tej samej reakcji wymiany ulega dwuhydrocytydyna i reszty dwuhydrocytydyny w kwasie oligo-dwuhydrocytydylowym. Reakcja zachodzi z całym szeregiem aminokwasów, jak również z dwu i trójpeptydami dając 4-peptydylo--2-keto-5,6-dwuhydropirymidyno pochodne. Analogiczna reakcja zachodzi z innymi aminami, jak z histaminą, putrescyną, kadaweryną i semikarbazydem.

2. Powstałe 4-podstawione dwuhydropirymidyny wykazują znacznie większą stabilność w porównaniu z macierzystymi dwuhydrocytozynowymi pochodnymi. Ta podwyższona stabilność, która istnieje również w fizjologicznym pH, wskazuje na możliwość powstawania takich związków w organizmach żywych. Przedyskutowano przypuszczalną rolę tych związków.

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Addendum. Since submission of the foregoing manuscript, we have located in the literature several addition examples of natural compounds consisting of purines or pyrimidines containing an alanine residue. For example, *Lathyrus tingitanus* seeds have been shown to contain β-(2-aminopyrimidine-4-yl)-alanine (Bell, 1961; Bell & Foster, 1962; Nowacki & Przybylska, 1961). The mould *Fusarium* was also found to contain the riboside of 2-(α-propionoamino)-6-hydroxypurine (Ballio, Delfini & Russi, 1960), while the aglycon of this compound has been isolated from extracts of *Eremothecium ashbyii* (Al-Khalidi & Greenberg, 1961).

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RECENZJE KSIĄŻEK

PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY (J. A. V. Butler & H. E. Huxley, eds.) vol. 15. Pergamon Press, Oxford-London-Edinburgh-New York-Paris-Frankfurt, 1965; str. 230, cena 80s.

Kolejny tom tego seryjnego wydawnictwa jest prawie w całości poświęcony problemom biosyntezy, struktury oraz funkcji białek i struktur podkomórkowych, a także roli elementów genetycznych. Autorem pierwszego artykułu, zatytułowanego „Biosynteza białek", jest P. N. Campbell. W ostatnim czasie ukazało się tak wiele świetnych przeglądów tego aktualnego tematu, że — jak stwierdza sam autor — trudno uniknąć powtarzania myśli innych. Problem ten daleki jest jednak od wyczerpania, a artykułowi Campbella, wybitnego specjalisty w tej dziedzinie, nie można zarzucić braku oryginalności. Największy nacisk położył on bowiem na sprawy, na które dotychczas zwracano stosunkowo najmniej uwagi, a mianowicie na korelację pomiędzy strukturą i funkcją poszczególnych organelli komórkowych oraz na kodowanie i syntezę niektórych białek naturalnych. Pierwsze etapy biosyntezy białek, jak również badania z użyciem sztucznych messengerów przedstawiono w wielkim skrócie, co należy uznać za słuszne wobec wspomnianej obfitości piśmiennictwa na ten temat.

Stosunkowo krótki artykuł J. Bracheta o roli kwasów nukleinowych w rozmnażaniu i rozwoju jest chyba jednym z pierwszych artykułów poglądowych na ten temat. Autor oparł się głównie na pracach dotyczących funkcji DNA, RNA i histonów u płazów, jeżowców oraz u *Acetabularia*. Dochodzi on do wniosku, że aczkolwiek należy zgromadzić jeszcze wiele faktów doświadczalnych, zanim można będzie wysnuć zadowalającą teorię różnicowania, to jednak już dziś można powiedzieć, że informacyjne RNA odgrywa zasadniczą rolę w różnicowaniu. Najważniejszym zagadnieniem, które należy rozstrzygnąć w przyszłych pracach, jest problem regulacji aktywności genów a także roli struktury i organizacji różnych elementów pozajądrowych, których wpływ na embriogenezę został wykazany w szeregu prac nad różnicowaniem jaj jeżowców.

B. B. Hyde omawia w swym artykule ultrastrukturę chromatyny. Oceniając krytycznie hipotezę t. zw. wieloniciowej budowy chromatydy, dochodzi on do wniosku, że zarówno u zwierząt, jak i u roślin wyższych podstawowa nić chromatydowa składa się z dwu pojedynczych nici zbudowanych z nukleohistonu. Poglądy te pozostają w zgodności ze współczesnym pojęciem kolinearności kodu genetycznego z pierwszorzędową strukturą białek, które dominują w genetyce bakterii i fagów. Artykuł jest napisany w sposób dostępny i niewątpliwie zainteresuje nie tylko specjalistów.

Artykuł D. R. Daviesa opisuje osiągnięcia ostatnich kilku lat dotyczące rentgenograficznych badań nad strukturą polipeptydów. Opisane są wyniki uzyskane głównie na wysuszonych preparatach szeregu białek fibrylarnych. Ostatnia część artykułu jest jednakże poświęcona najnowszym wynikom uzyskanym przy pomocy metody rozpraszania promieni rentgenowskich w roztworach. Praca ta napisana jest niewątpliwie dla specjalistów.

D. Steve Bacciarelli opisuje budowę i rozwój wirusów zwierzęcych. Replikacja wirusów zahacza o podstawowe zagadnienia biologii molekularnej, gdyż stanowi jeden z najprostszych modeli przekazywania informacji genetycznej oraz regulacji syntezy kwasów nukleinowych i białek.

Budowa i replikacja wirusów zwierzęcych jest zbadana w znacznie mniejszym stopniu niż bakteriofagów, i piśmiennictwo dotyczące tego problemu jest uboższe; dlatego też próby zebrania nagromadzonych faktów oraz wskazania kierunków dla dalszych badań należy przywitać z uzna-

niem. Dziwne wydaje się połączenie w jednym artykule zagadnień struktury i funkcji wirusów. Zagadnienia te są obecnie tak obszerne, że mogą z powodzeniem stanowić temat dwu odrębnych przeglądów.

V. P. Whittaker w obszernym artykule omawia zastosowanie techniki frakcjonowania komponent podkomórkowych do badań nad funkcją mózgu. Praca ta, oparta w znacznej mierze na badaniach z laboratorium autora, zawiera szereg sekcji dotyczących otrzymywania i własności synaptosomów i innych cząstek podkomórkowych z tkanki mózgowej, wewnątrzkomórkowego rozmieszczenia amin biogennych oraz enzymów biorących udział w ich przemianach, a także składu chemicznego różnych frakcji podkomórkowych tkanki nerwowej.

Wydaje się, że autor potrafił rozwiązać szereg problemów dotyczących podejścia metodycznego do zagadnienia biochemicznego badania funkcji mózgu, które do niedawna wydawały się niemożliwe do rozwiązania przy pomocy współczesnych metod badawczych. Na szczególną uwagę zasługuje zastosowanie przez autora kontroli przy pomocy mikroskopii elektronowej preparatów uzyskanych drogą ultrawirowania w gradiencie gęstości. Pozwoliło to na wyciągnięcie wniosków dotyczących składu, struktury oraz funkcji poszczególnych elementów. Praca jest ilustrowana szeregiem wykresów, tablic i doskonałych elektronogramów, ułatwiających zrozumienie tekstu.

Tradycyjnie doskonała szata graficzna całego tomu nie tylko ułatwia, lecz i uprzyjemnia jego czytanie.

Michał Bagdasarian

R. Vogel, I. Trautschold & E. Werle, NATÜRLICHE PROTEINASEN-INHIBITOREN G. Thieme-Verlag, Stuttgart, 1966; str. 106, cena 29,70 DM.

Recenzowana książka przedstawia dzisiejszy stan wiedzy o inhibitorach proteinaz i podaje obszerne piśmiennictwo poświęcone temu zagadnieniu; zestawienie prac ogłoszonych na ten temat obejmuje ponad 900 pozycji. Autorzy omawiają najpierw inhibitory roślinne, potem pochodzenia zwierzęcego; inhibitory uzyskane z tkanek i płynów ustrojowych człowieka są omówione szczególnie starannie. Ostatni rozdział jest poświęcony zastosowaniu terapeutycznemu inhibitorów proteinaz. Książka jest pisana zwięźle, ale przedstawia wyczerpująco poszczególne zagadnienia. Jako monografia z serii poświęconej zagadnieniom biochemicznym w klinice jest publikacją bardzo pożyteczną i aktualną.

Włodzimierz Mozołowski

E. Kálmán, L. Sztanyik, E. Unger & V. Várterész, STRAHLENBIOLOGIE. (V. Várterész, ed.) Akadémiai Kiadó, Budapest, 1966; str. 603.

Recenzowana książka jest wszechstronnym przewodnikiem z zakresu zagadnień dotyczących działania promieniowania jonizującego na układy biologiczne. Z obszernej problematyki ujętej w 13-tu rozdziałach wydzielić można pod względem tematycznym kilka grup zagadnień, łączących się w logiczną całość, jakkolwiek opracowanych nie jednakowo wyczerpująco. Przedmiotem pierwszych dwóch rozdziałów są elementarne informacje dotyczące rodzajów promieniowania jonizującego, praw rozpadu pierwiastków promieniotwórczych, dozymetrii i klasycznych fizycznych teorii efektu promieniotwórczego. Rozdział trzeci zawiera podstawowe wiadomości z zakresu radiobiochemii i omawia wpływ promieniowania jonizującego na chemiczne składniki ustrojów żywych, przemianę energetyczną i poszczególne cykle metaboliczne. Problematyka fizyczna i biochemiczna składająca się na pierwsze trzy rozdziały książki ujęta jest w zakresie elementarnym i stanowi wprowadzenie do właściwej tematyki monografii.

Następną grupę zagadnień, poruszonych w rozdziałach 4 - 7, stanowią wyczerpująco omówione problemy z zakresu patologii ogólnej uszkodzeń popromiennych. Uwzględniają one zmiany morfologiczne w poszczególnych narządach i tkankach, problem wrażliwości i oporności rodzajowej i osobniczej, jak też i wpływ różnych czynników na efekt promieniowania. Bardzo wyczerpująco

omówiona została w tej grupie tematycznej patologia funkcjonalna narządów oraz ogólnobiologiczne zmiany, w szczególności immunobiologiczne, będące następstwem działania promieniowania na organizmy żywe.

Rozdziały 8 - 9 poświęcone są patologii szczegółowej i terapii uszkodzeń popromiennych. Pierwszy z nich obejmuje opis obrazów klinicznych i stosowanych zabiegów leczniczych u osób dojrzałych, które uległy chorobie popromiennej wskutek nieszczęśliwych wypadków, jak również w wyniku eksplozji atomowych. Rozdział 9-ty omawia wyłącznie uszkodzenia powstałe w życiu płodowym. Szczególnie cenne jest zebranie dotychczas opublikowanych danych dotyczących kliniki ostrych zachorowań wynikłych wskutek awarii reaktorów atomowych i innych urządzeń na przestrzeni lat 1945 - 1960. Syntetyczna ocena kryteriów diagnostycznych, zwłaszcza wyników badań laboratoryjnych, oraz stosowanych zabiegów leczniczych posiada duże znaczenie dla lekarza, który nie znajduje odpowiednich informacji w klasycznych podręcznikach patologii szczegółowej chorób wewnętrznych.

Kolejnym zagadnieniem poruszonym w rozdziale 10-tym monografii jest mutagenne działanie promieni jonizujących. Omówiono w nim wyniki badań nad mutacjami powstającymi w organizmach żywych od najniższych (wirusy, fagi, mikroorganizmy) do najwyższych (człowiek). Szczególnie interesujące jest zebranie danych dotyczących częstości występowania zaburzeń genetycznych u ludzi, powstających w obecnym okresie jako następstwo promieniowania. Informacje dotyczące tego zagadnienia są jeszcze niezwykle skąpe, lecz posiadają doniosłe znaczenie dla epidemiologii schorzeń popromiennych. Ze względu na wciąż aktualną, chociaż formułowaną w różnych wariantach, teorię mutagenną etiologii nowotworów, tematyka ta łączy się z treścią następnego rozdziału, omawiającego kancerogenne działanie promieniowania jonizującego. Zawiera on opis schorzeń nowotworowych wywołanych przez kontaminację izotopami radioaktywnymi lub ekspozycję na promieniowanie.

Odrębnym zagadnieniem z zakresu szczegółowej patologii uszkodzeń wywołanych ciałami promieniotwórczymi jest radiotoksykologia, t.j. zespół zmian spowodowanych kontaminacją wewnętrzną izotopami radioaktywnymi. Problemowi temu poświęcony jest wyczerpująco opracowany rozdział 12-ty, który omawia zarówno wpływ szeregu parametrów toksykologicznych, jak i biologiczno-kinetycznych warunkujących działanie danego izotopu, typy uszkodzeń klinicznych oraz postępowanie lecznicze w przypadku kontaminacji wewnętrznej. Opis poszczególnych metod ratownictwa, polegających z jednej strony na zahamowaniu wchłaniania, z drugiej na przyspieszeniu wydalania, daje przegląd stosowanych obecnie zabiegów w leczeniu zatruć izotopami radioaktywnymi.

Ostatni rozdział monografii omawia związki chemiczne stosowane dotychczas w ochronie radiologicznej i mechanizm ich działania.

Wartość omawianego dzieła podnosi bardzo bogate piśmiennictwo. W tej postaci jest ono godnym polecenia, wyczerpującym kompendium dla wszystkich zainteresowanych całokształtem problematyki związanej z biologicznym działaniem promieniowania jonizującego.

Kazimierz Spett

M. Florkin, A MOLECULAR APPROACH TO PHYLOGENY. Elsevier Publ. Co, Amsterdam-London-New York, 1966; str. 176, cena Dfl. 35.

Autor omawianej książki — powszechnie znany biochemik zajmujący się biochemią porównawczą oraz współredaktor wielotomowych podręczników biochemii — wykłada w tej krótkiej monografii swoje poglądy na filogenezę molekularną. Tak bowiem możnaby nazwać gałąź nauki, której początki przedstawia autor w sposób zajmujący, żywy i nowy. Dwa spośród dziesięciu rozdziałów książki są poświęcone wprowadzeniu w zagadnienie i podstawowym pojęciom molekularnej filogenezy. Następny rozdział dotyczy filogenezy peptydów i białek, dalej następują rozdziały zatytułowane: „Biosynteza a filogeneza", „Chityna, chitynoliza a filogeneza", „Końcowe produkty metabolizmu azotowego, aspekty ekologiczne i filogenetyczne", „Osmotyczne efektory

hemolimfy w filogenezie owadów", „Definicja specjalnych cech metabolizmu węglowodanów u owadów w ujęciu ewolucji molekularnej", „Białka kopalne", oraz końcowy rozdział, zawierający pewne uogólnienia pt. „Organizmy i molekuły ulegające ewolucji".

Idea przewodnia omawianej monografii polega na tym, że w rozumowaniu filogenetycznym nie jest ważne — jak to przyjmowano dotychczas — istnienie określonych końcowych produktów przemian, czy też ciągów reakcji. Najważniejsze jest podobieństwo czy wręcz identyczność sekwencji nukleotydów w genowych kwasach nukleinowych, a co za tym idzie — podobieństwo czy identyczność sekwencji aminokwasów w białkach. Takie podejście stało się możliwe dzięki ostatnim imponującym zdobyczom biologii molekularnej. Zrozumiałe jest jednak równocześnie, że w chwili obecnej liczba danych doświadczalnych, które umożliwiłyby porównanie sekwencji aminokwasów w homologicznych białkach różnych gatunków — jest znikoma; nie mówiąc już o możliwości porównywania sekwencji nukleotydów w genowym DNA. Wciąż także ciąży na wszelkich badaniach filogenetycznych (także na poziomie molekularnym) ograniczenie prawidłowości wyciąganych wniosków polegające na tym, że badamy — i porównujemy ze sobą — gatunki współcześnie żyjące. Wiadomo zaś, że tak jak całe organizmy „pierwotne" dostępne dzisiaj badaniu mogą być znacznie różne od ich odległych przodków, tak i wielkie cząsteczki chemiczne współczesnych organizmów mogą być różne od tych sprzed milionów lat. W związku z tym autor wskazuje w rozdziale 9-tym zatytułowanym „Paleoproteins" na możliwość badania białek pierwotnych organizmów kopalnych. Może to mieć oczywiście bardzo doniosłe znaczenie dla rozwoju naszych pojęć o ewolucji molekularnej.

Nie wszystkie rozdziały omawianej książki są potraktowane jednolicie. Wobec niezwykle małej liczby danych doświadczalnych dotyczących pierwszorzędowej struktury DNA i białek enzymatycznych, autor musiał ograniczyć się do przedstawienia skutków działania powstających białek enzymatycznych, to znaczy do przedstawienia dróg metabolicznych. W taki tradycyjny sposób jest ujęty m.in. rozdział 6-ty zatytułowany „Końcowe produkty metabolizmu azotowego, aspekty ekologiczne i filogenetyczne". Ale i w tym rozdziale autor przedstawił nowy punkt widzenia. Zestawia mianowicie fakty, które mają świadczyć o tym, że rozwinięcie się ureotelizmu u zwierząt jest związane z osmoregulacją, a nie — jak to się od wielu lat przyjmowało — z obfitością lub brakiem wody w okresie życia płodowego danego gatunku. Nie może to być wniosek jednoznaczny; można z nim dyskutować. Zresztą rozważania autora podane na str. 81, a dotyczące związku amonio- ureo- lub uriko-telizmu z dostępnością środowiska wodnego, zdają się być w pewnej sprzeczności z postulatem wysuniętym na str. 65. Postulat ten głosi, że należałoby zaniechać łączenia ureogenezy z jakimikolwiek aspektami dostępności wody w czasie życia płodowego.

Książka jest wydana bardzo starannie, w ładnej oprawie graficznej; do tekstu zakradły się nieliczne tylko pomyłki korektorskie, np. na str. 37 w wierszu 15 od dołu jest „plants" zamiast „animals", a na str. 160 w wierszu 4 od góry jest znak dodawania zamiast mnożenia. Monografia prof. M. Florkina jest dalszym krokiem naprzód w zbliżeniu do siebie porównawczego podejścia w badaniu żywych organizmów metodami morfologicznymi oraz biochemicznymi. Bowiem, jak kończy autor swoje dzieło: „...można stwierdzić, że przeciwieństwo stwarzane czasami pomiędzy całościowym (organizmowym) a molekularnym podejściem do filogenezy jest bez znaczenia".

Omawianą książkę będą z przyjemnością czytać biolodzy, biochemicy oraz wszyscy, którzy interesują się zjawiskiem życia na ziemi.

Mariusz Żydowo

TRANSPORT FUNCTION OF PLASMA PROTEINS. West-European Symposia on Clinical Chemistry. (P. Desgrez & P. M. Traverse, eds.) Elsevier Publ. Co.; Amsterdam, 1966; str. VIII+184, cena 70 s. lub Dfl. 35.00.

Zachodnio-europejskie sympozja klinicznej chemii odbywają się od 1960 roku i były one poświęcone: przemianie wodnej i elektrolitowej (Amsterdam, 1960 i 1963), chemii klinicznej monoamin (Manchester, 1962), enzymom w chemii klinicznej (Ghent, 1964), a w 1965 w Paryżu funkcji transportowej białek osocza. Recenzowana książka zdaje sprawę z tego ostatniego sympozjum.

W czternastu wykładach są ujęte najważniejsze zagadnienia funkcji transportowej białek osocza. W pierwszym H. Bennhold daje krótki ale bardzo przejrzysty wstęp historyczny, a także podaje wyniki badań nad transportem barwników i żelaza w osoczu. Zamyka książkę artykuł, w którym G. Biserte zbiera wyniki obrad i wyciąga ogólne wnioski mające znaczenie dla chemii klinicznej. Między tymi dwoma wykładami, ujmującymi w jedną całość zagadnienia omawiane w książce, mamy wykłady, które omawiają transport na białkach osocza: lipidów, hormonów tyroidowych, związków o aktywności insulinowej, kortykosteroidów, witamin, enzymów, bilirubiny, leków i związków toksycznych; osobne rozdziały są poświęcone zagadnieniom: wiązań między białkami a przenoszonymi cząsteczkami, zastosowania izotopów w badaniu transportu na białkach, a także klinicznego znaczenia transportowej funkcji białek osocza. Książka jest wydana starannie, jak tego wymaga tradycja Elseviera; ukazała się bardzo prędko po odbyciu sympozjum, dane wykładów są więc aktualne. Książkę można polecić zarówno klinicystom, jak biochemikom i fizjologom.

Włodzimierz Mozołowski

GLYCOPROTEINS, THEIR COMPOSITION, STRUCTURE AND FUNCTION (A. Gottschalk, ed.); B. B. A. Library vol. 5; Elsevier Publ. Co.; Amsterdam-London-New York, 1966; str. 628.

Książka ta, razem z poprzednio wydaną jako tom 6-ty B. B. A. Library pozycją p.t. *Mucopolysaccharides*, omawia całokształt biochemii wielocukrowców w różny sposób związanych z częścią białkową. W *Mucopolysaccharides* omówiono głównie takie wielocukrowce, które bardzo luźno wiążą się z białkiem i które wobec tego łatwo dają się izolować bez komponenty białkowej. W recenzowanym tomie 5-tym zajęto się głównie glikoproteidami, t.j. związkami, które po wyizolowaniu zawierają komponentę białkową i węglowodanową.

Jest to praca zbiorowa pod redakcją A. Gottschalka, badacza pracującego przez wiele lat z F. M. Burnetem w Melbourne nad kwasem sjalowym w glikoproteidach oraz udziałem tych związków w hemaglutynacji krwinek czerwonych przez wirus grypy.

Treść tej książki można podzielić na dwie części. Pierwsza obejmuje rozdziały omawiające różnego rodzaju metody identyfikacji, oznaczania składu aminokwasowego, badania struktury, oznaczania masy cząsteczkowej oraz ilościowej analizy składników węglowodanowych. W dalszych rozdziałach natomiast omówiono kolejno znane glikoproteidy.

Poszczególne rozdziały są napisane przez ludzi zajmujących się przez wiele lat chemią i biochemią glikoproteidów i mających na tym polu znaczny dorobek. Rozdziały omawiające zagadnienia metodyczne napisali J. E. Eastoe, R. A. Gibbons, A. Gottschalk, R. D. Marshall i A. Neuberger. Albuminę jaja kurzego, jej metody oczyszczania, skład, własności fizyczne i biologiczne omówili A. Neuberger i R. D. Marshall, owomukoid M. D. Melamed, kazeinę P. Jollés, glikoproteidy surowicy E. R. B. Graham, R. W. Jeanloz, E. M. Press, R. R. Porter, A. G. Bearn i W. C. Parker; glikoproteidy ślinianek podszczękowych W. Pigman i A. Gottschalk, glikoproteidy moczu M. Maxfield; substancje grupowe krwi W. M. Watkins; tyreoglobulinę M. T. McQuillan i V. M. Trikojus; glikoproteidy z aktywnością biologiczną H. Papkoff; inne glikoproteidy omówił E. Buddecke.

Książka jest zakończona rozdziałem omawiającym ogólnie metabolizm aminocukrów i związków zawierających aminocukry, napisanym przez L. Warrena, oraz rozdziałem napisanym przez A. Gottschalka i A. Neubergera p.t. „Retrospect and Outlook", w którym autorzy zastanawiają się, jakie znaczenie ogólne mają glikoproteidy w przyrodzie i jakie trudności czekają badaczy w dalszych pracach dotyczących wyjaśnienia budowy i funkcji tych substancji.

Rozdziały omawiające poszczególne glikoproteidy są zrozumiałe bez czytania rozdziałów opisujących metodykę badań, dlatego też recenzowana książka jest nie tylko bardzo pożyteczna dla badaczy zajmujących się tą dziedziną wiedzy, ale i dla tych, którzy chcą zapoznać się bliżej z interesującym ich jakimś glikoproteidem. Może też być ona traktowana jako encyklopedia, gdzie łatwo w razie potrzeby można znaleźć wyczerpująco opisane interesujące nas zagadnienia.

Wiktor Rzeczycki