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TRYPSIN INHIBITOR FROM RYE SEEDS

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1. In aqueous extracts from rye seeds, a trypsin inhibitor of protein nature was found. 2. In the seed extract no proteolytic activity could be demonstrated; it appeared only after adsorption of the inhibitor at pH 4.5 on cellulose or seed coats. 3. The inhibitory and proteolytic activities were separated by ammonium sulphate fractionation at pH 4.5. 4. During early germination, a rapid decrease in the activity of the inhibitor was observed.

Seeds of many plants are known to contain, in addition to some proteolytic enzymes, proteins capable of binding trypsin to form inactive complexes. Seeds of leguminous plants are especially rich in trypsin inhibitor. Among the cereals, Shyamala, Kennedy & Lyman (1961) and Learmonth & Wood (1963) found in wheat grain the trypsin inhibitor which was later purified and characterized by Shyamala & Lyman (1964). All the trypsin inhibitors obtained from seeds are proteins with a molecular weight of several thousands. Their role so far remains unknown; their ability to form inactive complexes with trypsin, an enzyme of animal origin, may indicate that they have a similar function with respect to analogous plant proteases.

The aim of the present work was to determine the trypsin inhibitor activity in aqueous extract from rye seeds and to get some insight into its physiological role.

MATERIALS AND METHODS

Material. Rye seeds (*Secale cereale*) variety "Ludowe" stored at 14 - 18° at a relative moisture of the air 45 - 55% for a period not exceeding one year, were obtained from the Experimental Agricultural Station of the Higher School of Agriculture in Wrocław.

The air-dry seeds (14 - 16 g.) were ground in a coffee-mill five times for 30 sec., with 1 - 2 min. intervals. The flour was treated with 10 vol. of water for 1 hr. with constant shaking, then centrifuged for 20 min. at 12 000 rev./min. and the supernatant collected. For some experiments, the seeds were germinated on soft filter paper in Petri dishes at 18 - 20°. The germination capacity of the seeds was 98%.

For preparation of seed coats, rye bran obtained from the mill "Sułkowice" (Wrocław) was passed through analytical sieves. The fraction of the bran which passed through the sieve with 0.43 mm. mesh, and was retained on 0.3 mm. mesh, was collected and purified by a stream of air. For experiments the lightest fraction was used; it was washed successively with water, 0.9% solution of NaCl and 0.2% solution of NaOH until free of protein, then washed thoroughly with water and dried at 105° for 3 hr.

Analytical methods. Protein was determined by the turbidimetric tannin micromethod (Mejbaum-Katzenellenbogen, 1955), the turbidity being measured in Pulfrich photometer with a S-61 filter. Gelatin (Grübler, Leipzig, Germany) dried to constant weight in a vacuum desiccator over sulphuric acid and assayed by nitrogen determination, was used as standard.

Proteolytic and inhibitor activities were determined according to Mejbaum-Katzenellenbogen, Wilusz & Polanowski (1966) by measuring the decrease in total protein in the incubation mixture. One unit of the proteolytic activity was defined as the amount of the enzyme which during 5 min. at 30° degraded 20 μg. of casein to products not reacting with tannin. This corresponded to the activity of 1 μg. of protein of the trypsin preparation of Merck. One unit of the trypsin inhibitor activity was defined as the amount of protein which inhibits the activity of 1 μg. of protein of the trypsin preparation.

Reagents. Trypsin (Merck A. G., Darmstadt, Germany), activity 20 000 Fuld-Gross units/g.; white soluble casein (British Drug Houses, Poole, England); tannic acid (Politechnika Śląska, Gliwice, Poland) commercial preparation was checked for the content of pentadigalloyloglucose by ethyl acetate extraction according to Armitage *et al.* (1961) and by paper electrophoresis according to Mejbaum-Katzenellenbogen & Kudrewicz-Hubicka (1966).

RESULTS

The inhibition of trypsin activity by the aqueous extract from rye seeds is shown in Fig. 1. The rate of casein degradation decreased with the increasing amount of seed protein in the incubation mixture. With a tenfold excess of extract protein in relation to trypsin, the rate of the reaction was inhibited by a half. The effect of the seed extract on the time-course of casein degradation by trypsin is shown in Fig. 2. During 30 min. of incubation the inhibitory activity of the seed extract remained unaltered; this indicates that the inhibitor is resistant to digestion by trypsin.

The trypsin inhibitor from rye seeds was extracted with water, with 0.9% solution of NaCl, and 1% solution of acetic acid. It was not soluble in 5% solution of trichloroacetic acid or 0.15 M-sulphosalicylic acid (Table 1). The same amounts of the inhibitor were extracted with water and with sodium chloride, but as the NaCl extract contained more protein, the activity of the inhibitor per milligram protein was lower.

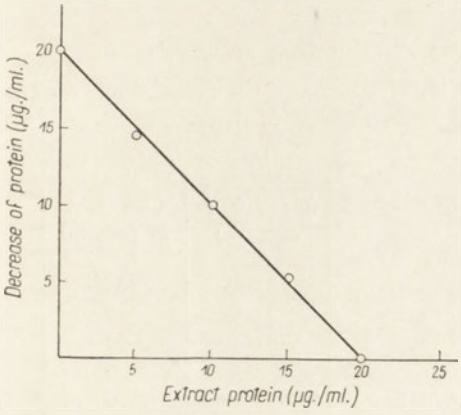

Fig. 1

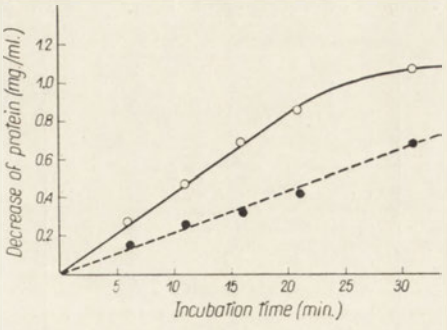

Fig. 2

Fig. 1. The effect of concentration of proteins of aqueous extract from rye seeds on the rate of casein degradation by trypsin. The incubation mixture contained in 1 ml.: 80 μg. of casein in 0.1 M-Na, K-phosphate buffer, pH 7.6; 1 μg. of trypsin, and increasing amounts of seed extract protein. After 5 min. at 30°, the reaction was stopped by adding 1 ml. of the tannin reagent, which started the assay of protein.

Fig. 2. Time-course of casein degradation by trypsin: (○), without the inhibitor; (●), with the inhibitor added. The incubation mixture contained in 1 ml.: 2.5 mg. of casein in 0.1 M-Na,K-phosphate buffer, pH 7.6, and 5 μg. of trypsin; in expt. (●), 50 μg. of protein of the aqueous extract from seeds was present. The incubation was at 30°. The reaction was stopped by adding 9 ml. of 0.3 N-HCl. The results are calculated per 1 ml. of the incubation mixture.

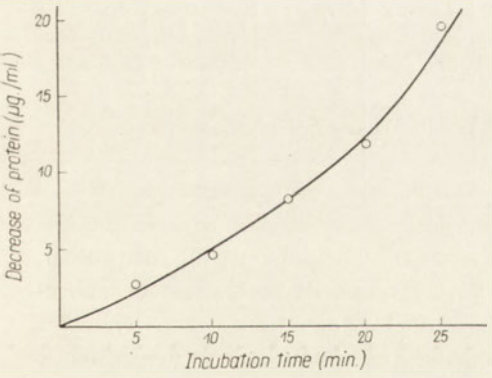

Fig. 3

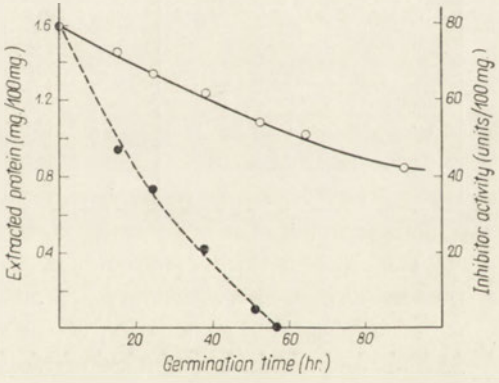

Fig. 4

Fig. 3. Time-course of casein degradation by rye protease. The incubation mixture contained in 1 ml.: 80 μg. of casein in 0.1 M-Na,K-phosphate buffer, pH 7.6, and 1 μg. of protein of the 0.5 - 1.0 ammonium sulphate sat. precipitate. The incubation was at 30°. The reaction was stopped by adding 1 ml. of the tannin reagent.

Fig. 4. Protein in the aqueous extract and trypsin inhibitor activity in rye seeds during imbibition and early phase of germination. Samples of the seed, 1 g., were germinated on soft filter paper in Petri dishes at 18 - 20°, and after the indicated periods weighed and ground with a tenfold volume of water for 1 hr. In the extract, (○), the content of protein and (●), the inhibitor activity were determined. The results are expressed per 100 mg. of air-dry material.

Table 1

Extraction of the trypsin inhibitor activity from rye seeds

Samples of ground seeds, 1 g., were extracted for 1 hr. with 10 ml. of the indicated solvent. The inhibitor activity was determined as described in Methods.

Extraction with	Protein extracted from 100 g. of seeds (g.)	Inhibitor activity	
		(units/100 g. of seeds)	(units/mg. of protein)
Water	1.36	48 000	35
Sodium chloride, 0.9%	2.45	49 000	20
Acetic acid, 1%	1.00	35 000	35
Trichloroacetic acid, 5%	0.42	0	0
Sulphosalicylic acid, 0.15 M	0.47	0	0

Table 2

Adsorption of seed extract proteins on seed coats

Rye seed coats washed free of protein, 1 g., were equilibrated with water or 0.05 M-sodium acetate buffer of pH 4.5, for 24 hr. After centrifuging, the seed coats were mixed with 3 ml. of aqueous seed extract, left for 6 hr., then in the supernatant the content of protein, trypsin inhibitor activity and proteolytic activity toward casein were determined.

Adsorption medium	Before adsorption			After adsorption		
	Protein (mg.)	Trypsin inhibitor activity (units/mg. of protein)	Proteolytic activity (units/mg. of protein)	Protein (mg.)	Trypsin inhibitor activity (units/mg. of protein)	Proteolytic activity (units/mg. of protein)
Water	3.6	45	0	2.6	0	0
0.05 M-acetate buffer, pH 4.5	3.6	45	0	2.3	0	40

The aqueous extract from 1 g. of air-dry seeds contained 14 - 16 mg. of protein, possessing inhibitor activity of 35 - 50 units/mg. Heating of the extract at 75° resulted in a gradual coagulation of the protein and loss of inhibitor activity. After 25 min. of heating, about 25% of the protein was precipitated, and the inhibitor was completely inactivated.

When the proteins of the extract were submitted to paper electrophoresis in 0.05 M-sodium acetate buffer, pH 4.5, only 60% could be eluted with 0.2 M-phosphate buffer, pH 7.6. In the eluate, no inhibitor activity was detected, but distinct proteolytic activity was found to appear. Twenty μg. of the protein of the eluate possessed an activity corresponding to 1 μg. of the trypsin preparation. The same results were obtained when the extract was treated with Whatman chromatographic cellulose equilibrated with 0.05 M-sodium acetate buffer, pH 4.5. After 6 hr. about 30% of the protein became adsorbed; the supernatant contained no inhibitory acti-

vity, and at the same time proteolytic activity appeared. The attempts at eluting the inhibitor from the cellulose with different solvents were unsuccessful.

As cellulose is the main constituent of the cell wall and of seed coat, for adsorption of the inhibitor purified seed coats were applied. The seed extract after treatment with seed coats equilibrated with water, contained neither inhibitory nor proteolytic activity, whereas after treatment with seed coats equilibrated with a buffer of pH 4.5, it lost the trypsin inhibitor activity but showed proteolytic activity (Table 2). Attempts to elute the proteins adsorbed on seed coats were unsuccessful.

The above results indicated that at pH 4.5 the complex of the proteolytic enzyme with the inhibitor dissociated. Therefore ammonium sulphate fractionation was performed at pH 4.5 (Table 3). When the extract was brought to pH 4.5, a precipitate appeared which contained about a half of the protein material. From the protein remaining in the supernatant, 70% was precipitated at 0.5 ammonium sulphate saturation and it contained the whole trypsin inhibitor activity of the extract. The proteolytic activity remained in the supernatant. The course of digestion of casein by rye protease is shown in Fig. 3.

Table 3

Separation of the inhibitor and protease from rye seeds

Aqueous extract from ground seeds was brought to pH 4.5 with 0.1 and 0.01 N-HCl, left for 12 - 14 hr. at 4°, then centrifuged for 15 min. at 10 000 rev./min. To the supernatant, heated to 18°, ammonium sulphate *in substantia* was added gradually to 0.5 saturation. After 2 hr., the precipitated protein was centrifuged and dissolved in 0.9% NaCl solution. The supernatant was saturated with ammonium sulphate, left for 2 hr., then centrifuged and the protein dissolved in water.

Preparation	Protein		Trypsin inhibitor		Protease	
	(mg./g. of seeds)	(% of extracted protein)	(units/g. of seeds)	(units/mg. of protein)	(units/g. of seeds)	(units/mg. of protein)
Aqueous extract	13.1	100	448	37	0	0
pH 4.5 supernatant	6.9	52.5	414	60	0	0
Ppt. at 0.5 ammonium sulphate sat.	4.8	36.5	439	91	0	0
Ppt. at 0.5 - 1.0 ammonium sulphate sat.	2	15	0	0	414	200

The separated inhibitor was able to react again with the proteolytic enzyme. On addition of 10 μg. of the globulin fraction to the incubation mixture containing 1 μg. of the albumin fraction, the activity of protease was completely inhibited. The inhibitor was resistant to digestion by seed protease as well as by trypsin.

To locate the inhibitor in the seed, the germ, endosperm and seed coat (together with the aleurone layer) were isolated, ground and assayed. The amounts of protein extracted with water from the particular parts of the seed, as well as the trypsin inhibitor activity, are presented in Table 4. The greatest amount of water-soluble

proteins was found in the germ and in the aleurone layer. In the endosperm, the amount of water-soluble proteins per gram was almost the same as in the whole seed. The trypsin inhibitor was found only in the endosperm.

Table 4

Distribution of the trypsin inhibitor activity in rye seeds

Aqueous extract from	Protein (% of dry wt.)	Trypsin inhibitor (units/mg. of protein)
Whole seed	1.56	45
Germ	8.60	0
Seed coat (with aleurone layer)	2.85	0
Endosperm	1.45	50

Changes in the content of water-soluble protein and of trypsin inhibitor activity during imbibition of seed and in the early phase of germination, are shown in Fig. 4. A gradual decrease in the amount of water-soluble protein was accompanied by a rapid decrease of inhibitor activity. After about 56 hr. of germination, aqueous extracts exhibited no inhibitor activity. The amount of protein extracted with water decreased during this time by about 30%. This could by caused by degradation of protein to products not reacting with tannin, or by loss of protein solubility.

DISCUSSION

In the aqueous extract the protein of rye seeds did not undergo autolysis and no proteolytic activity toward casein was observed. The extract was found to contain a trypsin inhibitor of protein character, as judged by its thermolability, insolubility in 5% trichloroacetic acid and 0.15 M-sulphosalicylic acid, and formation of precipitate with tannin. The aqueous extract, after treatment with cellulose at pH 4.5, lost the inhibitor activity, and in the eluate appeared the proteolytic activity. This indicates the presence in rye seeds of a complex of protease with the inhibitor, which dissociates at pH 4.5. The affinity of the inhibitor to cellulose, which in plants is the structural material of cell walls, may indicate the physiological role of cellulose cell structures in activation of the enzyme-inhibitor complex.

The inhibitor from rye seeds does not require preincubation to form the inactive complex with trypsin, and it is not digested by trypsin. Similar properties were observed in the majority of the so far described natural trypsin inhibitors, excepting ovomucoid (Gorini & Audrain, 1952, 1953) and pancreatic inhibitor of Kazal (Laskowski & Wu, 1953) which are digested by trypsin and therefore their complexes are unstable, and the pancreatic inhibitor of Kunitz & Northrop (1936) which requires preincubation to form an inactive complex (Green & Work, 1953).

Engeln & Heins (1947) who studied the distribution of proteolytic enzymes in cereal seeds, found that the endosperm contained no proteases, or their activity

was so low that it could not be detected. The results of the present work explain to some extent why in the endosperm of the seed the proteolytic activity could not be detected. The enzyme is bound to the inhibitor, and the proteolytic activity can be demonstrated only when the complex is dissociated and the two components separated. The mechanism of activation may be regulated by physico-chemical changes occurring during after-ripening of seeds. Mori (1944) observed that during storage the acidity of the seed increased. In freshly harvested seed the pH was 6.5, whereas after a period of storage it decreased to about pH 4. Under these conditions, the enzyme-inhibitor complex present during anabiosis of the seed, dissociates. Internal translocation of the components may be facilitated by increased water content during the imbibition phase, and the inhibitor may diffuse toward the external layers of the seed where it becomes adsorbed on cellulose of the seed coats.

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INHIBITOR TRYPSYNY Z ZIARN ŻYTA

Streszczenie

1. W białkach wyciągów wodnych z ziarn żyta wykazano aktywność antytrypsynową.

2. Białka wyciągu po adsorpcji na celulozie lub wypreparowanych okrywach nasiennych w pH 4,5 tracą aktywność antytrypsynową, a w eluatach pojawia się aktywność proteolityczna, której nie można było wykazać w wyciągach.

3. Aktywność inhibitorową i proteolityczną rozdzielono po dysocjacji w pH 4,5 przy pomocy siarczanu amonu.

4. Pęcznienie oraz wczesną fazę kiełkowania ziarna cechuje gwałtowne obniżanie się aktywności antytrypsynowej.

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SYNTHETIC POLYRIBONUCLEOTIDE ATTACHMENT TO BACTERIAL RIBOSOMES

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1. Poly-5,6-dihydrouridylic acid and poly-*N*-methyluridylic acid which do not form complexes with poly-adenylic acid and are not active as templates in the RNA-polymerase system and in the amino acid incorporating system, are capable of binding ribosomes in a manner similar to poly-uridylic acid. The template inactive polymers and poly-uridylic acid are attached to the same ribosomal binding sites. 2. Poly-ribothymidylic acid which forms complexes with poly-adenylic acid and is active as messenger, binds ribosomes at temperatures above its ordered state - random coil transition. 3. The foregoing testifies to the non-essentiality of the base in the process of synthetic messenger - ribosomal interaction and stresses the significance of polymer conformation.

The association of 70s ribosomal particles and m-RNA[1] is a prerequisite of protein synthesis (Spyrides & Lipmann, 1962). The nature of this binding, forces responsible for it, and the mechanism of polysome formation are still unclear. The interaction of extracted ribosomal RNA with synthetic polyribonucleotides has been shown to depend upon the availability of unbound bases in the polynucleotide and in the r-RNA, and to proceed through complementary base-pairing regardless of whether the r-RNA was derived from the 50s or the 30s subunit (Millar, Cukier & Nirenberg, 1965). The interaction of intact ribosomes appears to proceed by a different mechanism. Binding occurs at a Mg^{2+} concentration not less than 5×10^{-3} M and only the 30s subunit is involved. No enzymic factor or energy source has been demonstrated to be required. Any kind of deviation from a random coil configuration of the synthetic messenger causes strong inhibition of the association process (Takanami & Okamoto, 1963a). The use of intact ribosomes in the study of m-RNA - ribosome interaction seems to offer a more promising approach than the use of ribosomal RNA since it avoids the possibility of random complex formation between the two interacting RNA species.

[1] *Abbreviations used in this text*: m-RNA, messenger RNA; r-RNA, ribosomal RNA; poly-U, poly-uridylic acid; poly-*N*-MeU, poly-3-methyluridylic acid; poly-DHU, poly-5,6-dihydro-uridylic acid; poly-DHU-U(7 : 3), a copolymer containing 5,6-dihydrouridylic acid and uridylic acid in the proportion indicated; poly-rT, poly-ribothymidylic acid; poly-A, poly-adenylic acid; T_m, midpoint of temperature profile.

An attempt was made to investigate this problem employing model poly-ribonucleotides, analogues of poly-U, with *a priori* well defined self-pairing, complementary base-pairing and template properties, and to correlate the above properties with the ability to interact with ribosomal particles. The following ^{14}C-labelled polynucleotides were synthesized: poly-*N*-MeU, poly-DHU and its copolymers with poly-U, and poly-rT. Their interaction with *E. coli* 70s ribosomes was investigated by means of sucrose gradient centrifugation. The analogues chosen, along with [^{14}C]poly-U, represent a set of polymers which, while chemically closely related, differ among themselves in their structural properties and functional behaviour. Both poly-DHU and poly-*N*-MeU possess a random coil configuration under any conditions, do not form complexes with poly-A (although each for different reasons) and do not promote the incorporation of phenylalanine into acid-insoluble products in the cell-free system (Cerutti, Miles & Frazier, 1966; Szer & Shugar, 1961; Rotman & Cerutti, 1966; Wahba *et al.*, 1963). Also, poly-*N*-MeU does not serve as a template for poly-A synthesis in the RNA-polymerase system (Krakow & Ochoa, 1963). Yet, both poly-DHU and poly-*N*-MeU when mixed with ribosomes give optical density - radioactivity distribution patterns along the gradient similar to those of poly-U, the latter's interaction with ribosome being used as a reference system. Neither the attachment to 70s particles, nor the formation of heavier polysomal particles are affected despite the changes in the base moiety. In contrast to the former polymers, poly-rT attains a rigid, double helix-like ordered state with a sharp, temperature induced transition at 34° in 10^{-3} M-Mg^{2+}; it forms a complex with poly-A with a T_m about 20° higher than that formed by poly-U with poly-A (Szer, Świerkowski & Shugar, 1963) and is capable of promoting the synthesis of polyphenylalanine in the cell-free system above about 30° (Szer & Ochoa, 1964). Poly-rT is active as a template in the RNA-polymerase system (Krakow, Szer & Ochoa, unpublished results). The binding of [^{14}C]poly-rT to ribosomes was found to be temperature dependent, i.e. the polymer becomes attached above a critical temperature consistently with its ordered state - random coil transition.

A single-stranded helix type of structure, e.g. that currently believed to occur in the neutral form of poly-A (Leng & Felsenfeld, 1966) and likely to be encompassed in part in m-RNA, is not represented by any of the polymers tested. Other obvious limitations of the employed approach concern the model nature of the polymers and, particularly, the absence of specific initial attachment sites, which presumably occur in natural m-RNA at the starting point of the translation process (Takanami, Yan & Jukes, 1965).

MATERIALS AND METHODS

E. coli strain B was grown in a medium containing 0.5% glucose, 0.5% yeast extract (Difco, Detroit, Mich.) 0.5% Casamino acids (Difco), 0.12 M-Na_2HPO_4, 0.12 M-KH_2PO_4, pH 7.0. Cells were harvested during early logarithmic phase and the S-30 fraction was isolated and preincubated according to Nirenberg & Matthaei (1961). The ribosomal fraction was then prepared and washed twice after Lengyel, Speyer

& Ochoa (1961). The ribosomal pellet was resuspended in 0.01 M-tris, pH 7.6, containing 0.25 mM-magnesium acetate - 6 mM-mercaptoethanol and dialysed against this buffer for 12 hr. at 4°. The magnesium acetate concentration was raised to 5 mM and dialysis was continued for another 12 hr. Thus prepared ribosomes were divided into portions and stored on solid CO_2 for up to three weeks. Protein concentration was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Each fresh batch of ribosomes was tested for its ability to associate with [^{14}C]poly-U prior to experiments with the artificial polymers.

^{14}C-Labelled 5′-nucleoside pyrophosphates were synthesized by the following methods. 5′-rTDP was prepared from commercial [^{14}C]ribosethymine (Calbiochem, Luzerna, Switzerland). 50 μc of ribosethymine (17 mc/m-mole) was diluted to sp. act. 0.02 with unlabelled ribosethymine (Calbiochem.) and a stepwise synthesis was carried out through the 2′,3′-isopropylidene derivative, and the 5′-monophosphate as described in detail for non-radioactive 5′-rTDP (Szer *et al.*, 1963). ^{14}C-Labelled 5′-*N*-MeUDP was obtained starting from commercial [^{14}C]uridine (Amersham Centre, Buckinghamshire, England). 100 μc of uridine (243 mc/m-mole) was diluted to sp. act. 0.02 with unlabelled uridine (Light Co., Colnbrook, England) and a stepwise synthesis was carried out through the 2′,3′-isopropylidene derivative, the *N*-methyl derivative and the 5′-monophosphate as described earlier for unlabelled 5′-*N*-MeUDP (Szer & Shugar, 1961). [^{14}C]UDP (7.78 mc/m-mole) was a commercial product (Schwartz Bioresearch, Orangeburg, N.Y.). It was diluted prior to polymerization with unlabelled UDP (Calbiochem.) to sp. act. 0.02. Labelled nucleoside 5′-pyrophosphates were polymerized with *Azotobacter vinelandii* polynucleotide phosphorylase under standard conditions (Basilio & Ochoa, 1963) and isolated as described elsewhere (Szer & Ochoa, 1964). Poly-DHU and a copolymer containing DHU-U residues at a ratio of 7 : 3 were obtained by photochemical reduction of [^{14}C]poly-U as first described for the unlabelled polymer by Cerutti, Ikeda & Witkop (1965). In order to remove possible short fragments, polymers were dissolved prior to use in 2 M-KCl at a concentration of about 3 - 5 mg./ml. and precipitated with one-third volume of ethanol at -15°. After two washings with 90% ethanol the precipitate was dissolved in minimum water and dialysed twice for 3 hr. against glass-distilled water. Thus prepared polymers were stored in solution at -60° in small portions. The concentrations of polymer solutions were estimated spectrophotometrically. The distribution pattern of [^{14}C]poly-U alone along the gradient is shown in Fig. 1d. All the other polymers gave similar patterns, first traces of radioactivity making their appearance in the 17th - 18th tube, near the meniscus. A low background (less than 2 counts/min.) Nuclear-Chicago gas-flow counting system was used.

Sucrose gradient centrifugation, fractionation and analysis of the fractions for absorption and radioactivity distribution were performed by routine methods (e.g. Takanami & Okamoto, 1963a). Twenty three fractions were usually collected. A 5 to 20% linear gradient was used throughout in 0.01 M-tris, pH 7.6, containing 5 mM-magnesium acetate. Centrifugation was carried out for 2 hr. at 100 000 *g* using Spinco rotor SW39 at rotor temperature about 5°, unless otherwise specified

in the text. A 0.2 ml. sample containing from 0.25 to 1.0 mg. of ribosomes and an appropriate amount of polymer was preincubated 10 min. at 20°, unless otherwise described in legends to Figures, and was layered on the top of a prechilled gradient. Several mixing ratios of polymer : ribosome from 1 : 30 to 1 : 200 (by weight) were tested. Decreasing the proportion of polymer relative to ribosomes shifted somewhat the distribution of radioactivity toward the polysomal region, as observed earlier by Takanami & Okamoto (1963a); in none of our experiments, however, did we find a shift of two-thirds of the ribosomes to the "heavy" region, amounting to an almost total disappearance of the principal 70s and 100s peaks, as observed by these authors. A significant change in the distribution pattern of ribosomes manifested in the 260 mμ absorption profile of the gradient would greatly facilitate the expe-

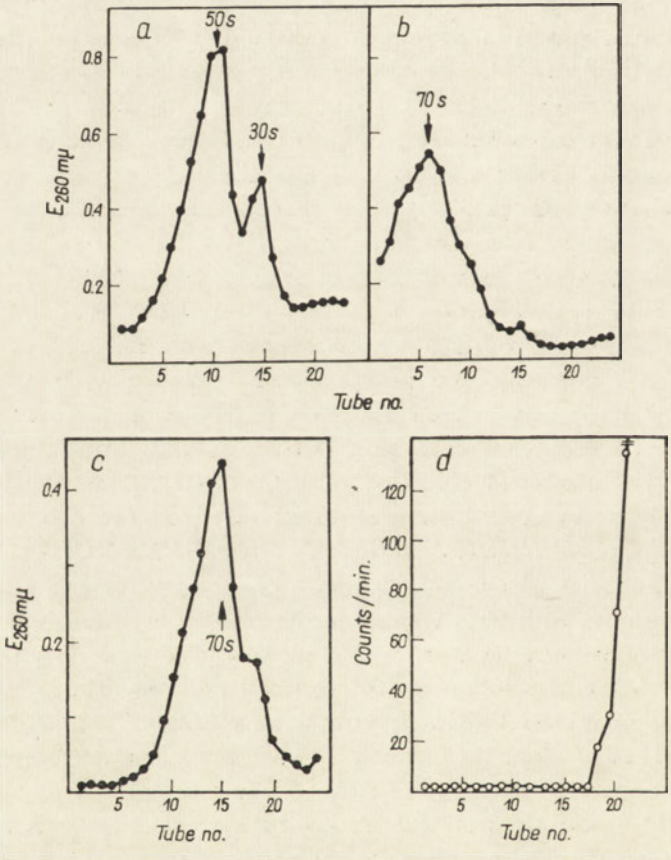

Fig. 1. Sedimentation analysis of ribosomes and polymers in a sucrose gradient (5 - 20%). *a*, Ribosomes (0.25 mg.) after dissociation into subunits (at 0.25 mM-Mg^{2+}); centrifugation for 3 hr. at 35 000 rev./min. *b*, Reformed ribosomes (0.25 mg., at 5 mM-Mg^{2+}); centrifugation as in *a*. *c*, Reformed ribosomes (0.20 mg., at 5 mM-Mg^{2+}); centrifugation for 2 hr. at 30 000 rev./min. Centrifugation of mixtures of polymers and ribosomes (cf. Figs. 2, 3 and 5) was carried out under these conditions. *d*, [^{14}C]Poly-U (30 μg., 0.02 mc/m-mole); centrifugation for 2 hr. at 30 000 rev./min. All other [^{14}C]polymers employed were run alone in the gradient and gave the same patterns.

rimental procedure since the employment of radioactive polymers might have been avoided in some instances. We therefore attempted to obtain such a system varying the conditions of bacterial growth by impoverishing the medium, prolonging preincubation periods of the S-30 fraction to remove endogenous m-RNA, and increasing the time of dissociation and recombination of ribosomal subunits. In all instances the distribution of radioactivity and absorption along the gradient were similar to the results obtained by Spyrides & Lipmann (1962) and Barondes & Nirenberg (1962). Experiments described in the next paragraph were performed at a polymer : ribosomes mixing ratio of 1 : 30 by weight. The patterns of ribosomes alone in 0.25 mM-Mg^{2+} and 5 mM-Mg^{2+} are shown in Fig. 1a and 1b, respectively. Other details are given in legends to Figures. Incorporation of [^{14}C]amino acids into the insoluble fraction was assayed by described methods (e.g. Szer & Ochoa, 1964). ^{14}C-Labelled phenylalanine, leucine, valine, serine, tyrosine and alanine (Schwartz, Orangeburg, N.Y.) of specific radioactivity (mc/m-mole) 2.5, 2.0, 2.5, 10.0, 10.0, 2.5, respectively, were used.

RESULTS

The interaction of m-RNA, natural or synthetic, with ribosomes is conveniently studied by sucrose density gradient technique. The distribution of radioactivity along the gradient is indicative of the synthetic polymer attachment to the 70s - 100s ribosomes, as well as of the formation of heavier polysomal particles. The preincubation procedure (Nirenberg & Matthaei, 1961) and the dissociation of 70s particles into 50s and 30s subunits with subsequent recombination, serve to remove residual pieces of endogenous m-RNA still attached to ribosomes (Takanami & Okamoto, 1963a) and to facilitate the binding of exogenous messenger. Fig. 2a presents the interaction of [^{14}C]poly-U with ribosomes. Poly-U is widely used as a reference polymer in binding studies. Its interaction with ribosomes is much stronger than that of other polyribonucleotides, and it easily forms polysomes within a temperature range from 8° to 37° (Moore, 1966a; Logan & Whitmore, 1966). Fig. 2b

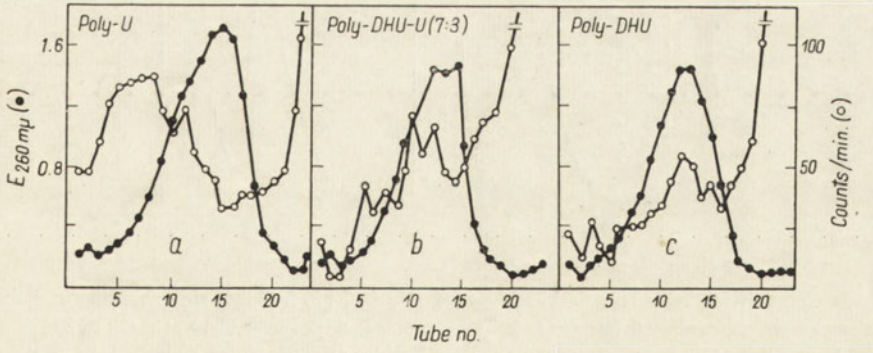

Fig. 2. Sedimentation analysis of mixtures of ribosomes and polymers, as indicated in the drawings. Centrifugation as in Fig. 1c. For details see Materials and Methods.

and 2c show representative examples of poly-DHU-U(7 : 3) and poly-DHU binding to ribosomes. It will be seen that the general pattern of absorption and radioactivity distribution along the gradient is similar to that in Fig. 2a. In several binding experiments, however, in which different batches of ribosomes were employed, we have repeatedly observed a somewhat diminished binding capacity of the hydrogenated polymers as compared to poly-U. The amount of radioactivity found near the bottom, i.e. in the polysomal region, was reduced. The decrease in polysome forming capacity may possibly be attributed to a decrease in the polymer chain length due to the hydrogenation procedure. It is recalled that a single ribosome binds strongly, and renders ribonuclease-resistant, a poly-U fragment containing an average of 25 to 30 nucleotides (Takanami & Zubay, 1964). Alternative explanations of the diminished polysome forming capacity are possible, but in view of the exchange experiments described below, it does not seem likely that the base modification is essential.

The positive outcome of binding experiments with hydrogenated polymers raised the question whether poly-U and poly-DHU are attached to the same ribosomal binding sites. Moore (1966a) has demonstrated that the association of poly-U and poly-C with ribosomes is mutually exclusive, and that the two polymers compete for the same binding sites. Poly-U is very effective in binding and can displace a part of bound poly-C. We have performed displacement experiments using subsequently non-labelled poly-DHU-U(7 : 3) plus [^{14}C]poly-U, and non-labelled poly-U plus [^{14}C]poly-DHU-U(7 : 3). Ribosomes were first preincubated with an excess of non-labelled poly-DHU-U(7 : 3); under these conditions all available binding sites were saturated and the formation of complexes containing no more than one or very few ribosomes per polymer molecule (monosomes) was favoured. Ribosomes preincubated in this way were indeed not capable of polysome formation upon subsequent addition of small amounts of [^{14}C]poly-U (Fig. 3a). It is also clear from Fig. 3a that the [^{14}C]poly-U added was hardly capable of displacing the

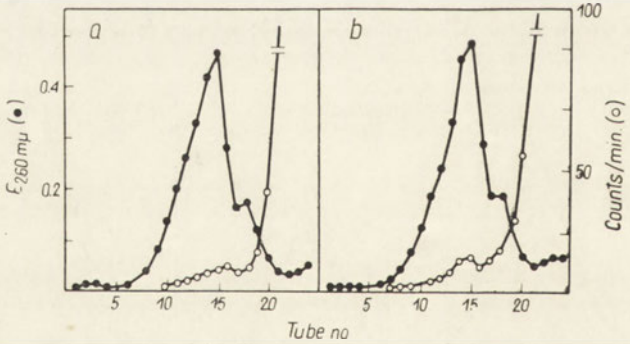

Fig. 3. Competition between poly-U and poly-DHU-U(7 : 3) for ribosomal binding. *a*, Ribosomes (0.2 mg.) were preincubated 10 min. at 20° with unlabelled poly-DHU-U(7: 3) (14 μg.), [^{14}C]poly-U (6 μg.) was added and incubation continued for 10 min. Centrifugation as in Fig. 1c. *b*, Ribosomes (0.2 mg.) were preincubated with unlabelled poly-U (28 μg.), and [^{14}C]poly-DHU-U(7 : 3) (6 μg.) was added. Other details as in *a*.

hydrogenated polymer. Only a trace of radioactivity (cf. Fig. 2a) is seen in the monosomal region of the gradient, indicating some exchange between the two polymers. The same experiment was then repeated using an excess of unlabelled poly-U for preincubation with the subsequent addition of a small amount of [^{14}C]poly-DHU-U (7 : 3); it will be seen from Fig. 3b that nearly the same pattern was obtained. We therefore conclude that the two polymers are attached to the same binding sites. Moreover, since neither polymer can displace the other one from the complex, their affinity for binding ribosomes is presumably of the same order, quite unlike that of poly-U *versus* poly-C.

Kinetic experiments on amino acid incorporation in a cell-free system have been also employed to demonstrate that the ribosome has the same binding site for various polymers. Haselkorn & Fried (1964) have shown that if poly-U and poly-C are present in the same system, phenylalanine is incorporated in preference to proline. This is most plausibly explained in terms of a ribosomal preference for binding poly-U. Poly-DHU lacks messenger activity and, although the binding properties of both polymers appear to be similar, one may anticipate that the addition of s-RNA and the supernatant fraction will considerably enhance the affinity of poly-U to ribosomes (Hatfield, 1965; Suzuka, Kaji & Kaji, 1966) but not that of poly-DHU. We have, however, carefully examined the initial period of phenylalanine incorporation in the presence of poly-DHU-U(7 : 3). In four consecutive experiments we have found the initial rate of poly-U directed phenylalanine incorporation somewhat diminished in the presence of poly-DHU-U(7 : 3) relative to controls without the hydrogenated polymer (Fig. 4). The inhibition was observed only during the first 2 to 5 min.; it ranged from 25 to 65% and was more pronounced

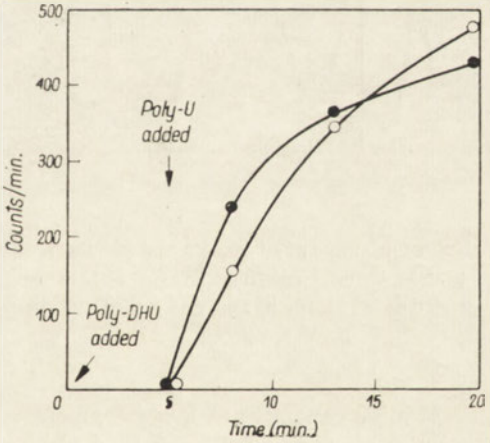

Fig. 4. Time-course of poly-U dependent phenylalanine incorporation: (○), in the presence of poly-DHU-U(7 : 3); (●), control without poly-DHU-U(7 : 3). 0.75 ml. of the standard incorporating mixture (containing 1.5 mg. ribosomal protein) was incubated for 5 min. at 37° with 12 μg. poly-DHU-U(7 : 3) prior to the addition of 66 μg. poly-U. A control was preincubated without poly-DHU-U(7 : 3). 0.2 ml. samples were withdrawn at time intervals indicated and assayed for phenylalanine incorporation.

with old (4 - 5 weeks stored) than with freshly isolated ribosomal preparations. Unexpectedly, a higher level of total incorporation (after 15 - 20 min.) was obtained in all cases when poly-DHU-U(7 : 3) was present in the system. No immediate explanation for this phenomenon is forthcoming. It is possible that the life-time of poly-U is somewhat prolonged in the presence of an additional substrate available for nucleolytic enzymes.

Parallel to the binding studies with the hydrogenated polymers we have tested their ability to promote phenylalanine incorporation in the cell-free system. We have also assayed a number of other amino acids, including those containing two U's in their respective codons. Neither phenylalanine, nor any of the amino acids tested were incorporated. During the course of this investigation several papers appeared dealing with the same problem (Rotman & Cerutti, 1966, Smrt, Škoda, Lisý & Šorm, 1966) and it was convincingly demonstrated that 5,6-dihydrouracil does not replace uracil or any other base in coding. We will therefore not repeat the details of our experiments, since they fully confirm the results of the foregoing authors.

The interaction of poly-*N*-MeU with ribosomes is shown in Fig. 5c; it is evident that *N*-methylation does not affect the ribosome-binding and polysome-forming capacity of the polymer. The interaction of poly-rT with ribosomes proved to be temperature-dependent (Fig. 5a and 5b). At 37° poly-rT exhibited a good ribosome-

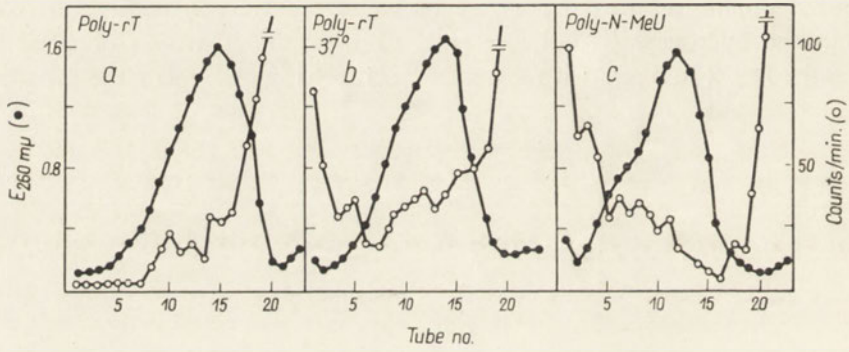

Fig. 5. Sedimentation analysis of mixtures of ribosomes and polymers, as indicated in the drawings. For details see Materials and Methods. Centrifugation for 2 hr. at 30 000 rev./min., except for *b* where preincubation was carried out at 37° for 10 min. and centrifugation for 1 hr. at 30 000 rev./min., rotor temperature 26°.

-binding and polysome-forming capacity; at lower temperatures when the polymer becomes ordered, its ability to bind ribosomes is sharply reduced. Some radioactivity still present in the 70s - 100s region (cf. Fig. 5a) can be possibly explained by the presence of short, unstructured fragments. Such fragments may be present in the rather polydisperse polymer preparation, or may result from degradation by ribosomal nucleases during the experiment. The lack of messenger activity exhibited by poly-rT below 30° (Szer & Ochoa, 1964) should be apparently attributed

to the interference of its ordered state with ribosomal attachment whereas the total lack of messenger activity of poly-*N*-MeU (Wahba *et al.*, 1963) is a result of later events, conceivably the lack of interaction at the codon-anticodon level.

DISCUSSION

Some remarks with respect to polymers containing non-complementary bases, *N*-methyluracil and 5,6-dihydrouracil, may be pertinent to this discussion. Poly--*N*-MeU does not form an ordered state under any conditions, nor is it capable of entering the twin stranded complex with poly-A. Steric hindrance of the *N*-methyl group is the most probable reason preventing any interaction in both instances. The inability of poly-DHU to complex with poly-A should be explained in quite different terms. It may be assumed that lack of aromaticity affects stacking of bases and weakens short range forces between them. Nevertheless, the two polymers form complexes with ribosomes at a minimum Mg^{2+} concentration required for the binding of poly-U. Displacement and kinetic experiments described in the preceding paragraph demonstrate that polymers, both active and inactive as templates, occupy the same ribosomal binding sites. This indicates clearly that the base moiety does not have any direct role in polynucleotide attachment to ribosomes and, hence, complementary base pairing as a source of this interaction is ruled out. This limits the number of alternatives but gives no positive indication as to what might be responsible for binding. Several investigators have proposed cationic bridges holding together phosphate groups on different molecules (for review see Watson, 1965). Moore (1966a) observed recently that the difference in size between such cations as Mg^{2+} and spermidine, both active in binding, makes this idea doubtful. On the other hand, if it is assumed that cations serve in this interaction only to neutralize the backbone charges, then they would permit close approach of the ribosome and m-RNA, but not binding. Yet, the reversibility of the binary m-RNA - ribosome complex upon divalent cation concentration changes in bacterial systems, suggests the involvement of secondary, cation-dependent forces and, thus, the participation of the phosphate groups, one way or another, is obligatory. Intermolecular hydrogen bonding derived from the 2′-OH group has been advanced recently by Arnott *et al.* (1966) as a possible factor. It should be recalled, however, that single-stranded DNA is also capable of association with ribosomes (Takanami & Okamoto, 1963b). Since neither the base nor sugar moieties appear to matter, the phosphate group remains as the most plausible site of interaction.

On the ribosomal side, the mere fact that the 30s subunit is active while the 50s is not, indicates that some features of ribosomal tertiary structure are involved. From the point of ribosomal groups essential for binding at least two possibilities are to be considered: amino groups of basic ribosomal protein or ribosomal RNA amino groups. A number of basic proteins, natural and synthetic, have been shown in model investigations to bind effectively polynucleotide phosphate and to render the bound fragment less susceptible to ribonuclease digestion (cf. Sober, Schlossman, Yaron, Latt & Rushizky, 1966). The affinity of a polyvalent cation for binding

polynucleotide phosphate increases with an increase in the number of positively charged groups per molecule (Felsenfeld, 1962; Szer, 1966). For instance, polylysine is known to displace quantitatively divalent metal ions from polynucleotides (Felsenfeld, 1962). While this appears an attractive idea, recent experimental evidence points rather to r-RNA amino groups as being responsible for the interaction with m-RNA. Moore (1966b) investigated the effect of selective chemical pretreatment of ribosomes on their binding activity. He demonstrated that reagents affecting r-RNA amino groups destroy the binding capacity whereas dinitrofluorobenzen, an amine reagent assumed to react only with ribosomal protein, has no effect. This undoubtedly constitutes important evidence but it appears to us that chemical pretreatment might also affect some unknown features of ribosomal structure. An unambiguous identification of binding sites and chemical groups directly involved in binding m-RNA requires further clarification of the structure of ribosomes and their subunits.

Experiments on the temperature-dependent binding of poly-rT to ribosomes give strong support to the already expressed view that polymer configuration is a factor of primary importance in this interaction (Takanami & Okamoto, 1963). Above 35° poly-rT behaves as a random coil while below its T_m it exhibits properties typical of a double helix-like structure (Szer, 1966) and is almost inactive in binding. It has been shown elsewhere (Szer & Ochoa, 1964) that poly-rT replaces poly-U in promoting polyphenylalanine synthesis but profound differences between the behaviour of the two polymers in the *E. coli* system have been found. For instance, poly-U remains a fairly active messenger on decreasing temperature to 20° while the activity of poly-rT ceases below 30°. It is now apparent that no interaction occurs between the ribosome and the polymer until the latter exists in the ordered state.

While the base does not appear to play any direct role in binding, it nonetheless determines secondary structure properties of a polymer, and they, in turn, constitute the key factor in determining the affinity of a given polymer for binding ribosomes. When the base is involved in a hydrogen bonded structure, as in poly-(A+U) or in poly-rT, no association takes place. Poly-C, which is currently believed to form a single stranded helix with stacked bases at neutral pH, has been recently shown to interact with ribosomes less effectively than poly-U. It presumably binds one ribosome per polymer molecule and is hardly capable of polysome formation (Moore, 1966a). Taking into account that (i) the availability of the base for direct interaction does not matter, (ii) the phosphate residue is exposed outside the helix and is therefore available for interaction even in the ordered state, it may be concluded that the main feature preventing entirely ribosomal binding is the existence of the ordered state. By virtue of its rigid shape it cannot be accommodated on the ribosomal surface. A single stranded helix with a less restricted configuration is capable of forming monosomes while polymers exhibiting a random coil configuration easily form polysomes regardless of the properties of their constituent bases. The lack of mutual displacement ability by poly-U and poly-DHU supports the view that the affinity of polynucleotides for ribosomes is closely related to configurational factors.

In conclusion, the reported results suggest strongly that the interaction of the synthetic messenger and the ribosomal particle is unspecific in the sense that it is not predetermined by any particular base, or base sequence.

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PRZYŁĄCZANIE SYNTETYCZNYCH POLIRYBONUKLEOTYDÓW DO BAKTERYJNYCH RYBOSOMÓW

Streszczenie

1. Kwasy poli-5,6-dwuhydrourydylowy i poli-*N*-metylourydylowy, które nie tworzą kompleksów z kwasem poli-adenylowym i są nieaktywne jako matryce w układzie polimerazy RNA oraz w układzie syntezy białka *in vitro*, mają zdolność wiązania rybosomów bakteryjnych. Obydwa polimery przyłączają się do rybosomów w sposób podobny do kwasu poli-urydylowego i zajmują na rybosomie te same miejsca wiążące.

2. Kwas poli-5-metylourydylowy, który tworzy kompleksy z kwasem poli-adenilowym i posiada własności matrycowe, wiąże się z rybosomami w temperaturach powyżej swego przejścia ze stanu uporządkowanego w stan bezładnego kłębka.

3. Dane te świadczą o tym, że zasada nie bierze bezpośredniego udziału w procesie współdziałania syntetycznego informacyjnego RNA z rybosomem; uwypuklają one znaczenie konformacji polimeru w tym procesie.

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DIKETOPIPERAZINES, THE NON-ASSIMILABLE COMPONENTS OF PANCREATIC HYDROLYSATES OF PROTEIN FOR INFUSION

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1. Diketopiperazines and pyroglutamic acid were found to be present in the hydrolysates obtained by *in vitro* digestion of egg-white, casein and soluble soya-bean protein with pancreatic enzymes. 2. The quantities of these compounds, as well as the structure of diketopiperazines depend on the kind of protein used and may account for differences observed in the degree of utilization (after intravenous infusion) of bound amino acids of the hydrolysates of different proteins.

It has previously been shown that about 50% of bound amino acids of enzymic bovine blood protein hydrolysate (used for intravenous infusion) is found in the form of diketopiperazines and pyroglutamic acid (Bułhak & Chmielewska, 1964; Chmielewska, Bułhak & Toczko, 1966). It has been also established that these compounds constitute the main part of so-called non-assimilable peptides of the preparation (Chmielewska, Toczko & Szumiel, 1964). It seemed interesting to find out to what extent the presence of this type of compounds can be related to the reactions which accompany the hydrolysis of the protein with pancreatic enzymes, and to what degree their quantity and composition depend on the kind of protein used. Egg-white, casein and water-soluble soya-bean protein were chosen for the experiments, because they are most frequently used as starting materials in production of preparations for parenteral amino acid feeding.

EXPERIMENTAL

A sample of about 40 g. of dry denatured protein (egg-white, or casein, or soluble soya-bean protein), equivalent to 6 g. of nitrogen, was ground with 600 ml. of water saturated with toluene. The suspension was adjusted to pH 7.0 and after addition of 9 g. of minced and activated fresh porcine pancreas and about 50 ml. of toluene left to stand for 5 days at 37°. Two further samples of 9 g. of pancreas were added to the suspension on the second and third day of incubation. The pH was maintained between 6.5 and 7.0. After five days the hydrolysate was adjusted to pH 4.5, heated for 15 min. at 90°, cooled, filtered, and the solution freeze-dried.

The hydrolysate of 27 g. of porcine pancreas in 600 ml. of water was prepared in the same way to evaluate autodigestive processes.

The non-ampholytic amino acid derivatives (fraction *I*) of the hydrolysate were isolated on an Amberlite IR-120 (H^+) column and were further separated into three subfractions: neutral, acidic and strongly-acidic (*a*, *b* and *c*, respectively) on Dowex 2X8 (acetate form) column, as described previously (Chmielewska *et al.*, 1966).

The complete acid hydrolysis was carried out in sealed ampoules in 6 N-HCl for 20 - 24 hr. at 105 - 110°.

The alkaline hydrolysis was performed in 0.1 N-NaOH for 17 hr. at 37°. The sample was then applied on an Amberlite IR-120 (H^+) column. After washing the column with water (effluent) the peptides were eluted with 4 N-ammonia (eluate). The effluent and eluate were concentrated in a rotatory evaporator at 30 - 35° and analysed.

The effluent was hydrolysed in acid and the resulting hydrolysate was used for amino acid analysis. The peptides from the eluate were separated by high-voltage electrophoresis in pyridine - acetic acid - water buffer, pH 6.2, as described previously (Chmielewska *et al.*, 1966).

Amino acids in the hydrolysates of individual dipeptides were identified by electrochromatography. The molar ratios of amino acids were determined by the DNP-method of Levy, as described by Toczko, Szumiel & Manicki (1965).

Total nitrogen was determined by the Kjeldahl semimicromethod. Determinations of amino nitrogen were carried out by the manometric ninhydrin - CO_2 method of Van Slyke, MacFayden & Hamilton (1943) with the use of Thomas-Van Slyke manometric apparatus.

Results are expressed in g. or mg. of nitrogen per 10 g. of total (sum of free and bound) amino nitrogen of hydrolysate.

RESULTS AND DISCUSSION

In conditions of enzymic hydrolysis applied in these experiments, the egg-white protein was digested in 92%, casein in 86%, and the soya-bean protein in 77%.

The quantities of different nitrogen forms in each enzymic hydrolysate and the amino nitrogen content in subfractions *a*, *b* and *c* are given in Table 1.

The amino nitrogen in fraction *I* (sum of subfractions *a*, *b* and *c*) from the hydrolysate of egg-white amounts to 22%, of casein to 26% and of soya-bean protein to 13% of total bound amino nitrogen, the rest of it being found in true peptides. The quantity of bound amino nitrogen originating from pancreas selfdigestion amounted to not more than 2 - 4% of amino nitrogen of fraction *I*. This indicated that the *N*-substituted amino acids, which were found in the hydrolysates, derived from the investigated proteins.

It has been established earlier (Chmielewska *et al.*, 1966) that under the above described conditions of hydrolysate fractionation the subfraction *a* contains neutral compounds, including all neutral diketopiperazines, whereas subfraction *b* the

Table 1

Nitrogen forms in protein digest

For details see text.

Form of nitrogen	Egg-white	Casein	Soya-bean protein
	Nitrogen (g./10g. of total amino N)		
Total N	11.77	11.96	12.37
Amino N of amino acids	7.97	6.21	6.84
Bound amino N	2.03	3.79	3.16
In fraction *I*	0.41	1.07	0.40
In subfraction *a*	0.13	0.07	0.01
In subfraction *b*	0.25	0.67	0.35
In subfraction *c*	0.03	0.33	0.04

Table 2

Diketopiperazines and pyroglutamic acid in protein digest

For details see text.

Subfraction	Compound	Egg-white	Casein	Soya-bean protein
		Nitrogen (mg./10 g. of total amino N)		
a	Neutral diketopiperazines	31	74	9
b	Acidic diketopiperazines	155	320	120
	Pyroglutamic acid	94	352	230

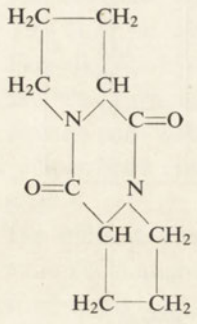
acidic compounds, such as pyroglutamic acid and all acidic diketopiperazines. For that reason compounds of subfraction *c* were not examined.

For further study, subfractions *a* and *b* were submitted to alkaline hydrolysis, during which diketopiperazines were split to dipeptides, and in much lesser degree to amino acids, whereas pyroglutamic acid was not decomposed. The compounds were then analysed and identified. The results of this analysis showed that the diketopiperazines were the only components of subfraction *a* of both casein and soya-bean protein hydrolysates, whereas the diketopiperazines in the corresponding subfraction from egg-white accounted for only 25% of amino nitrogen of this subfraction.

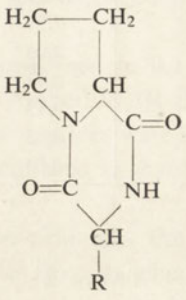
Subfraction *b* of each of the three hydrolysates proved to contain only acidic diketopiperazines and pyroglutamic acid (Table 2). The quantities of pyroglutamic acid and diketopiperazines in the hydrolysates varied greatly, depending on the protein used. The ratio of amino nitrogen of diketopiperazines to the amino nitrogen of pyroglutamic acid was equal to 2 : 1 for egg-white, 1 : 1 for casein, and 1 : 2 for soya-bean protein. In each preparation the acidic diketopiperazines were prevailing over the neutral ones.

Proline was the main amino acid among those found in neutral diketopiperazines, especially in casein hydrolysate. In acidic diketopiperazines, in addition to dicarboxylic amino acids, appreciable quantities of following amino acids were found: serine and glycine in egg-white hydrolysate, proline and serine in the casein hydrolysate, and glycine and proline in the soya-bean protein hydrolysate.

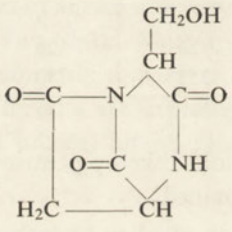
The structure of the main diketopiperazines from the investigated hydrolysates is given in Scheme 1.



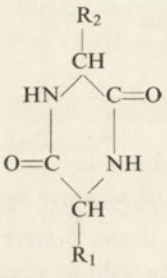
Compound I



Compound II: R=—H
IV: R=—CH_2CH_2COOH



Compound III



Compound V: R_1=—CH_2COOH; R_2=—CH_2OH
VI: R_1=—CH_2CH_2COOH; R_2=—CH_2OH
VII: R_1=—CH_2CH_2COOH; R_2=—H
VIII: R_1=—CH_2CH_2COOH; R_2=—$CHOHCH_3$

Scheme 1. Structure of identified diketopiperazines.

The content of respective compounds in subfractions *a*, and *b*, as well as their percent contribution to a given subfraction are shown in Table 3.

The egg-white hydrolysate contains considerable amounts of diketopiperazines composed of serine and aspartic acid (compounds III and V) and of glycine and glutamic acid (compound VII). The compounds III and V are the most characteristic feature of this preparation, and are not found in the two other hydrolysates.

Table 3

Diketopiperazines in protein digest

For details see text. The results are expressed in mg. of nitrogen per 10 g. of total amino N and as percentages of the total amino N of diketopiperazines of the respective subfraction.

Sub-fraction	Diketopiperazine	Compound no. *	Egg-white		Casein		Soya-bean protein	
			(mg.)	(%)	(mg.)	(%)	(mg.)	(%)
a	Total		31.0	100.0	74.0	100.0		
	Pro-Pro	I	5.4	17.4	41.4	55.9		
	Pro-Gly	II	5.0	16.1	6.2	8.4		
	Ser-Asp	III	8.6	27.9				
b	Total		155.0	100.0	320.0	100.0	120.0	100.0
	Pro-Glu	IV	29.2	18.8	173.8	54.3	37.4	31.2
	Ser-Asp	V	44.4	28.6				
	Ser-Glu	VI			48.4	15.1	18.0	15.0
	Gly-Glu	VII	46.2	29.8	17.2	5.4	43.0	35.8
	Thre-Glu	VIII	6.4	4.1	40.6	12.7	13.8	11.5

* See Scheme 1.

Predominance of neutral and acidic diketopiperazines containing proline (compounds I, II and IV) is a peculiarity of the casein hydrolysate. Amino nitrogen of these compounds amounts to 62% of the total amino nitrogen of all diketopiperazines present in this preparation. In the soya-bean protein hydrolysate the diketopiperazines composed of glycine and glutamic acid (compound VII) and of proline and glutamic acid (compound IV) are prevailing.

The above presented results indicate that diketopiperazines and pyroglutamic acid are regular components of hydrolysates obtained by *in vitro* digestion of protein with pancreatic enzymes, and consequently they are present also in preparations for intravenous infusion, produced in this way. It is likely, therefore, that the degree of utilization of bound amino acids of a given preparation can be related to the kind of the protein used for its production.

Our thanks are due to Mr. Jan Kanabus for the amino nitrogen determination. This work was supported partly by the Department of Medical Sciences of the Polish Academy of Sciences.

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DWUKETOPIPERAZYNY, NIEPRZYSWAJALNE SKŁADNIKI ENZYMATYCZNYCH HYDROLIZATÓW BIAŁKOWYCH DO WLEWAŃ DOŻYLNYCH

Streszczenie

1. Stwierdzono występowanie dwuketopiperazyn i kwasu piroglutaminowego w hydrolizatach, otrzymanych w wyniku trawienia *in vitro* białka jaja, kazeiny i rozpuszczalnych w wodzie białek soi enzymami trzustki.

2. Ilość tych związków, jak również budowa dwuketopiperazyn zależy od rodzaju białka użytego do hydrolizy i może być przyczyną różnic w stopniu wykorzystania aminokwasów związanych hydrolizatów różnych białek, stosowanych do wlewań dożylnych.

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TRANSLATION PROCESSES IN THE *E. COLI* CELL-FREE SYSTEM IN THE PRESENCE OF NATURAL MESSENGER RNA's

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1. Analysis of the product synthesized in the *E. coli* cell-free system under the influence of RNA from the middle portion of silkglands of the silkworm *Bombyx mori* showed that the product newly synthesized under these conditions is not sericin, a silk protein. 2. This product was compared with those synthesized in the same system under the influence of *E. coli* RNA and with no RNA added. The addition of heterologous as well as homologous RNA to the *E. coli* cell-free system prompted the synthesis of proteins which, when compared by autoradiography of tryptic peptide fingerprints, appeared to be similar to the protein newly formed in the *E. coli* system. 3. It was shown by immunoprecipitation, however, that the *de novo* formed proteins are immunologically different. 4. The overall findings point to differences between translation *in vivo* and *in vitro*, and indicate that the translation *in vitro* can be disturbed by addition of heterologous RNA or even of homologous RNA to the *E. coli* cell-free system.

Experiments with synthetic polynucleotides as templates have led to the conclusion that ribosomes are non-specific structures which can synthesize protein directed by messenger RNA (mRNA) they contain at a given moment. This idea has been supported by investigations on the messenger role of the viral RNA in cell-free systems. It has been demonstrated that f2 group of RNA coliphages such as f2, R17, MS2 stimulate the synthesis of phage protein in the bacterial extracts (Nathans, Notani, Schwartz & Zinder, 1962; Nathans, 1965; Yamazaki & Kaesberg, 1966; Capecchi, 1966; Nathans, Oeschger, Eggen & Shimura, 1966). Directed synthesis of f2 phage coat was also reported in *Euglena gracilis* cell-free system (Schwartz, Eisenstadt, Brawerman & Zinder, 1965).

Positive results were also obtained with systems from higher organisms. Arnstein, Cox & Hunt (1964) demonstrated that the ribosomes from rabbit reticulocytes synthesize *in vitro* rabbit haemoglobin under the influence of homologous RNA. Similar results were published by Kruh, Dreyfus & Schapira (1964) and Ganoza, Williams & Lipmann (1965). However, many controversial results exist. The experiments of Schaeffer, Favelukes & Schweet (1964) on directed haemoglobin synthesis in the *E. coli* cell-free system have led to contradictory conclusions. Drach & Lingrel (1966) proved that although one fraction of reticulocyte RNA does stimulate protein synthesis in *E. coli* extracts, the *de novo* synthesized protein is not a globin, but

is rather similar to the *E. coli* protein. Negative results were also obtained with tobacco mosaic virus (TMV) RNA-directed protein synthesis in *E. coli* system as shown by Aach, Funatsu, Nirenberg & Fraenkel-Conrat (1964), who did not detect the synthesis of viral coat. The experiments of Schapira, Padieu, Maleknia, Kruh & Dreyfus (1966) also speak in favour of the limited non-specificity of ribosomes. The authors have shown that the ribosomes of rabbit and guinea pig reticulocytes in cross experiments with ribosome-free supernatants, synthesize two kinds of haemoglobin: one specific for the organism the ribosomes derived from and the other one specific for the organism the supernatant derived from.

In the work presented here, analysis of the protein synthesized in the *E. coli* cell-free system under the influence of RNA from the middle portion of the silkgland of the silkworm *Bombyx mori* L., is described. The analysed products have been compared with those synthesized in the *E. coli* cell-free system in the presence of homologous RNA. A part of this work has been reported at the International Symposium on Biochemistry of Ribosomes and Messenger-RNA is Schloss Reinhardsbrunn, Germany, May 23 - 26, 1967.

EXPERIMENTAL

Reagents. All the chemicals used were commercial products: sodium adenosine triphosphate (ATP), sodium guanosine triphosphate (GTP), pyruvate kinase, sodium dodecyl sulphate (SDS) of Sigma (St. Louis, Mo., U.S.A.); deoxyribonuclease of Worthington Biochemical Corporation (Freehold, N.J., U.S.A.); 2-mercaptoethanol, DL-valine, DL-tyrosine of British Drug Houses (Poole, England); DL-alanine, L-arginine, glycine, DL-histidine, DL-isoleucine, L-lysine, DL-methionine, DL-tryptophan of Merck (Darmstadt, Germany); L-glutamic acid, DL-leucine of Fluka (Buchs, Switzerland); L-aspartic acid, L-cysteine, L-hydroxyproline, L-phenylalanine, DL-proline of Light (Colnbrook, England); DL-serine and DL-threonine of Chemapol (Prague, Czechoslovakia); 1-phenyl,2-thiourea of Eastman Organic Chemicals (Rochester, N.Y., U.S.A.); albumin bovine plasma cryst. A grade of Calbiochem (Los Angeles, Calif., U.S.A.) and millipore filters of VCHZ Synthesia (Uhřiněves, Czechoslovakia), DEAE-cellulose of Whatman (England). Sodium phosphoenolpyruvate (PEP) was prepared from barium salt obtained from Reanal (Budapest, Hungary). Trypsin, commercial product of Nutritional Biochemical Corporation (Cleveland, Ohio, U.S.A.) was purified by 2-day dialysis against 0.001 N-HCl at 4°; the insoluble residue was discarded by centrifugation and the supernatant was freeze-dried and stored at -20°.

The following uniformly ^{14}C-labelled amino acids were used (the activity in mc/m-mole is indicated in parentheses): glycine (2.2 and 67), L-leucine (0.59), L-lysine (4.76), L-phenylalanine (9.8), L-serine (11), DL-tryptophan (9.17), L-tyrosine (13.73), L-valine (4.8) products of the Radiochemical Centre (Amersham, England); L-arginine (4.38), L-glutamic acid (40.8) products of Nuclear Chicago Corporation (Chicago, U.S.A.); L-alanine (65.5), L-aspartic acid (2.6), glycine (22.7), L-serine (24 and 61) products of the Institute for Research, Production and Utilization of

Radioizotopes (Prague, Czechoslovakia). [2(*ring*)-^{14}C]L-Histidine (2.62 mc/m-mole) was a product of California Corporation for Biochemical Research (Los Angeles, Calif., U.S.A.).

Methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Ribonucleic acid was estimated spectrophotometrically, assuming that the extinction at 260 mμ of 1 mg. of RNA is 24. Radioactivity was measured with a liquid scintillation counter type SE-2.

Ribonucleic acid. Silkworm *Bombyx mori* L. RNA and *E. coli* RNA were used for the incorporation experiments. Silkworm RNA was prepared from the middle portion of the silkgland at the fifth instar, 7 - 5 days before spinning. The glands were homogenized in three volumes of 0.01 M-sodium acetate buffer, pH 5.1, containing 0.5% SDS and 0.08% 1-phenyl,2-thiourea, kindly given by Dr. J. Ilan from the Rockefeller University. RNA was isolated according to the SDS-phenol method of Scherrer & Darnell (1962). The sedimentation coefficients, calculated from the centrifugation in Spinco model E analytical ultracentrifuge, were found to be within the range 4s - 14s. *E. coli* RNA freed of sRNA with 1.5 M-NaCl was prepared by the same method and kindly given to us by Dr. S. Perzyński from our Institute.

Sericin. Silkworm cocoons were boiled for 1 hr. in 1% sodium deoxycholate solution and insoluble fibroin was discarded by centrifugation. To the supernatant equal volume of 10% trichloroacetic acid (TCA) was added, the precipitate of sericin centrifuged, washed with ethanol and ether and stored at 4°. For experiments, dry sericin was dissolved in boiling water to the required concentration.

The Escherichia coli *incorporating system. E. coli* B. cells were grown on the glucose medium according to Baldwin & Shooter (1963) and harvested at the logarithmic phase. The cells were washed with cold 0.2 M-KCl, then 0.01 M-$MgCl_2$ - - 0.005 M-mercaptoethanol - 0.01 M-tris-HCl buffer, pH 7.8. The centrifuged pellet was suspended in three volumes of 0.01 M-$MgCl_2$ - 0.06 M-KCl - 0.006 M-mercaptoethanol - 0.01 M-tris-HCl buffer, pH 7.8, frozen in solid CO_2 and disrupted in an Eaton press (Eaton, 1962). The suspension of the disrupted cells was adjusted to pH 7.5 and 1 μg. of deoxyribonuclease added per 1 ml. The suspension was centrifuged for 10 min. at 16 000 *g* at 4°, cell debris was discarded and the supernatant centrifuged for 5 min. at 30 000 *g*. The supernatant (S-30), containing usually 10 - - 17 mg. of protein per ml., was used on the same day.

For the incorporation experiments the S-30 supernatant was preincubated for 2 hr. at 37° in a mixture containing in 1 ml. the following components (in μmoles): KCl, 80; $MgCl_2$, 6; NH_4Cl, 80; ATP, 6; tris buffer pH 7.8, 40; 2-mercaptoethanol, 12; each of 19 non-radioactive amino acids, 0.2; 0.7 ml. of S-30 supernatant (7 - - 12 mg. protein). The preincubated S-30 supernatant was dialysed overnight at 4° against 0.01 M-$MgCl_2$ - 0.06 M-KCl - 0.006 M-mercaptoethanol - 0.01 M-tris-HCl buffer, pH 7.8, and centrifuged for 5 min. at 30 000 *g* at 4°. The incorporation was performed for 30 min. at 37° in a system containing in 1 ml. the following components (in μmoles): KCl, 38; $MgCl_2$, 3.4; NH_4Cl, 30; ATP, 0.7; GTP, 0.17; tris buffer pH 7.8, 14; 2-mercaptoethanol, 4.3; PEP, 4.7; pyruvate kinase, 20 μg.; each of 18 non-radioactive amino acids, 0.11; one ^{14}C-labelled amino acid, 0.04 - 0.1;

silkworm or *E. coli* RNA, 860 μg., and 0.7 ml. of preincubated S-30 supernatant from *E. coli* (5 - 8.5 mg. protein).

To the sample of the incubation mixture, an equal volume of 10% TCA was added, the precipitated protein was washed (Szafrański, Lutowicz & Pużyńska, 1964) and radioactivity measured.

The stimulatory effect of the silkworm RNA on the incorporation of ^{14}C-labelled serine into *E. coli* protein is shown in Fig. 1. To solubilize the newly synthesized radioactive proteins, ultrasonic disintegration was applied.

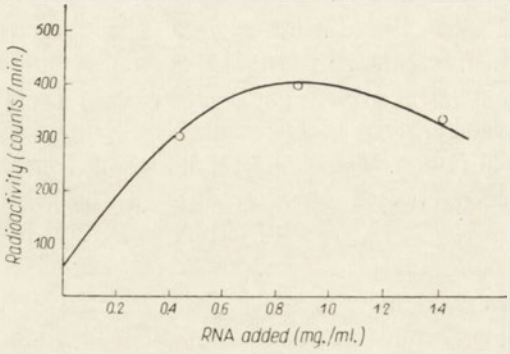

Fig. 1. Stimulation of ^{14}C-serine incorporation into protein in the *E. coli* cell-free system by RNA from the middle portion of silkglands of the silkworm. One ml. of the incubation mixture contained 0.057 μmole of [U-^{14}C]serine (spec. act. 24 mc/m-mole).

Ultrasonic disintegration. The incubation mixture after the incorporation of amino acids was disintegrated in the MSE 100 watt ultrasonic disintegrator for 10 min. in an ice bath. The suspension was freed of ribosomes by centrifugation for 1 hr. in the MSE "Superspeed 40" ultracentrifuge at 105 000 *g* and the supernatant (DS-105) used for the immunological assays.

Preparation of rabbit antisera. The rabbits were immunized according to Simkin, Skinner & Seshadri (1964) either with sericin obtained from *Bombyx mori* cocoons or with *E. coli* S-30 supernatant. The antisera were heated at 56° for 30 min. and the sediments were discarded by centrifugation at 40 000 *g* for 10 min. The presence of antibody in the serum was shown by agar double diffusion analysis, based on the technique of Ouchterlony (1953). The optimum amount of antibody *versus* antigen was established in the usual way (Kabat & Mayer, 1964).

Immunological assays for sericin. To 1 ml. of the radioactive 105 000 *g* supernatant obtained after disintegration (DS-105), 100 μg. of sericin and 1 ml. of the antisericin rabbit serum were added and allowed to stand overnight at 4°. The antigen-antibody precipitate was collected by centrifugation, washed three times with 0.9% NaCl in 0.01 M-tris buffer, pH 7.4, and dissolved in 1 N-NaOH containing non-radioactive serine and glycine. The protein was precipitated with TCA, dried with ethanol, ether, dissolved in concentrated formic acid and the radioactivity was measured.

The results presented in Table 1 show that in the case of protein synthesis stimulated by silkworm RNA as well as *E. coli* RNA, immunoprecipitates contained only a small part of radioactivity present in whole DS-105 supernatant. This indicates that the protein newly synthesized under the influence of silkworm RNA might be not a sericin. Further evidence in favour of this supposition was provided by fractionation of radioactive proteins on DEAE-cellulose and isolation of the fraction corresponding to sericin.

Table 1

Comparison of radioactivity of synthesized protein with that present in immunoprecipitates specific for sericin

The incorporation of ^{14}C-labelled amino acids into protein was performed in 1.4 ml. with *E. coli* preincubated S-30 fraction. In the first experiment [^{14}C]serine (11 mc/m-mole) and [^{14}C]glycine (22.7 mc/m-mole), in the second experiment [^{14}C]glycine (22.7 mc/m-mole), in the third experiment [^{14}C]glycine (67 mc/m-mole) were used, each in amount 0.05 μmole/ml. of the incubation mixture. The samples of S-30 after incorporation of amino acids and DS-105 containing proteins solubilized by sonication were withdrawn, protein precipitated with trichloroacetic acid, washed and its radioactivity measured (see *The* E. coli *incorporating system*). The remaining part of DC-105 was used for immunological precipitation (see *Immunological assays for sericin*).

Expt. no.	Radioactivity of S-30 proteins (counts/min./ml.)			Radioactivity of DS-105 proteins (counts/min./ml.)			Radioactivity of immunoprecipitates (counts/min./ml.)		
	no RNA added	silk-gland RNA	*E. coli* RNA	no RNA added	silk-gland RNA	*E. coli* RNA	no RNA added	silk-gland RNA	*E. coli* RNA
1	860	1890	5970	600	1391	4210	127	118	270
2	495	1640	4000	260	1376	2940	65	70	138
3	875	2600	8450	664	2175	6668	106	103	263

Fractionation of sonicated 105 000 *g supernatant (DS-105) on DEAE-cellulose.* Radioactive DS-105 proteins freed of amino acids on Sephadex G-25 column (1 cm. × 15 cm.) with 0.005 M-tris-HCl buffer, pH 7.4, were fractionated on DEAE-cellulose column with the linear NaCl gradient concentration from 0 to 2 M in 0.05 M-ammonium acetate buffer, pH 5.8. Fractions of 4 ml. were collected. Two drops of 0.5% aqueous solution of bovine plasma albumin were added to each fraction, the protein was precipitated with TCA and collected on millipore filters. The radioactivity of proteins on the filters was measured. The results are presented in Fig. 2.

Fractions 5 to 12 could be expected to contain newly synthesized sericin since, as it had been established in control experiments, native sericin was eluted from the column in this range. Therefore these fractions were submitted to immunological assay. The fractions were pooled, concentrated by freeze-drying and dialysed against 0.01 M-tris-HCl buffer, pH 7.4. Sericin as a carrier and antiserum were added to the dialysed solution as described in *Immunological assays for sericin.*

No radioactivity was found in the antigen-antibody precipitate which indicates that the isolated material is not sericin.

As can be also seen from Fig. 2, the distribution of radioactivity did not correspond in general to *E. coli* protein peaks. On the other hand, very similar patterns of radioactivity were obtained with no RNA added to the system or with addition of *E. coli* RNA. These results have suggested similarity between the proteins newly

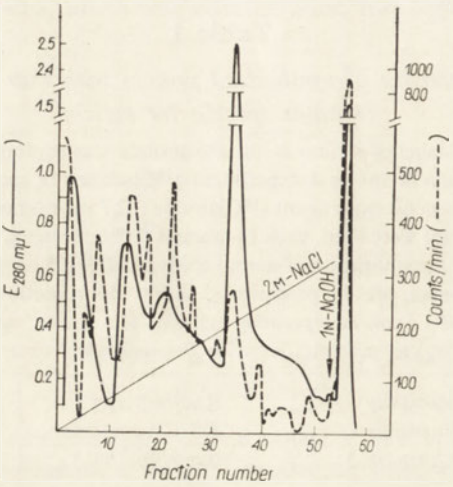

Fig. 2. Fractionation of proteins synthesized in the *E. coli* cell-free system under the influence of silkgland RNA, on DEAE-cellulose. Incorporation of [^{14}C]glycine (67 mc/m-mole) was performed with *E. coli* preincubated S-30 fraction in 4 ml. total volume (see *The* E. coli *incorporating system*). Incubation mixture was sonicated and centrifuged for 1 hr. at 105 000 *g*. DS-105 supernatant freed of amino acids on Sephadex G-25, was applied on DEAE-cellulose column (0.9 × 10 cm.) and eluted with linear NaCl gradient concentration in ammonium acetate buffer.

synthesized in the *E. coli* system in the presence of silkworm RNA and endogenous RNA. To elucidate this possibility and to demonstrate by another method the difference between sericin and the proteins *de novo* formed in the *E. coli* system, fingerprint analysis and autoradiography were performed.

Fingerprint analysis and autoradiography. The radioactive proteins of the DS-105 fraction formed in the *E. coli* cell-free system under the influence of silkworm RNA or *E. coli* RNA as well as with no RNA added, were digested overnight at 37° with trypsin (100 μg./ml.) in the presence of 0.01 M-calcium chloride. Then undigested proteins were discarded by precipitation with an equal volume of 10% TCA. TCA was removed by extraction with ether and the digests were freeze-dried. Digest of the *E. coli* protein obtained in the experiment with no RNA added was considered as *E. coli* native protein digest. The freeze-dried peptides were dissolved in a small volume of water and applied on Whatman no. 1 paper for chromatography. In the first run butanol - acetic acid - water (4 : 1 : 1, by vol.) and in the second 2,6-lutidine - water (132 : 60 v/v) were employed. The chromatograms were stained

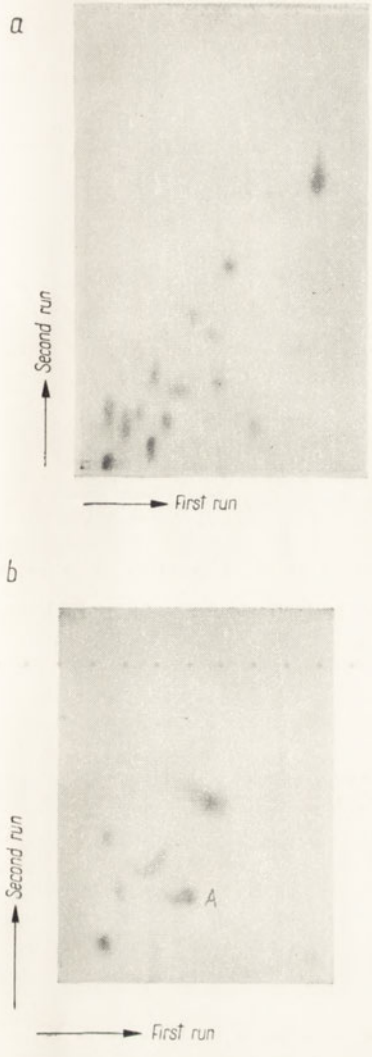

Fig. 3

Fig. 3. The ninhydrin-positive tryptic peptide fingerprints of (*a*), sericin and (*b*), *E. coli* protein.

Fig. 4. Autoradiograms of the tryptic peptide fingerprints of the protein synthesized in the *E. coli* cell-free system under the influence of (*a*), silkgland RNA; (*b*), *E. coli* RNA; (*c*), with no RNA added. The protein was labelled with [U-^{14}C]glycine (spec. act. 24 mc/m-mole). Spot *1* corresponds to free [^{14}C]glycine.

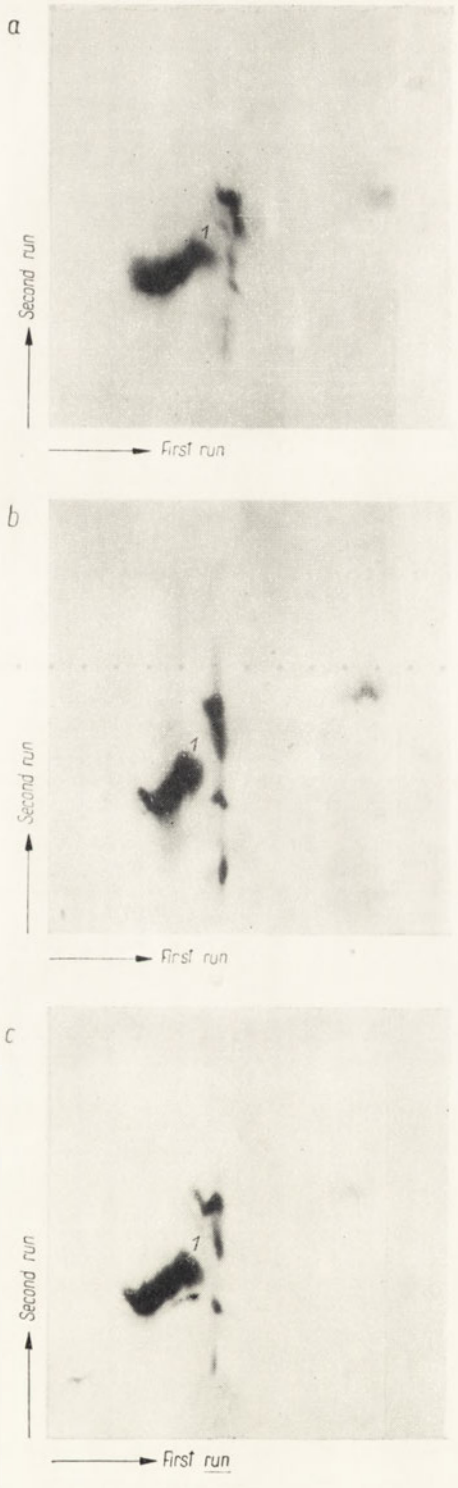

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Fig. 4

with 0.2% ninhydrin in acetone containing 2% pyridine. Native sericin was submitted to the same procedures of digestion and chromatography.

The radioactive fingerprints were superimposed on X-ray plates and left in darkness for 20 days. The plates were developed in a usual way. Tryptic peptide fingerprints of sericin and *E. coli* protein are presented in Fig. 3 and autoradiograms in Fig. 4.

The ninhydrin-positive tryptic peptide fingerprint of sericin (Fig. 3a) differs very much from the autoradiogram of the tryptic peptide fingerprint of the proteins synthesized in the *E. coli* cell-free system in the presence of silkworm RNA (Fig. 4a). On the other hand, this autoradiogram is very similar to the autoradiograms of the tryptic peptide fingerprints of the proteins synthesized in the *E. coli* cell-free system in the presence of added *E. coli* RNA and with no RNA added (Figs. 4b, c). Comparison of the autoradiograms with Fig. 3b shows that the majority of radioactive spots do not correspond to the ninhydrin-positive tryptic peptide fingerprint of *E. coli* protein. This discrepancy does not depend on the lack of glycine in the ninhydrin-positive spots of peptides, since peptide *A* (Fig. 3b), which does not correspond to any of the spots on autoradiograms, does contain glycine, as revealed by hydrolysis in 6 N-HCl, followed by paper chromatography. Thus it appears that only a small part of *E. coli* protein which can be detected by the fingerprint technique is synthesized in a cell-free system.

Table 2

Comparison of radioactivity of synthesized protein with that present in immunoprecipitates specific for E. coli *protein*

The incorporation of ^{14}C-labelled amino acids was performed in 1.4 ml. with the *E. coli* preincubated S-30 fraction. The incubation mixture contained in 1 ml. 0.04 - 0.1 μmole of each of the following ^{14}C-labelled amino acids (the activity in mc/m-mole is given in parentheses): L-alanine (65.5), L-arginine (4.38), L-aspartic acid (2.6), L-glutamic acid (40.8), glycine (2.2), L-histidine (2.62), L-leucine (0.59), L-lysine (4.76), L-phenylalanine (9.8), L-serine (61), DL-tryptophan (9.17), L-tyrosine (13.73), L-valine (4.8) and 0.04 - 0.1 μmole of each of the six non-labelled amino acids. The samples of DS-105 fractions containing proteins solubilized by sonication were withdrawn, protein precipitated with trichloroacetic acid, washed and its radioactivity measured (see *The* E. coli *incorporating system*). From the remaining part, 0.5 mg. of each sample was used for immunological precipitation (see *Immunological assays for* E. coli *protein*).

Expt. no.	Radioactivity of DS-105 proteins (counts/min./sample)			Radioactivity of immunoprecipitates (counts/min./sample)			Radioactivity precipitated (%)		
	no RNA added	silk-gland RNA	*E. coli* RNA	no RNA added	silk-gland RNA	*E. coli* RNA	no RNA added	silk-gland RNA	*E. coli* RNA
1	1128	1932	2208	666	354	333	59	18	15
2	583	1052	1279	189	96	168	32	9	13
3	511	924	1122	153	151	252	30	16	22

The similarity of the three autoradiograms leads to the conclusion that the addition of heterologous or homologous RNA to the *E. coli* cell-free system stimulates the synthesis of a protein which is in many respects similar to the protein synthesized in the *E. coli* system with no RNA added. To answer whether different RNA's stimulate the synthesis of protein specific for *E. coli*, the immunological assays with the antiserum prepared against *E. coli* protein were performed.

Immunological assays for E. coli *protein.* Incorporation of ^{14}C-labelled amino acids into proteins in the *E. coli* cell-free system was performed with no RNA added or with addition of either silkworm RNA or *E. coli* RNA. The radioactive *E. coli* DS-105 supernatant was added to the rabbit antiserum prepared against *E. coli* proteins. The supernatant containing 0.5 mg. of protein in 0.1 - 0.2 ml. was added to 2.5 ml. of antiserum, incubated at 37° for 1 hr. and left at 4° overnight. The antigen-antibody precipitates were washed and their radioactivity measured as described in *Immunological assays for sericin.*

The data presented in Table 2 show that whereas the incorporation of ^{14}C-labelled amino acids into TCA-insoluble precipitates increased on addition of RNA to the *E. coli* cell-free system, the radioactivity of immunoprecipitates decreased. In the first experiment, 59% and in the second and third about 30% of radioactive proteins were precipitated with antiserum prepared against *E. coli* proteins. After addition of silkworm RNA or even homologous RNA to the system, the radioactivity of immunoprecipitates dropped markedly.

It can be concluded on the basis of the presented experiments that the addition of silkworm RNA or *E. coli* RNA to the *E. coli* cell-free system results in synthesis of proteins whose structure resembles proteins formed in this system when no RNA was added, from which, however, they differ immunologically.

DISCUSSION

In our previous report (Szafrański *et al.*, 1964) it has been demonstrated that RNA from the middle portion of silkglands of the silkworm *Bombyx mori* L. stimulates in the *E. coli* cell-free system incorporation of amino acids into TCA-insoluble precipitate. Since RNA which stimulates protein synthesis in cell-free systems is considered to be messenger RNA, it could be expected that the synthesized product would be sericin, a protein with very high serine and glycine content, formed by the middle portion of the silkglands. However, the analysis of the newly synthesized products by serological methods, DEAE-cellulose chromatography, fingerprinting and autoradiography showed no similarity between newly formed protein and sericin.

Protein synthesis in the *E. coli* cell-free system was two- to threefold stimulated by silkworm RNA and up to ninefold by *E. coli* RNA. In both cases only a small portion of the *de novo* synthesized protein was precipitated with antisericin rabbit serum. Immunoprecipitation performed with the fraction corresponding to sericin isolated on DEAE-cellulose, also gave negative results. The autoradiogram of the

tryptic peptide fingerprint of protein synthesized in the *E. coli* cell-free system under the influence of silkworm RNA was found to be different from the tryptic peptide fingerprint of sericin, too. This autoradiogram, however, was very similar to the autoradiograms of fingerprints of proteins formed in the *E. coli* system with addition of homologous RNA or with endogenous RNA. Still the majority of the radioactive spots of these three autoradiograms did not correspond to the ninhydrin-positive tryptic peptide fingerprint of *E. coli* native protein, although peptides on this fingerprint did contain non-labelled glycine. This indicates that only some of *E. coli* proteins are synthesized from ^{14}C-labelled amino acids in a cell-free system.

Protein synthesis in the *E. coli* system increased upon addition of silkworm or *E. coli* RNA's, while the radioactivity of immunoprecipitates specific for *E. coli* protein decreased, as compared with that when no RNA was added to the system. It can be concluded that RNA from the silkworm as well as homologous RNA stimulate in the *E. coli* system the synthesis of a protein similar, as indicated by autoradiography, to the protein formed in this system without addition of RNA, but differing from it in the structure responsible for immunoprecipitation.

Results similar to ours were reported by Shigematsu, Shiio, Takeshita, Onodera & Maruo (1966) who did not detect the synthesis of fibroin in silkworm cell-free system by immunological method. Drach & Lingrel (1966) have also demonstrated that the protein synthesized in the *E. coli* cell-free system under the influence of one of the RNA fractions from rabbit reticulocytes differs from globin electrophoretically and on Dowex-50 chromatography. On the other hand, they found a similarity between the tryptic peptides of proteins newly synthesized in the *E. coli* system and tryptic peptides of *E. coli* protein. Similarly, Aach *et al.* (1964) did not obtain the tobacco mosaic virus RNA-directed protein synthesis in *E. coli* cell-free system, as shown by fractionation of the newly formed proteins on DEAE-cellulose, Dowex-1 and by serological methods.

It is possible that the natural messenger RNA-directed protein synthesis in the cell-free system depends on an additional factor absent in the above mentioned as well as in our experiments. Weisberger & Armentrout (1966) described the isolation of ribonucleoprotein complex (mRNP) from ribosomes of deer reticulocytes, which stimulated the synthesis of deer globin in rabbit reticulocytes cell-free system. Ribonuclease as well as deproteinization of mRNP abolished its stimulatory effect. Eisenstadt & Brawerman (1966) in the cross experiments with *E. coli* and *Euglena gracilis* cell-free systems on the f2 phage RNA-directed protein synthesis also showed the requirement of a protein factor. Revel & Gros (1966) reported the presence of a similar protein factor linked with DNA. The lack of such a factor in our experiments cannot be excluded since silkworm and *E. coli* RNA's were prepared by phenol method.

The obtained results have shown that silkworm RNA prompted in the *E. coli* cell-free system the synthesis of a protein which is not a silk protein. The addition of heterologous as well as homologous RNA to this system stimulated the synthesis of proteins which, when compared by autoradiography of tryptic peptide fingerprints, appeared to be similar. The nucleolytic hydrolysis during the preparation

of the RNA's cannot be excluded; it does not, however, explain why such different RNA's stimulated the synthesis of similar proteins. The finding that the autoradiography of the tryptic peptide fingerprints of the ^{14}C-labelled proteins formed in a cell-free system did not correspond to the ninhydrin-positive tryptic peptide fingerprint of *E. coli* protein indicated the differences between translation processes *in vivo* and *in vitro*. A similar suggestion was also made by Capecchi (1966). The immunological experiments with the anti-*E. coli* protein rabbit serum indicate that the translation *in vitro* can be also disturbed by addition of silkworm or *E. coli* RNA to the *E. coli* cell-free system.

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SYNTEZA BIAŁKA W BEZKOMÓRKOWYM UKŁADZIE Z *ESCHERICHIA COLI* W OBECNOŚCI NATURALNYCH INFORMACYJNYCH RNA

Streszczenie

1. Analiza związku powstałego w bezkomórkowym układzie z *E. coli* pod wpływem RNA ze środkowej części gruczołów przędnych jedwabnika morwowego *Bombyx mori* wykazała, że produkt ten nie jest serycyną — białkiem jedwabiu.

2. Porównano go z produktami syntetyzowanymi w tym samym układzie pod wpływem RNA z *E. coli* i bez dodanego RNA. Dodanie homologicznego lub heterologicznego RNA do układu bezkomórkowego powoduje syntezę białka, które, jak wynika z porównania autoradiogramów map peptydów trypsynowych, jest podobne do białka powstającego w układzie bez dodania RNA.

3. Przy pomocy immunoprecypitacji wykazano jednak, że powstałe *in vitro* białka różnią się pod względem immunologicznym.

4. Wyniki doświadczeń wskazują na różnice w przekazywaniu informacji *in vivo* i *in vitro* oraz że proces translacji *in vitro* może być zakłócony przez dodanie heterologicznego RNA lub nawet homologicznego RNA do układu bezkomórkowego z *E. coli*.

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SEPARATION AND PURIFICATION OF SOLUBLE ARYLSULPHATASES OF HUMAN PLACENTA

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1. Soluble arylsulphatases A and B (EC 3.1.6.1) of human placenta were separated and purified. 2. After electrophoretic separation, 80% of the starting activity of arylsulphatase A was found in the protein possessing the mobility of blood serum α_2-globulins, and 77% of the starting activity of arylsulphatase B was found in the protein corresponding to γ-globulins. 3. The precipitate obtained at 1.6 M-ammonium sulphate concentration contained 62% of the starting activity of arylsulphatase A; it was shown by electrophoresis to contain a small amount of proteins with the mobility of α_2-globulins. The precipitate at 1.6-2.8 M-ammonium sulphate concentration contained 72% of the starting activity of arylsulphatase B. This fraction contained very little of proteins corresponding to γ-globulins. 4. On chromatography on DEAE-cellulose, arylsulphatase B was eluted without NaCl (85% of the starting activity) and arylsulphatase A at 0.14 M-NaCl concentration (72% of activity). 5. The highest purification of the enzymes was achieved when the fractions precipitated at 1.6 M and 2.0-2.8 M-ammonium sulphate concentration, were submitted to paper electrophoresis.

It was found previously (Gniot-Szulżycka & Działoszyński, 1966) that soluble placental arylsulphatases (arylsulphate sulphohydrolase EC 3.1.6.1) could be separated by dialysis. Arylsulphatase B precipitated with the euglobulins while arylsulphatase A remained in solution. Electrophoresis of the placental extracts (Działoszyński, Gniot-Szulżycka & Jagusztyn, 1966) revealed that the mobility of arylsulphatase A corresponded to that of α_2 serum globulins and that of arylsulphatase B agreed with the cathode front of γ-globulins.

This work is a continuation of our study on the properties of the soluble arylsulphatases of human placenta and contains new results on isolation and purification of those enzymes by ammonium sulphate precipitation, paper electrophoresis and chromatography on DEAE-cellulose.

MATERIAL AND METHODS

Water extracts of the acetone-dried fraction obtained from autolysed placenta served as the source of enzymes. Autolysis of the placental tissue was carried out according to Ahmed & King (1960) with a modification which consisted in the addition of ammonium acetate up to 0.2 M and lowering the concentration of ace-

tone down to 10%. It was shown by Błeszyński & Działoszyński (1965) that addition of ammonium acetate increased the extractability of arylsulphatases from autolysed ox brain. A lower concentration of acetone was used to prevent the precipitation of arylsulphatase B.

Acetone-dried fraction and its extracts. Fresh human placenta was homogenized in 0.2 M-ammonium acetate, containing heparin, acetone, toluene and ethyl acetate, the mixture was autolysed at 0 - 4° for 72 hr. and filtered on Büchner funnel through muslin. The clear filtrate was adjusted to pH 5.0 with acetic acid, and acetone was added up to 60% in order to precipitate the arylsulphatases. The precipitate separated by centrifugation was washed a few times with acetone solutions of increasing concentration (70%, 80%, 90%) and pure acetone, and immediately dried *in vacuo* over P_2O_5. The dry powder was stored at 0° over P_2O_5. For further experiments, 5% water extracts were used.

Paper electrophoresis. Electrophoresis was carried out in veronal buffer pH 8.6 and ionic strength 0.1, on Whatman no. 3 paper. It lasted 3 hr. at a voltage gradient of 8.67 V/cm., the electrophoretic chamber being cooled with ice.

Two strips (2 cm. wide) with enzymic extracts (30 µl.) were dyed according to Mejbaum-Katzenellenbogen & Dobryszycka (1959) and the concentration of protein in each fraction was estimated. Two other strips with enzyme extracts were cut crosswise according to the protein fractions indicated on the dyed strip. Each section from both strips was shredded and incubated at 37° with 10 mM-2-hydroxy--5-nitrophenylsulphate for 1 hr. in 0.5 M-acetate buffer. Sections used for estimation of the activity of arylsulphatase A were incubated at pH 4.7, those used for determination of the activity of arylsulphatase B at pH 6.0. Additionally one strip with enzyme extract and another with human blood serum, were dyed according to Hardwicke (1954).

Fractionation with ammonium sulphate. In order to obtain a fraction rich in protein corresponding to the mobility of serum α_2- and γ-globulins, the method described by Bourrillon, Got & Levy (1962) was used. An appropriate amount of ammonium sulphate was added to the enzymic extract at -5° to -9°, the pH and the volume brought to the required values and the mixture left for 10 - 11 hr. at 0° to 4°, then centrifuged for 5 min. at 21 000 *g*. The precipitate was washed three times with ammonium sulphate solution of corresponding concentration and pH. Washings were added to the supernatant and the precipitation with higher concentration of ammonium sulphate performed as before.

Precipitation at pH 7.0 was carried out with the following concentrations of ammonium sulphate: 1.6 M, 1.6 - 2.0 M, 2.0 - 2.2 M and 2.2 - 2.4 M, and at pH 6 with 2.4 - 2.8 M and 2.8 - 4.0 M. Each precipitate was dissolved in a small volume (5 - 10 ml.) of water, dialysed and the activity of arylsulphatase A and B estimated. In all fractions protein was determined by the method of Mejbaum-Katzenellenbogen (1955) and electrophoresis on Whatman no. 3 paper was performed on every one in order to find what electrophoretic fraction they contained.

For further purification of the enzymes, fractions precipitated at 1.6 M (pH 7.0) and 2.0 - 2.8 M (pH 6.0) ammonium sulphate and containing correspondingly most

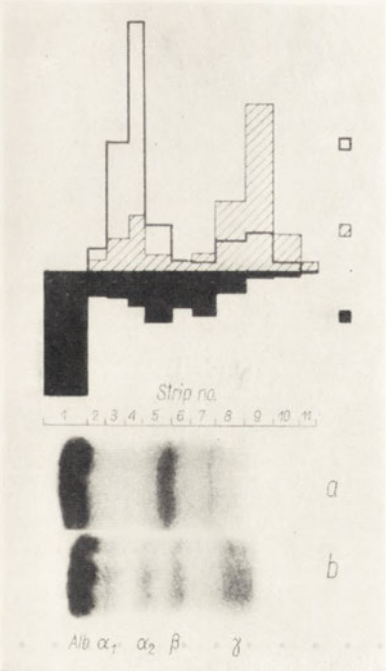

Fig. 1. Distribution of arylsulphatase A and B activity in proteins of extract from acetone-dried human placenta, separated by paper electrophoresis. ☐, Activity of arylsulphatase A; ////, activity of arylsulphatase B; ■, protein. The surface of the small squares represents, respectively, 1% of the starting enzyme activity or of the amount of protein. *a*, Electrophoretogram of human placental proteins; *b*, electrophoretogram of blood serum proteins.

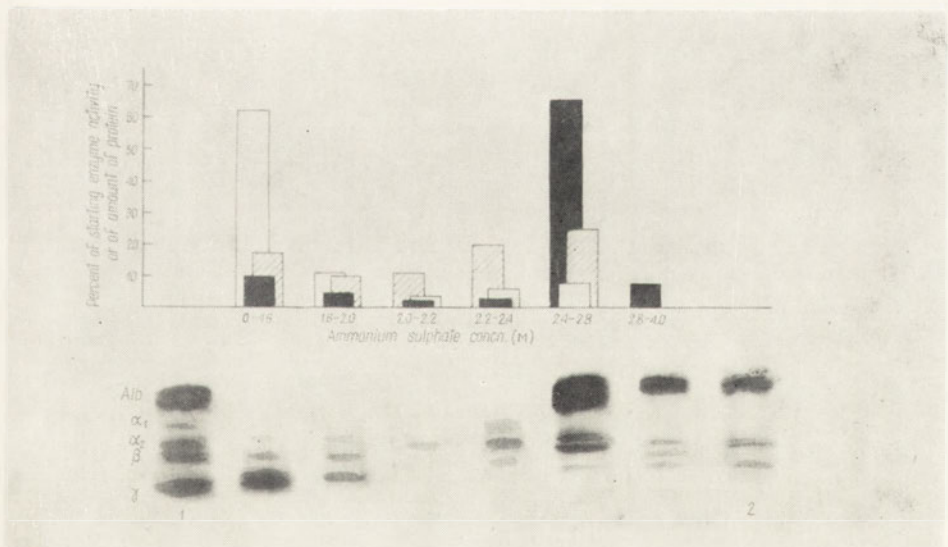

Fig. 2. Separation of arylsulphatase A and B from an extract of acetone-dried human placenta, by fractionation with ammonium sulphate. ☐, Arylsulphatase A; ////, arylsulphatase B; ■, protein. *1*, Electrophoretogram of human blood serum protein; *2*, electrophoretogram of the starting extract. The remaining electrophoretograms correspond to the particular fractions.

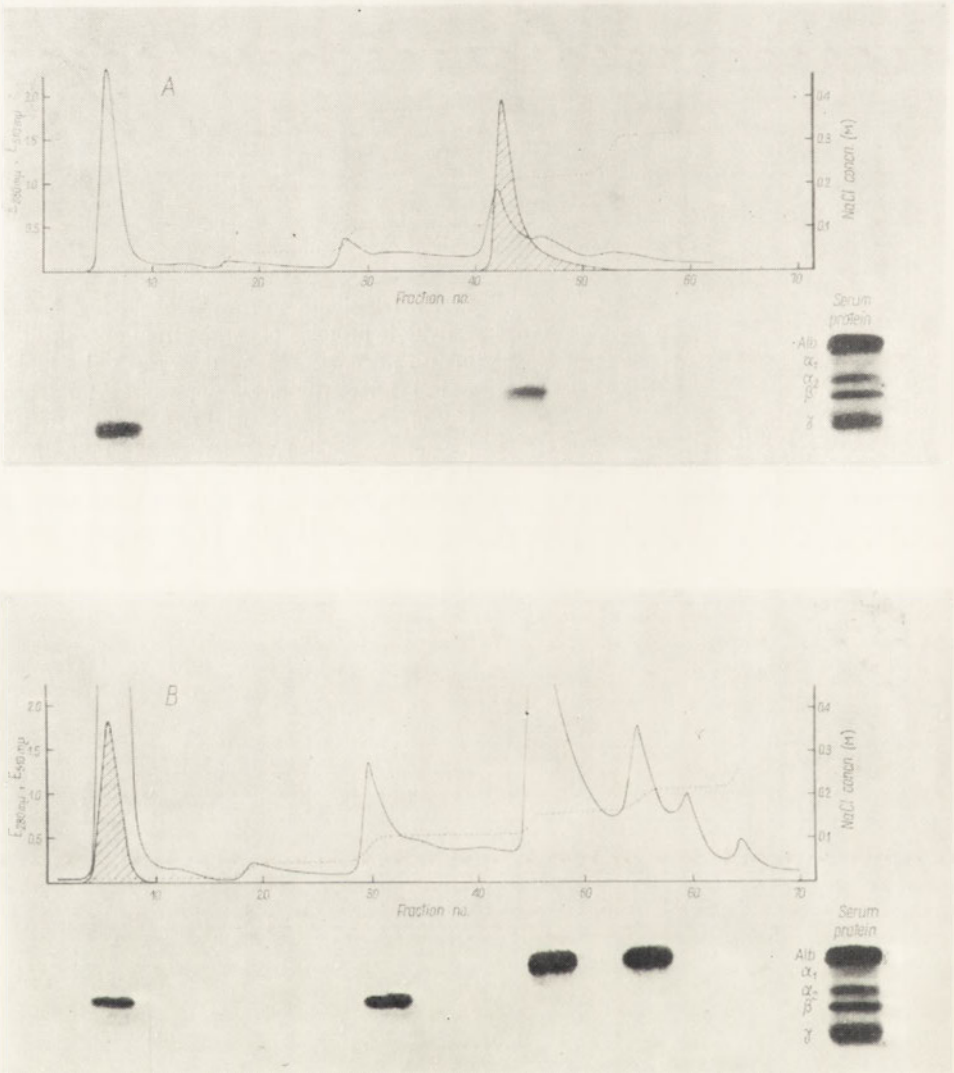

Fig. 3. Elution patterns obtained from the DEAE-cellulose column to which were applied: (*A*), the fraction precipitated at 0 - 1.6 M-ammonium sulphate concn.; (*B*), the fraction precipitated at 2.0 - 2.8 M-ammonium sulphate concn. (see Fig. 2). (- - -), NaCl concentration gradient; (———), protein (E_{280}). Enzyme activity (E_{510}) is indicated by hatching. The electrophoretograms of the respective fractions and blood serum protein are also presented.

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of arylsulphatase A and B, were collected from separate enzyme solutions, dialysed and concentrated 5 - 10-fold on silica gel. During concentration 6% of the enzyme activity was lost. About 800 µl. of the concentrated fractions were placed on a strip of Whatman 3 *MM* paper 21 cm. wide, and electrophoresis performed as described above.

Chromatography on DEAE-cellulose. DEAE-cellulose powder was washed with 0.1 N-HCl and 0.1 N-NaOH and then with water, and pH of the suspension brought to 6.8. It was now washed several times with veronal-HCl buffer, pH 6.8, and transferred to a column (20×2 cm.). Chromatographic separation was carried out with fractions precipitated from the enzymic extract at 1.6 M and 2.0 - 2.8 M-ammonium sulphate. NaCl of stepwise increased gradient was used for elution.

Determination of enzymic activity. The substrate, 2-hydroxy-5-nitrophenyl sulphate dipotassium salt prepared according to Roy (1958) was recrystallized several times from glass-distilled water. The activity of arylsulphatases was determined according to Robinson, Smith & Williams (1951). The red colour of the liberated 4-nitrocatechol was developed with alkaline mixture containing 5 N-NaOH, 10% Na_2SO_3 and 4% quinone in 0.1 N-HCl (1 : 1 : 0.1, by vol.). The activity was measured in 0.5 M-acetate buffer of pH 4.7 for arylsulphatase A and pH 6.0 for arylsulphatase B. The final concentration of the substrate was 10 mM. The temperatures and times of incubation are indicated in Tables 1 and 2.

RESULTS

The extraction of arylsulphatase A and B from human placenta. Tables 1 and 2 present the total and specific activities found at various stages of purification of the enzymes. The acetone powder extract contained 66% of the total activity of arylsulphatase A and 22% of the arylsulphatase B present in the original homogenate. The specific activity was 370 mµmoles nitrocatechol/mg. protein/60 min. for arylsulphatase A and 320 for arylsulphatase B, the degree of purification being 8 and 2.3, respectively. The extract was used for further purification.

Separation of soluble arylsulphatases A and B by electrophoresis and fractional precipitation with ammonium sulphate. Fig. 1 shows the distribution of arylsulphatase A and B in the electrophoretic fractions of the extract from acetone-dried placenta. It should be noted that in the described conditions no, or only small, losses of enzymic activities were observed. The fraction corresponding to blood serum α_2-globulins contained 80% (±8%) of the original arylsulphatase A activity and the fraction corresponding to the cathode front of serum γ-globulins, 77% (±15%) of the arylsulphatase B activity. Paper electrophoresis led to separation of the enzymes and marked increase of specific activity. Arylsulphatase A was purified 60-fold and arylsulphatase B 70-fold as compared with the activity of the homogenate.

The fractional precipitation did not cause any substantial losses of the enzymes, as altogether about 90% of the activity was recovered. Arylsulphatase A (Fig. 2) precipitated mainly at 1.6 M-ammonium sulphate concentration. This fraction

Table 1

Purification of arylsulphatase A from human placenta

Assays were made at 10 mM-substrate (dipotassium salt of 2-hydroxy-5-nitrophenyl sulphuric acid) concentration in acetate buffer, pH 4.7. The results are expressed as amount of nitrocatechol liberated. The relationship between the time of incubation and activity was direct only during 20 min. at 25°.

Material	Procedure	Total activity (m-moles)	Activity applied (m-moles)	Recovery (%)	Specific activity (mμmoles/mg. protein)	Purification	Conditions	No of expts.
Homogenate suspension		7940		100	46.8/hr.	—		
supernatant		7250		91	99/hr.	2.1	37°, 60 min.	2
Acetone powder extract		5300		66	369/hr.	7.9		
Acetone powder extract	Paper electrophoresis, strip section 1 - 11			102	—			
	3, 4, 5		370	80	1750/hr.	37.4	37°, 60 min.	11
	3, 4			65	2830/hr.	60.5		
Acetone powder extract	Fractionation with ammonium sulphate 0 - 4 M			89	—	—		
	0 - 1.6 M		518	62	725/hr.	15.5	37°, 60 min.	3
Ppt. at 1.6 M-ammonium sulphate concn.	Chromatography on DEAE-cellulose, fractions 41 - 49			72	—	—		
	42 - 46		70	55	497/min.	—	25°, 20 min.	2
Ppt. at 1.6 M-ammonium sulphate concn.	Paper electrophoresis wide strip			—	1419/min.	—		
	average		30—120	83	4800/min.	—	25°, 20 min.	7
	narrow strip			—	16 800/min.	—		

Table 2

Purification of arylsulphatase B from human placenta

Assays were made at 10 mM-substrate (dipotassium salt of 2-hydroxy-5-nitrophenyl sulphuric acid) concentration in acetate buffer, pH 6.0, at 37°. The results are expressed as amount of nitrocatechol liberated. The relationship between the time of incubation and activity was direct up to 60 min.

Material	Procedure	Total activity (m-moles)	Activity applied (m-moles)	Recovery (%)	Specific activity (mμmoles/mg. protein/min.)	Purification	Time of incubation	No. of expts.
Homogenate suspension		23 000		100	2.3	—		
supernatant		10 300		45	2.5	1.1	60 min.	2
Acetone powder extract		5120		22	5.3	2.3		
Acetone powder extract	Paper electrophoresis, strip section 1 - 11			98	—	—		
	8, 9, 10		320	77	87	37.8	60 min.	11
	9			41	161	70.0		
Acetone powder extract	Fractionation with ammonium sulphate 0 - 4 M			91	—	—		
	1.6 - 2.8 M		603	72	26.2	11.4	60 min.	3
	2.2 - 2.8 M			46	25.8	11.2		
Ppt. at 2.0 - 2.8 M-ammonium sulphate concn.	Chromatography on DEAE-cellulose, fractions 5 - 12			85	—	—		
	6, 7, 8		51	79	206.2	90.0	30 min.	2
Ppt. at 2.0 - 2.8 M-ammonium sulphate concn.	Paper electrophoresis wide strip			70	1172	510		
	average		12	57	2506	1090	30 min.	5
	narrow strip			38	5670	2426		

contained 62% of the starting activity and was purified 15.5-fold. Fractions precipitated between 1.6 M and 2.8 M-ammonium sulphate concentration contained 72% of the starting activity of arylsulphatase B, and the average increase in specific activity was 11.5-fold as compared with that of the homogenate. From the electrophoretic pattern presented in Fig. 2 it may be seen that the fraction precipitated at 1.6 M-ammonium sulphate, and very rich in arylsulphatase A, contained very little protein corresponding in mobility to serum α_2-globulins, which in the whole extract contained most of the arylsulphatase A activitv (see Fig. 1). Similarly, the fractions containing most of the arylsulphatase B activity, except that precipitated at 1.6 - 2.0 M-ammonium sulphate concentration, contained very little protein with the mobility of γ-globulins.

Purification of arylsulphatase A and B. Arylsulphatase A preparation precipitated at 1.6 M-ammonium sulphate concentration, and arylsulphatase B preparation precipitated at 2.0 - 2.8 M-ammonium sulphate concentration, were purified by paper electrophoresis or chromatography on DEAE-cellulose (Tables 1 and 2). After electrophoretic separation the average specific activity of arylsulphatase A was 4800 mμmoles/mg. protein/min., and that of arylsulphatase B, 2500. By cutting narrower sections from the electrophoretogram, and thus limiting the yield, higher specific activities of both enzymes could be achieved, respectively 16 800 and 5670. The mean recovery of arylsulphatase A was 83%, and that of arylsulphatase B 57%.

The degree of purification of the two arylsulphatases on DEAE-cellulose (Fig. 3) was much smaller than that obtained by electrophoresis. After chromatography, the specific activity in the peak of arylsulphatase A was 497, and that of arylsulphatase B 206 mμmoles nitrocatechol/mg. protein/min. Arylsulphatase B was eluted by buffer alone, while arylsulphatase A at 0.14 M-NaCl. Both fractions contained some protein impurities.

DISCUSSION

Many workers have used paper electrophoresis for the separation of arylsulphatases (for ref. see Działoszyński *et al.*, 1966). However, none of them gave any data on the recovery of the enzymes during the process of electrophoresis. The conditions of electrophoresis described by Działoszyński *et al.* (1966) made it difficult to estimate the activity of arylsulphatase B from human placenta. Dodgson & Spencer (1956) experienced similar difficulties with separation of arylsulphatase found in urine, and Wortman (1962) could not separate electrophoretically the enzymes from corneal extracts.

Electrophoretic examination of the placental extracts in conditions described in this paper made it possible not only to locate the activity of arylsulphatase A within the protein fraction possessing the mobility of serum α_2-globulins and that of arylsulphatase B in the protein corresponding to the cathode front of γ-globulins, as it was done in the previous work (Działoszyński *et al.*, 1966), but also to recover both enzymes in high yields (about 80%).

Paper electrophoresis of the fractions precipitated at 1.6 M- and 2.0 - 2.8 M--ammonium sulphate concentration, led to considerable purification of both arylsulphatases. The specific activity of the enzyme A was 16 800 mμmoles nitrocatechol/mg. protein/min. (at 25°) and that of the enzyme B, 5670 mμmoles nitrocatechol/mg. protein/min. (at 37°).

Fractional precipitation with ammonium sulphate gave somewhat unexpected results as the fraction containing mainly proteins corresponding to γ-globulins (0 - 1.6 M) had the highest activity of arylsulphatase A, while fractions containing little of this protein (2.0 - 2.8 M) possessed the activity of arylsulphatase B.

Chromatography on DEAE-cellulose led to separation of the two arylsulphatases. Although the conditions at which chromatography on DEAE-cellulose was performed, were not identical with those used by Wortman (1962) it may, nevertheless, be assumed that the fraction eluted without NaCl and called by Wortman *a* corresponded to arylsulphatase B, and that eluted with 0.14 M-NaCl and called by Wortman *d* was arylsulphatase A. Arylsulphatase-containing fractions found by Wortman in the corneas of rabbit and ox and designated as *b* and *c* could not be found in human placenta. Similarly fractions *b* and *c* could not be separated from ox liver by chromatography on DEAE-cellulose (Działoszyński, Błeszyński & Narloch, 1967), although they were found by Błeszyński (1967) in ox brain.

The serum γ-globulin-like character of placental arylsulphatase B to which point the following results: precipitation on dialysis (Gniot-Szulżycka & Działoszyński, 1966), electrophoretic mobility (Działoszyński *et al.*, 1966) and elution from DEAE--cellulose without NaCl gradient, explain to some extent the slower extraction of that enzyme from the tissues and its lower activity. The acetone-powder extract contains 66% of arylsulphatase A and 22% of arylsulphatase B found in the homogenate. The presence in human placenta of the insoluble arylsulphatase C (Gniot & Działoszyński, 1964) could be another factor lowering the activity of arylsulphatase B in the acetone powder, since the activity of the C enzyme is estimated in the homogenate, but the enzyme is presumably not solubilized on autolysis and therefore it is not found in the acetone powder.

According to Roy (1960) arylsulphatase B of ox liver is localized in the framework of the lysosomes while arylsulphatase A in the sap and this may explain the differences in the solubility of the two enzymes.

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ROZDZIAŁ I OCZYSZCZANIE ROZPUSZCZALNYCH ARYLOSULFATAZ ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Rozdzielono i oczyszczono arylosulfatazy A i B (EC 3.1.6.1) z łożyska ludzkiego.

2. Po elektroforezie bibułowej 80% wyjściowej aktywności arylosulfatazy A występuje we frakcji o ruchliwości α_2-globulin surowicy krwi, natomiast 77% wyjściowej aktywności arylosulfatazy B występuje w białku odpowiadającym γ-globulinom.

3. Frakcja białkowa strącająca się 1.6 M-siarczanem amonu zawiera 62% wyjściowej aktywności arylosulfatazy A; stwierdzono w niej znikome ilości białka o ruchliwości elektroforetycznej α_2-globulin. Frakcja białkowa strącająca się w zakresie 1.6 - 2.8 M-siarczanu amonu zawiera 72% wyjściowej aktywności arylosulfatazy B; elektroforetycznie stwierdzono w niej niewielką ilość białek odpowiadających γ-globulinom.

4. Z DEAE-celulozy enzym B eluuje się bez gradientu NaCl (84% aktywności wyjściowej), enzym A przy 0.14 M-NaCl (72% wyjściowej aktywności).

5. Największe oczyszczenie arylosulfataz A i B otrzymano przy stosowaniu elektroforezy bibułowej frakcji rozdzielonych siarczanem amonu.

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J. POPINIGIS

THE SODIUM-DEPENDENT EFFECT OF MITOCHONDRIAL BASIC PROTEIN ON ENERGY METABOLISM IN MITOCHONDRIA

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1. Basic protein isolated from hog-kidney mitochondria, at a low concentration (50 μg./ /ml.) stimulated the respiration of mitochondria in State 4, induced swelling, and inhibited the synthesis of citrulline in mitochondria, only in the presence of sodium ion. 2. The action of this protein on mitochondrial energy metabolism resembles the action of gramicidin.

Hillar (1965) reported that mitochondrial basic protein (MBP) isolated from hog kidney according to Rzeczycki, Grudzińska, Hillar & Wszelaki-Lass (1963) uncoupled the oxidative phosphorylation in rat-liver mitochondria. The stimulating effect of MBP on the oxidation of exogenous NADH in mitochondria (Popinigis & Rzeczycki, 1966) indicated that this protein influences the permeability of the mitochondrial membrane. The study of Hillar & Rzeczycki (1965) on the influence of MBP on swelling of mitochondria demonstrated that changes in the permeability of the mitochondrial membrane were dependent on the composition of the medium. MBP caused swelling of mitochondria in iso-osmotic solution of potassium chloride, and had no effect in sucrose medium. These experiments suggested that the effect of MBP on mitochondrial energy metabolism is dependent on the composition of the medium.

In the present work, a comparison was made of the effect of mitochondrial basic protein on respiration, swelling, and on synthesis of citrulline in rat-liver mitochondria, in the presence and absence of sodium ion.

MATERIALS AND METHODS

Preparation of mitochondria. The liver of a Wistar rat was homogenized manually in a Potter homogenizer in a medium containing 0.25 M-sucrose, 0.2 mM-tris chloride and 0.01 mM-EDTA, pH 7.3. The mitochondria were isolated according to Weinbach (1961), washed three times, suspended in 2.0 ml. of the above solution, and immediately used for experiments.

Estimation of swelling. The medium of pH 7.3 contained 0.17 M-sucrose, 40 mM--choline chloride, 1.25 mM-tris phosphate, 10 mM-tris glutamate and 10 mM-tris

succinate. To 3 ml. of the medium, mitochondria corresponding to 2.2 mg. of protein were added, and after 2 min. changes in extinction at 520 mμ were measured in a Unicam SP 500 spectrophotometer for a period of 10 min.

Determination of respiration. The Warburg manometric method was used. The mitochondria were incubated in 1 ml. of a medium of pH 7.3 containing 0.2 M--sucrose, 20 mM-tris chloride, 1.25 mM-tris phosphate, 10 mM-tris glutamate and 10 mM-tris succinate. The incubation was carried out at 25° for 25 min., the uptake of oxygen being measured between the 5th and 25th minute.

Synthesis of citrulline in mitochondria. This was assayed by incubating the mitochondria for 30 min. at 30° in 1 ml. of a medium of pH 7.3 consisting of 20 mM--tris L-glutamate, 20 mM-DL-ornithine, 5 mM-$MgCl_2$, 5 mM-tris phosphate, 50 mM--tris chloride, and 20 mM-NH_4HCO_3. The reaction was stopped by adding $HClO_4$, and the synthesized citrulline was determined using diacetylmono-oxime according to Archibald (1944).

Determination of protein in mitochondria. This was done by the biuret method (Layne, 1957) after adding 0.9 ml. of 0.1% sodium deoxycholate solution to 0.1 ml. of the suspension of mitochondria.

Preparation of mitochondrial basic protein (MBP). This was obtained from hog kidney according to Rzeczycki *et al.* (1963) by sulphosalicylic acid extraction and concentration of the protein by the tannin-caffeine procedure of Mejbaum-Katzenellenbogen (1955).

Reagents. Diacetylmono-oxime (Hopkin & Williams, LTD, Chadwell Heath, Essex, England), DL-ornithine (Lachema, Prague, Czechoslovakia), choline chloride (Merck, Darmstadt, Germany), citrulline (Light, Colnbrook, England), tris (Fluka, Buchs, Switzerland). Other reagents were products of Fabryka Odczynników Chemicznych (Gliwice, Poland) or Xenon (Łódź, Poland).

Tris succinate, tris phosphate, tris glutamate and tris chloride were prepared from aqueous solutions of the corresponding acids purified on Amberlite IRC-50 (H^+ form). Bidistilled water purified in the same way was used throughout.

RESULTS

The mitochondrial basic protein stimulated the respiration of mitochondria limited by the amount of the phosphate acceptor (State 4 of Chance & Williams, 1956). This is apparent from the results of experiments presented in Table 1, which show the effect of MBP on oxidation of glutamate and succinate by liver mitochondria. At low concentration of MBP (50 μg./ml.), the stimulation was observed only in the presence of sodium ion. At high MBP concentration, the stimulation was not Na^+ ion-dependent, although in the presence of Na^+ the respiration was greater.

Swelling of mitochondria was affected by MBP in a similar way (Fig. 1). At a concentration of 50 μg./ml., MBP induced swelling only when sodium ion was present, whereas at a concentration of 150 μg./ml. it caused swelling also in the absence of Na^+ ion.

Table 1

The effect of mitochondrial basic protein (MBP) on the respiration of mitochondria in the presence and absence of sodium ion

The respiration was measured in 1 ml. of a medium composed of 0.2 M-sucrose, 1.25 mM-tris phosphate buffer, 20 mM-tris HCl buffer, pH 7.3, and 10 mM-succinate and 10 mM-glutamate in the form of tris salts as substrates. MBP was added after addition of mitochondria.

Addition	Oxygen consumption (μl. O_2/20 min.)	
	Expt. 1 5.2 mg. protein/ml.	Expt. 2 4.5 mg. protein/ml.
None, control	40.8	31.2
NaCl, 30 μmoles	39.8	30.6
MBP, 50 μg.	38.0	32.4
MBP, 50 μg. + NaCl, 30 μmoles	55.1	42.2
MBP, 150 μg.	51.2	40.2
MBP, 150 μg. + NaCl, 30 μmoles	57.6	46.2

Table 2

The effect of mitochondrial basic protein (MBP) on the synthesis of citrulline by rat-liver mitochondria in the absence of sodium ion

The synthesis of citrulline was carried out in a medium composed of 20 mM-tris L-glutamate, 20 mM-DL-ornithine, 5 mM-$MgCl_2$, 5 mM-tris phosphate, 50 mM-tris chloride, 20 mM-NH_4HCO_3, at pH 7.3. Mean values from 3 experiments are given, with limit values in parentheses.

MBP concentration (μg./ml.)	Citrulline formed (μmoles/30 min./mg. of mitochondrial protein)
0	0.42 (0.41-0.44)
50	0.40 (0.39-0.41)
100	0.41 (0.40-0.43)
150	0.36 (0.33-0.40)
200	0.10 (0.09-0.12)
250	0.06 (0.03-0.09)

The effect of MBP on the synthesis of citrulline in the absence of sodium ion is shown in Table 2. MBP at a concentration of 50 - 100 μg./ml. had no effect on the synthesis, whereas concentrations exceeding 150 μg./ml. were inhibitory. In the presence of Na^+ ion, low concentration of MBP also inhibited the synthesis (Table 3). Sodium chloride alone did not inhibit the synthesis up to a concentration of 30 mM.

Table 3

The effect of mitochondrial basic protein (MBP) on the synthesis of citrulline in the presence of different sodium ion concentrations

The synthesis of citrulline was carried out as described in Table 2. Mean values from 5 experiments are given, with limit values in parentheses.

NaCl concentration (mM)	Citrulline formed (μmoles/30 min./mg. of mitochondrial protein)	
	without MBP	with MBP (50 μg./ml.)
0	0.38 (0.37-0.41)	0.37 (0.35-0.40)
3.3	0.37 (0.36-0.41)	0.34 (0.32-0.37)
10	0.37 (0.35-0.41)	0.32 (0.29-0.36)
30	0.36 (0.32-0.41)	0.29 (0.24-0.32)
60	0.31 (0.28-0.34)	0.26 (0.22-0.28)
90	0.28 (0.21-0.30)	0.20 (0.15-0.26)

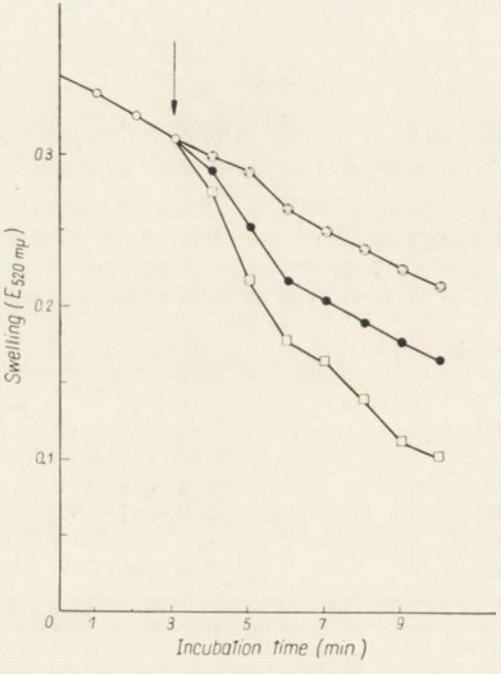

Fig. 1. The effect of mitochondrial basic protein (MBP) and Na^+ ion on swelling of mitochondria. The medium, 3 ml., was composed of 0.17 M-sucrose, 40 mM-choline chloride and 1.25 mM-tris phosphate, 10 mM-tris glutamate and 10 mM-tris succinate; pH 7.3. (○), Control. The arrow indicates the addition of: (△), MBP, 50 μg./ml.; (●), MBP, 50 μg./ml., and 30 mM-NaCl; (□), MBP, 150 μg./ml.

DISCUSSION

The results of the present work give further evidence of the effect of basic protein from kidney mitochondria on the permeability of the mitochondrial membrane. MBP, when applied at high concentration, acts as uncoupling agent: it stimulates the respiration with succinate and glutamate by rat-liver mitochondria in State 4, induces swelling and abolishes citrulline synthesis. This latter process in the absence of added ATP utilizes intramitochondrially generated ATP (Charles, Tager & Slater, 1967). MBP at low concentration acts as an uncoupling agent only when Na^+ ion is present in the medium. There is a similarity in action between gramicidin and MBP. Graven, Lardy, Johnson & Rutter (1966) reported that at higher concentration gramicidin induced ATPase activity in the absence of cation. Chappell & Crofts (1965) demonstrated that at low concentration in phosphate-containing medium, gramidicin acts as uncoupling agent: it stimulates respiration and induces swelling of mitochondria only in the presence of alkali-metal ions. They ascribed the action of gramicidin to the changes in the permeability of the mitochondrial membrane for these cations and utilization of energy in the process of their accumulation.

Mitochondrial basic protein, by changing the permeability of the mitochondrial membrane probably decomposes high energy intermediates (difference in membrane potential). Low concentrations of MBP require for their activity the presence of sodium ion. This points to the possibility of participation of basic protein from kidney mitochondria in processes of cation translocation.

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ZALEŻNY OD SODU WPŁYW MITOCHONDRIALNEGO BIAŁKA ZASADOWEGO NA PROCESY ENERGETYCZNE MITOCHONDRIÓW

Streszczenie

1. Białko zasadowe wyizolowane z mitochondriów nerki wieprza użyte w niskim stężeniu (50 μg./ml) stymuluje oddychanie mitochondriów znajdujących się w stanie metabolicznym 4, wywołuje ich pęcznienie, oraz hamuje zachodzący w nich proces biosyntezy cytruliny tylko w obecności jonu sodowego w środowisku.

2. Działanie tego białka na reakcje energetyczne mitochondriów wydaje się przypominać wpływ gramicydyny.

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RECENZJE KSIĄŻEK

THE USE OF INDUCED MUTATIONS IN PLANT BREEDING. Symposium Publications Division, Pergamon Press, Oxford, London, Edinburgh, New York, Paris, Frankfurt; str 832+ +VIII, cena 15£.

Omawiana książka stanowi suplement do tomu 5 "*Radiation Botany*" i zawiera referaty wygłoszone na Sympozjum, które odbywało się w dniach 25 maja - 1 czerwca 1964 r. w Rzymie i poświęcone było zagadnieniom związanym z zastosowaniem indukowanych mutacji w hodowli roślin. Sympozjum zorganizowane było przez FAO i IAEA przy współpracy EUCARPIA. Wzięło w nim udział 167 specjalistów reprezentujących 32 kraje oraz 2 organizacje międzynarodowe, FAO i EURATOM.

Wygłoszono 66 referatów podzielonych na następujące grupy tematyczne: A. Ogólny charakter mutacji; B. Wpływ mutagenów, wrażliwość roślin na te związki oraz kierowanie procesem mutacyjnym; C. Selekcja diplontyczna; D. Genetyka indukowanych mutacji, E. Zastosowanie indukowanych mutacji w hodowli roślin rozmnażanych płciowo; F. Zastosowanie indukowanych mutacji w hodowli roślin rozmnażanych wegetatywnie; G. Indukowanie zmian chromosomowych oraz techniki specjalne i H. Dozymetria neutronów. Najwięcej prac dotyczyło zagadnień objętych tematyką grup B, E i D. Teksty poszczególnych referatów podane są w językach oryginalnych, a każdy referat poprzedzony jest streszczeniami w językach angielskim, francuskim i hiszpańskim. Przebieg dyskusji po referatach, jak również przebieg końcowej dyskusji plenarnej zamykającej kongres podano *in extenso*.

Referowane na Sympozjum prace oryginalne, jak również wypowiedzi w dyskusjach wskazują na wyraźną zmianę poglądu na znaczenie indukowanych mutacji. Niewątpliwie bowiem w pewnych wypadkach indukowanie mutacji genowych bądź chromosomowych umożliwia osiągnięcie wyników, których nie można uzyskać innymi metodami, a w miarę poznawania nowych mutagenów, swoistości ich działania oraz ulepszania techniki identyfikacji i selekcji mutantów skuteczność indukowania mutacji jako metody hodowli roślin staje się coraz większa. Szczególnie duże znaczenie mają prace związane z wykrywaniem mutacyjnych zmian ilościowych.

Spontaniczne mutacje genowe uważa się za podstawowy czynnik ewolucji naturalnej, istnieją więc przesłanki by sądzić, że indukowane mutacje mogą odegrać wielką rolę w ewolucji w warunkach udomowienia. Metody indukowania mutacji nie należy więc przeciwstawiać innym metodom hodowli, lecz należy szukać możliwości tkwiących we wzajemnym uzupełnianiu się tych metod. Największe możliwości doskonalenia metody indukowania mutacji tkwią w ulepszaniu techniki stosowania mutagenów, zwiększaniu skuteczności selekcji oraz dokonywaniu szczegółowej analizy genetycznej.

Książka stanowi dobry przegląd wiedzy w zakresie hodowli mutacyjnej do 1964 r. ze szczególnym uwzględnieniem metod radiacyjnych.

Jan Bojanowski

N. Seiler: DER STOFFWECHSEL IM ZENTRALNERVENSYSTEM. Georg Thieme Verlag, Stuttgart 1966; str. 139, Cena 24 DM.—

Monografia ta w zwartej formie przedstawia główne procesy metaboliczne węglowodanów, aminokwasów i lipidów z uwzględnieniem roli regulacyjnej witamin, hormonów i elektrolitów w zdrowym i chorym mózgu. Podane w książce zwięzłe opisy lub schematy przemian dla tkanki

nerwowej nie różnią się w istocie swojej od podstawowych procesów metabolicznych w innych narządach. W krótkim wstępie omówiono procesy biochemiczne związane z zaopatrzeniem tkanki mózgowej w tlen i składniki odżywcze pod kątem widzenia istnienia bariery naczyniowo-mózgowej. W następnym rozdziale przedstawiono proces glikozy, cykl kwasu cytrynowego oraz cykl pentozowy z uwzględnieniem zaburzeń przemiany węglowodanowej. W tym też rozdziale można znaleźć dane dotyczące zmian w tkance mózgowej w niedotlenieniu i narkozie. Dużo uwagi poświęcił autor w kolejnym rozdziale przemianom aminokwasów do związków farmakologicznie czynnych w mózgu, jak adrenalinie, serotoninie i acetylocholinie. W tym rozdziale zestawiono schorzenia uwarunkowane blokiem enzymatycznym w przemianie aminokwasów. W rozdziale o kwasach nukleinowych i białkach przedstawiono proces biosyntezy białek w tkance nerwowej, a przemianę lipidów w mózgu ujęto w następnym rozdziale kilkoma przejrzystymi schematami. Na tle prawidłowych przemian w mózgu omawia autor zmiany w przebiegu reakcji enzymatycznych w stanach chorobowych. Stwierdzone zmiany stężeń metabolitów w tkance nerwowej przyporządkowano licznym objawom neurologicznym i psychiatrycznym. Szczególnie cenne w tej książce jest umiejętne powiązanie zmian czynnościowych w mózgu w stanie zdrowia i choroby z procesami metabolicznymi tkanki nerwowej.

Książka została zaopatrzona w bardzo obszerną i cenną bibliografię obejmującą 788 pozycji. Szata wydawnicza książki jest bardzo staranna.

Leon Żelewski

J. Leggett Bailey: TECHNIQUES IN PROTEIN CHEMISTRY, II wydanie poprawione i uzupełnione. Elsevier Publ. Co., Amsterdam - London - New York, 1967; 406 str., cena Dfl. 55.

Gdy w 1962 r. ukazało się pierwsze wydanie książki Bailey'a, zostało ono przyjęte ze zrozumiałym zainteresowaniem jako jedna z pierwszych prób zwięzłego i krytycznego przeglądu praktycznych metod badania substancji białkowych.

Szybki rozwój chemii i biochemii białek oraz konieczność stosowania metod badania białek w wielu dziedzinach nie tylko teoretycznych lecz także praktycznych w pełni usprawiedliwiają potrzebę nowego, rozszerzonego wydania książki, stanowiącej podręczne dzieło przydatne w każdym laboratorium związanym z badaniem białek.

Autor utrzymał pierwotny układ książki, dodając tylko jeden rozdział o chemicznej syntezie niektórych prostych peptydów. Objętość poszczególnych rozdziałów musiała jednak ulec zwiększeniu. Tak na przykład rozdział o chromatograficznym rozdzielaniu aminokwasów powiększył się o opis nowoczesnych metod chromatografii cienkowarstwowej oraz dokładniejszy niż w poprzednim wydaniu opis automatycznej analizy mieszanin aminokwasowych. Pozostawiono niestety bardzo obszerne opisy ilościowego oznaczania aminokwasów przy pomocy jednokierunkowej chromatografii bibułowej — metody, która jest bez wątpienia wypierana przez bardziej nowoczesne ilościowe metody.

Rozszerzony został rozdział o analizie grup końcowych białek i peptydów. Trudno jednakże zgodzić się z opinią autora umieszczoną we wstępie, że czytelnik może korzystać w praktyce z opisów metodycznych zamieszczonych w książce bez potrzeby sięgania do źródeł. Tak na przykład metoda kolumnowej chromatografii dwunitrofenylowych pochodnych aminokwasów opisana przez Methesona z pewnością nie będzie mogła zostać powtórzona na podstawie przytoczonego opisu. Autor nie zaznaczył też, że metoda ta jest jedną z najlepszych metod rozdziału, dającą całkowicie ilościowe wyniki; utrudnia to bez wątpienia wybór tym, którzy nie mają zbyt dużego własnego doświadczenia.

Znacznie rozszerzony został rozdział dotyczący filtracji na żelu. Podane są metody praktyczne i teoria rozdziału. Autor wspomina nawet o tak nowych osiągnięciach jak stosowanie agarozy, przemilcza jednakże istnienie Bio-gelu.

Niesłusznym wydaje się także przemilczenie istnienia DEAE-Sephadexu i CM-Sephadexu w rozdziałach, gdzie szczegółowo omawia się odpowiednie pochodne celulozowe.

W przedmowie autor usprawiedliwia się, że nie umieścił w swej książce rozdziału o ultrawirowaniu białek ze względu na to, że odpowiednie metody są opisane w instrukcjach do przyrządów.

Należy jednakże podkreślić, że w instrukcjach MSE oraz Spinco z ubiegłego roku nie ma żadnych informacji z wyjątkiem powołania na prace źródłowe odnośnie tak ważnej i szeroko używanej metody jak wirowanie w gradientach gęstości. Pewną niekonsekwencją jest także szczegółowy opis filtracji na żelu, chociaż w tym przypadku rzeczywiście informacje producenta docierające do każdego biochemika dają wyczerpujący opis teorii i praktyki oraz pełne piśmiennictwo.

Rozdział o elektroforezie zonalnej został znacznie unowocześniony przez wprowadzenie opisów rozdziału na żelu akryloamidowym i octanie celulozy.

W sumie należy powiedzieć, że drugie wydanie *Techniques in Protein Chemistry* jest rzeczywiście znacznie unowocześnione w porównaniu z pierwszym i aczkolwiek stanowi podręcznik dotyczący w głównej mierze bardziej klasycznych metod chemii i biochemii białek, tym niemniej jest pozycją bardzo wartościową, która niewątpliwie ułatwi pracę w tysiącach laboratoriów zajmujących się badaniem białek.

Michał Bagdasarian

C. J. Duncan: THE MOLECULAR PROPERTIES AND EVOLUTION OF EXCITABLE CELLS. International Series of Monographs in Pure and Applied Biology (Zoology Division), vol. 35. Pergamon Press, Oxford 1967; str. 253, cena 70 s.

W ciągu ostatnich 10 lat nagromadzono olbrzymi materiał doświadczalny przemawiający za tym, że fizykochemiczne właściwości błony komórkowej komórek pobudliwych są kontrolowane przez czynne procesy enzymatyczne, a więc że te procesy determinują tak podstawowe zjawiska jak pobudzenie i pobudliwość. Do najważniejszych czynników w tym zakresie należy wykrycie w błonach komórkowych mechanoenzymów — kurczliwych białek zbliżonych do aktomiozynu i posiadających własności ATP-azowe.

Duncan przedstawia w swej monografii fascynującą hipotezę unifikującą mechanizm funkcji wszystkich komórek pobudliwych i ich poszczególnych różniących się funkcjonalnie części: elementów wejściowych — receptorów, przewodzących — aksonów oraz wyjściowych — wydzielniczych błon presynaptycznych oraz błon postsynaptycznych. Te poszczególne elementy występujące u zwierząt wyższych rozwinęły się z prostego układu receptorowo-efektorowego występującego u ameb. Mechaniczna deformacja błony komórkowej tych zwierząt wywołuje zmianę w stosunku membranowej ATP-azy do jej substratu powodując bezpośrednią zmianę jej przestrzennej konfiguracji i efekty ruchowe. W toku rozwoju membranowa ATP-aza jednokomórkowców uzyskała inne funkcje, a przede wszystkim mechaniczną kontrolę średnicy porów błony komórkowej (a więc jej przepuszczalności dla jonów) oraz czynny transport jonów. Głównym przedmiotem zainteresowania autora jest mechanizm zmian biernej przepuszczalności błony komórkowej dla jonów, będących przyczyną generowania potencjałów czynnościowych w odpowiedzi na działające na nie bodźce. Konsekwentnie i logicznie autor dokonuje analizy olbrzymiego materiału doświadczalnego nagromadzonego w literaturze, upoważniającej go do przypuszczenia, że we wszystkich rozpatrywanych przez niego elementach komórek pobudliwych bodziec powoduje zmianę stosunku mechanoenzym-substrat doprowadzającą do strukturalnego przekształcenia enzymu i zmiany średnicy porów błony. Niesłychanie cennym i atrakcyjnym elementem hipotezy Duncana jest wykorzystanie danych dotyczących właściwości cholinesterazowych membranowych ATP-az. Byłyby to miejsca receptorowe mechanoenzymu warunkujące jego wrażliwość na acetylocholinę, a tym samym warunkujące chemowrażliwość np. błony postsynaptycznej na mediator.

Hipoteza Duncana oparta jest na wnikliwej, krytycznej analizie ogromnego materiału, stanowiącego cały prawie dorobek współczesnej fizjologii i biochemii komórek pobudliwych. Monografia jego jest więc niezwykle cennym przeglądem literatury w tym zakresie, tym cenniejszym że prace, na których jest oparta, są rozproszone po setkach czasopism różnych specjalności i wobec tego trudne do wyśledzenia przez przeciętnego czytelnika.

Monografia Duncana jest syntezą jednego z najbardziej fascynujących kierunków badań współczesnej biologii, leżącego na pograniczu fizjologii, biochemii i biofizyki, i zapełnia dotkliwą lukę w monograficznej i podręcznikowej literaturze tych zagadnień.

Bohdan Lewartowski

Organisch-chemische Arzneimittel und ihre Synonyma

Eine tabellarische Übersicht

Von Dr. MARTIN NEGWER

3., neubearbeitete, und stark erweiterte Auflage 1966. VIII, 1224 Seiten — gr. 8° — Lederin MDN 110,—

Diese tabellarische Übersicht ist in den wenigen Jahren seit dem Erscheinen ihrer ersten Auflage zu einem unentbehrlichen Standardwerk für all jene geworden, die mit Arzneimitteln zu tun haben.
Die dritte Auflage mußte wegen der seit Herausgabe der zweiten Auflage im Jahre 1961 in großer Zahl neuentdeckten Arzneimittel wiederum stark erweitert werden. Der Verfasser bemühte sich außerdem, noch vorhandene Lücken bei der Wiedergabe der Synonyma aus allen Ländern der Erde weitgehend zu schließen. Dadurch hat sich der Umfang des gebotenen Materials gegenüber der zweiten Auflage von 2600 chemisch einheitlichen Arzneistoffen mit mehr als 16 000 Synonyma auf über 3 900 Arzneistoffe mit etwa 26 000 Synonyma erhöht.
Darüber hinaus nahm der Verfasser verschiedene weitere Verbesserungen vor. So wurde die Wiedergabe der Strukturformeln der Steroide und der quartären Ammoniumverbindungen modernisiert. Die systematischen Namen wurden weitgehend der I U PAC-Nomenklatur angepaßt, daneben jedoch die älteren, noch häufig verwendeten Bezeichnungen auch weiterhin aufgeführt. Bei den Synonyma wurde eine unterschiedliche Kennzeichnung der von der Weltgesundheitsorganisation (WHO) vorgeschlagenen und der endgültig festgelegten internationalen Freinamen vorgenommen. Bei zahlreichen Arzneistoffen wurde eine eingehendere „Charakterisierung bzw. therapeutische Verwendung" angegeben.
Dem Buch wurde zusätzlich zum Synonymaregister ein über 800 Stichworte enthaltendes Gruppenregister beigefügt, durch das chemisch und zum Teil auch pharmazeutisch miteinander verwandte Arzneistoffe leichter aufgefunden werden können.

AUS DEN ZAHLREICHEN FACHURTEILEN

...Die in dem Werk angewandte Anordnung der Arzneimittel nach steigenden Summenformeln hat sich ausgezeichnet bewährt. Sie erlaubt, jedes Arzneimittel ohne Kenntnis eines Synonyms leicht aufzufinden.

Freiheit, Halle/Saale, 1962

...stellt ein ausgezeichnetes Nachschlagewerk dar, das eine schnelle Orientierung über die chemische Zusammensetzung eines Arzneimittels, seine im In- und Ausland gebräuchlichen Synonyma sowie über die therapeutische Versendung ermöglicht. Druck und Ausstattung des Buches sind lobenswert.

Pharmazeutische Zentralhalle, Dresden, 1962

...Die vorliegende Neubearbeitung wird jedem Pharmazeuten, pharmazeutischen Chemiker, Pharmakologen und Mediziner von großem Nutzen sein.

Archiv der Pharmazie, Heidelberg, 1962

...Das Werk ist ein ganz vorzüglicher, auch wissenschaftlich wertvoller Führer durch die Fülle unserer Arzneimittel. Es wird jedem Chemiker, Pharmakologen, Pharmazeuten und Mediziner, der auf den Gebieten der Erforschung, der Anwendung oder der Herstellung chemisch-pharmazeutischer Präparate tätig ist, nützlich sein, denn es gehört zu den besten Orientierungs- und Nachschlagewerken, die je für das Gebiet der Arzneimittel geschrieben worden sind.

Arzneimittel-Forschung, Aulendorf/Württemberg, 1962

Bestellungen durch eine Buchhandlung erbeten

Zamówienia można składać w księgarniach prowadzących sprzedaż wydawnictw importowanych oraz w Ośrodku Rozpowszechniania Wydawnictw Naukowych PAN (Warszawa, Pałac Kultury i Nauki, wejście główne od ul. Marszałkowskiej).

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