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INCORPORATION OF [^{14}C]LYSINE INTO DIFFERENT FRACTIONS OF HISTONES FROM BRAIN CELL NUCLEI

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1. Rabbit cerebral histone fractions were isolated 4 and 46 h after [^{14}C]lysine injection into the cerebellomedullary cistern. 2. The highest radioactivity calculated per 1 μg of lysine was found in fraction *f 4*, and in fraction *f 1* the lowest. 3. Differences in the turnover of the histone fractions may indicate their different role in the DNA--histone complex.

Cell division in adult brain is rare. Both neurons and glial cells are known to incorporate but traces of radioactive precursors into DNA which indicates a considerable stability of DNA, whereas, as reported by Borkowski (1962) the metabolism of specificated RNA is very high.

Changes in the content and composition of both RNA and protein in the nervous system have been demonstrated under a variety of conditions, such as physiological stimulation and during learning (Glassman, 1969). In view of the role ascribed to RNA in the function of the brain, and the possibility of involvement of cell nuclear proteins in the transcription of genetic information (Huang & Bonner, 1962; Allfrey, Littau & Mirsky, 1963), the presented experiments were undertaken to characterize the histones isolated from brain cell nuclei, and to determine the rate of incorporation of radioactive lysine into different histone fractions.

MATERIALS AND METHODS

The experiments were carried out on adult rabbits of mixed breed. For one experiment, four animals were used. The rabbits were anaesthetized by intravenous administration of Eunarcone (Riedel de Haen AG, Seelze-Hannover, D.B.R.); then L-[^{14}C]lysine (spec. act. 3 mCi/mmole; N. V. Phillips Duphar, Amsterdam, The Netherlands) in isotonic NaCl solution was injected into the cerebellomedullary cistern in an amount corresponding to 50 μCi per animal. After 4 and 46 h, the rabbits were killed by air embolism and the brain was removed rapidly. All further

steps of the procedure were carried out at 0 - 4°C. Cell nuclei were isolated by centrifugation in 1.5 M-sucrose solutions as described by Mardell, Harth, Borkowski & Mandel (1961), and their purity was checked by phase contrast microscopy.

From the nuclei, basic proteins were extracted by the method of Hnilica (1966) with some modifications: the nuclei were suspended in 20 volumes of 0.14 M-NaCl - - 0.01 M-sodium citrate, homogenized for 45 sec at 2500 rev./min in a Unipan (Warszawa, Poland) homogenizer and centrifuged at 1000 *g* for 10 min. The obtained sediment was washed five times with the same solution, then twice with 70% ethanol. From the sediment, which contained deoxynucleoproteins, histones were extracted twice with 0.25 N-HCl. The combined extracts were clarified by centrifugation for 30 min at 20 000 *g*, dialysed against water and precipitated with trichloroacetic acid added to 20% concentration. The histone precipitate was washed with an ethanol - ether (1:2, v/v) mixture, then with ether, and dried under vacuum; then it was dissolved in 0.1 M-Na-acetate buffer and fractionated on CM-cellulose column according to Johns, Phillips, Simson & Butler (1960). The obtained fractions were precipitated with trichloroacetic acid, then the radioactivity was measured and the amino acid composition determined.

Analytical methods. Protein was determined by the spectrophotometric method of Wadell & Hill (1956), and total nitrogen according to Bürck (1960). Whole histone and histone fractions after hydrolysis in 6 N-HCl were subjected to electrochromatographic separation according to Fishl & Segal (1963), and lysine, arginine and acidic amino acids were determined according to Fischer & Dörfel (1953).

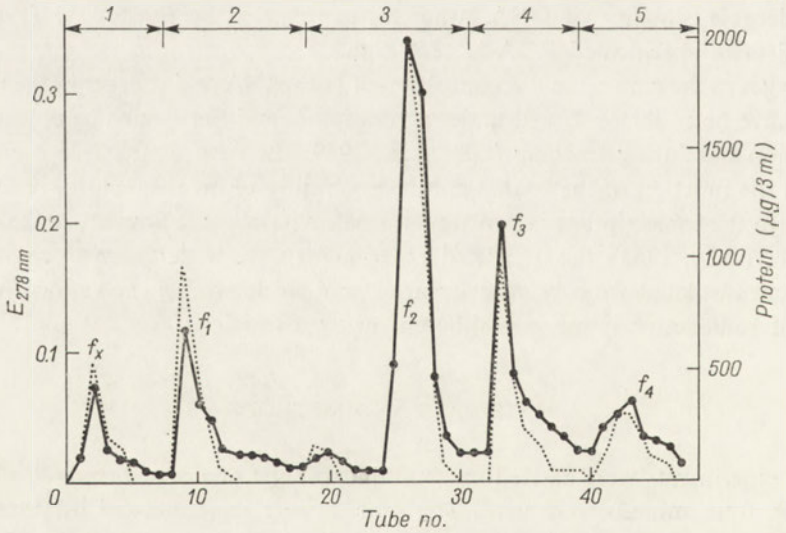

Fig. 1. Elution pattern of rabbit brain histones on CM-cellulose column (0.8 × 8.0 cm). The histone applied to the column, 10 mg, was dissolved in 0.1 M-Na-acetate buffer, and eluted with: (*1*), 0.1 M- -Na-acetate buffer, pH 4.2; (*2*), 0.1 M-acetate buffer, pH 4.2, containing 0.35 M-NaCl; (*3*), 0.01 N- -HCl; (*4*), 0.02 N-HCl; (*5*), 0.5 N-HCl. Fractions of 3 ml were collected at a flow rate of 0.5 ml/min. ——, Extinction at 278 nm; •••, protein content determined by the method of Wadell & Hill (1956).

For radioactivity determinations, the histone solution was spotted on discs of Whatman no. 3 paper, 3.0 cm in diameter (Rubin, Kelners & Goldstein, 1967), and radioactivity was measured in a scintillation counter USB-2 [scintillation liquid: 10 mg of 1,4-di-(5-phenyloxazol-2-yl)benzene (Koch-Light, Colnbrook, Bucks., England), 400 mg of 2,5-diphenyloxazole (Reanal, Budapest, Hungary), 100 ml of toluene].

RESULTS

As nuclei of brain cells, when examined under a phase contrast microscope, exhibited a high degree of purity, it may be supposed that the extracted histones were relatively uncontaminated by cytoplasmic proteins. From 100 g of brain tissue, 42.8 - 48.6 mg of whole nuclear histone was obtained. The total nitrogen content in whole histone was 17.6%. By CM-cellulose column chromatography (Fig. 1), four main fractions were obtained corresponding to histone fractions *f 1*, *f 2*, *f 3* and *f 4*, and a fraction designated *fx* which consisted of protein non-adsorbed on the column. The percentage distribution of histone fractions *f 1* - *f 4*, and the content of lysine, arginine and acidic amino acids, are presented in Table 1.

On the basis of the content of lysine and arginine, fraction *f 1* may be considered a lysine-rich fraction, fractions *f 2* and *f 4* moderately lysine-rich fractions, and fraction *f 3*, an arginine-rich one. The ratio of basic to acidic amino acids was the highest in fraction *f 1*, and the lowest in fraction *f 4*. Histones of brain cell nuclei showed a rather high incorporation of radioactive lysine. In the 4-h experiment, the radioactivity of whole histones corresponded to 11.5% of the total radioactivity determined in cell nuclei. In the 46-h experiment, this value was 14%.

Table 1

Characteristics of brain nuclear histones separated on CM-cellulose

The results are mean values from 6 - 10 determinations, ± S.D.

Fraction	Distribution (%)	Lysine (mg%)	Arginine (mg%)	Ratio of lysine to arginine	Ratio of basic to acidic amino acids
Whole histone	—	16.46 ±0.35	10.17 ±0.52	1.55 ±0.11	1.66 ±0.17
f 1	20.35 ±1.01	23.38 ±1.25	4.67 ±0.39	5.14 ±0.11	2.93 ±0.22
f 2	43.57 ±3.37	13.65 ±0.58	8.01 ±1.03	1.72 ±0.18	2.44 ±0.12
f 3	23.94 ±2.80	11.45 ±0.47	12.38 ±2.18	0.94 ±0.16	1.70 ±0.064
f 4	5.72 ±1.54	10.10 ±0.25	4.26 ±0.21	2.60 ±0.40	1.18 ±0.512

Table 2

Incorporation of [^{14}C]*lysine into the main fractions of cerebral histones*

The results are mean values from 5 experiments, ±S.D. The relative specific activity per 1 μg of lysine is expressed in relation to the activity of fraction *f 1*.

Fraction	4 hours			46 hours		
	counts/min/mg protein	counts/min/μg lysine	relat. spec. act.	counts/min/mg protein	counts/min/μg lysine	relat. spec. act.
Whole histone	2077 ±265	12.32 ±1.51	1.15 ±0.02	2893 ±283	17.69 ±1.80	1.42 ±0.074
f 1	2517 ±212	10.82 ±0.89	1.00	2972 ±515	12.57 ±2.25	1.00
f 2	1500 ±153	11.02 ±1.08	1.04 ±0.081	2091 ±490	15.21 ±3.71	1.29 ±0.11
f 3	1655 ±125	13.50 ±1.01	1.25 ±0.21	2412 ±364	20.06 ±3.06	1.63 ±0.20
f 4	2320 ±148	19.40 ±1.18	1.80 ±0.28	2875 ±438	27.73 ±5.08	2.24 ±0.24

In the present work, to facilitate penetration of labelled lysine into the brain tissue (Sylverstone, 1958), the isotope was introduced into the cerebellomedullary cistern.

On the basis of determination of radioactivity of lysine separated by electrochromatography of protein hydrolysates, it was found that all the radioactivity of the analysed fractions was derived from the incorporated [^{14}C]lysine. The values of radioactivity calculated per 1 mg protein and per 1 μg of lysine in whole histone and in its fractions at 4 and 46 h after the injection of radioactive lysine, are presented in Table 2. At 4 h, the incorporation of labelled lysine into all histone fractions was observed. The highest radioactivity per 1 μg of lysine was found in fractions *f 4* and *f 3*, the lowest in fraction *f 1*. After 46 h, a corresponding increase in the incorporation of lysine was observed but it was not proportional with time. Similarly as in 4-h experiments, the highest radioactivity after 46 h was observed in fraction *f 4*.

DISCUSSION

A number of communications indicate that the metabolic activity of histones is low, as the incorporation of labelled amino acids into histones was found to be much lower than into other proteins. Piha, Cuenod & Waelsch (1966) demonstrated that the metabolism of whole histone from brain cell nuclei is very low, and concluded that it is closely associated with the high stability of DNA.

In the present work it was found that the incorporation of radioactive lysine into individual fractions of cerebral histones was not uniform. Both in the 4-h and 46-h experiments, the lowest activity per 1 μg of lysine was observed in the lysine-rich fraction *f 1*. The activity of fraction *f 4* was twice as high as that of *f 1*, and of the arginine-rich fraction *f 3*, 1.5 times as high. No such differences were observed by Laurence & Butler (1965) and Dick & Johns (1969) who in experiments *in vivo* found a uniform distribution of activity of [^{3}H]lysine in the main fractions of histones isolated from liver, tumours and thymus. However, according to Hnilica & Busch (1963) and Hnilica, Kappler & Hnilica (1965), the arginine-rich fraction obtained from liver tissue showed higher metabolic activity as compared with other fractions. Also the results of Ohly, Mehta, Mourkides & Alivisatos (1967) and Ord, Raaf, Smit & Stocken (1965) point to differences in the metabolism of histone fractions. Chalkley & Maurer (1965) have demonstrated that in those tissues in which synthesis of DNA is very low, only the arginine-rich histone fraction is metabolized.

In the present work, the turnover of the particular histone fractions was found to differ, which may indicate the different role of these fractions in the DNA-histone complex. Fraction *f 1*, more metabolically stable, could play a structural role, whereas fractions *f 3* and *f 4* could participate in regulation of the function of genome (Spelsberg, Tankersley & Hnilica, 1969).

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INKORPORACJA [^{14}C]LIZYNY DO RÓŻNYCH FRAKCJI HISTONÓW Z JĄDER KOMÓREK MÓZGOWYCH

Streszczenie

1. Z mózgów króliczych w 4 i 46 godzin po wstrzyknięciu dozbiornikowym [^{14}C]lizyny izolowano 4 frakcje histonów.

2. Najwyższą aktywnością specyficzną wyrażoną w imp/min/μg lizyny charakteryzowała się frakcja *f4* a najniższą frakcja *f1*.

3. Różnice w metabolizmie poszczególnych frakcji histonów z jąder komórek mózgowych mogą wskazywać na ich niejednakową rolę w kompleksie DNA-histon.

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SIMILARITY OF *SALMONELLA TYPHI* AND *ESCHERICHIA COLI* MUREINS

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The structure of *Salmonella typhi* murein was compared with the well-known murein of *Escherichia coli* B. The products of digestion of both mureins by lysozyme proved to be identical in chromatographic and electrophoretic assays. The identity of the cross-linking bond in both mureins is discussed on the basis of Vi-phage II endopeptidase action.

The murein of Gram-positive and Gram-negative bacteria is built according to the same general plan but differs in essential details (Ghuysen, 1968). Investigation of many Gram-positive species showed that variation in the murein structure concerns mainly cross-linking bridges and composition of diamino acids (Schleifer, 1970). Among Gram-negative bacteria, the murein of *E. coli* B is the most thoroughly investigated one (Martin, 1966; Weidel & Pelzer, 1964). The examinations extended to a rather small number of other Gram-negative bacteria seem to suggest close similarity of the isolated mureins (Schocher, Bayley & Watson, 1962; Weidel, Frank & Leutgeb, 1963; Martin, 1964; Mardarowicz, 1966). However, the murein of *Proteus mirabilis* is digested by lysozyme to a lesser extent than that of *E. coli* (Katz & Martin, 1970) which may indicate differences in structure. Chromatographic studies of Weidel *et al.* (1963) show that although the muropeptide pattern of the lysozyme digestion products of *Salmonella gallinarum* is similar to that of *E. coli* murein, an additional strong spot (denoted X) appears on chromatograms of the *Salmonella* digest, which might reflect different composition of the *Salmonella* murein. The purpose of our work was to compare the structure of *Salmonella typhi* and *Escherichia coli* B mureins.

MATERIAL AND METHODS

Reagents. Lysozyme 3× crystallized was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), Visking dialysing tubing from Serva (Heidelberg, West Germany), MN 300 cellulose for thin-layer chromatography and MN 2214 FF paper for preparative chromatography, from Macherey & Nagel Co. (Düren, West

Germany); the latter was washed four days in the solvent system used for chromatography. Whatman no. 1 paper was purchased from W. & R. Balston Ltd. (England). Pyridine (Zakłady Koksochemiczne, Chorzów, Poland) was dried over NaOH pellets and distilled after short heating with a small amount of ninhydrin (Gerday, Robyns & Gosselin-Rey, 1968). Isobutanol, *n*-propanol (Kutnowskie Zakłady Przemysłu Spirytusowego, Kutno, Poland) and *n*-amyl alcohol (British Drug Houses, Poole, England) were purified by distillation.

Preparation of murein and its digestion with lysozyme. The mureins from *Salmonella typhi* 21802, phage type A, and *Escherichia coli* B were prepared as described by Taylor, Kwiatkowski & Antadze (1969) and stored as a suspension in chloroform-saturated water (5 mg/ml). Before use, the murein was dialysed against 0.1 M-ammonium acetate, pH 6.6, for 3 h. Then 1 mg of lysozyme in 0.5 ml of ammonium acetate was added and the digestion was carried out at 37°C, with simultaneous dialysis against 20 ml of a fresh solution of ammonium acetate containing few drops of chloroform; the dialysis vessel was covered with polyethylene foil. After 24 hours the external solution was changed, 0.5 ml of lysozyme solution was added to the reacting mixture and the digestion was continued for another 18 h.

The non-diffusible fraction was freed from ammonium acetate by dialysis against four changes of water. These diffusates were combined with those obtained during the digestion procedure, concentrated and dried. Ammonium acetate was removed by sublimation under reduced pressure over NaOH pellets in a desiccator, for four days. From the non-diffusible fraction, the slight insoluble residue was centrifuged off and dried. The supernatant of this fraction was concentrated under reduced pressure to 2 ml and lysozyme was precipitated by adding 2 ml of 10% trichloroacetic acid, and removed by centrifugation. Trichloroacetic acid was then repeatedly extracted with ethyl ether until the aqueous phase became neutral. The solution of non-diffusible substances was concentrated and dried in a desiccator.

The control sample, which did not contain the murein, was handled in the same way.

Chromatography. MN 300 cellulose plates were prepared according to Gerday *et al.* (1968). For chromatography, the following systems were used: pyridine - water - *n*-amyl alcohol - *n*-propanol - isobutanol (3:3:1:1:1, by vol.) and *n*-butanol - acetic acid - water (4:1:5, by vol.). Spots were localized with 0.3% ninhydrin in acetone.

Electrophoresis. This was carried out in the following buffers, containing in a final volume of 1500 ml: 1), 90 ml of acetic acid and 21 ml of formic acid (pH 1.9); 2), 9 ml of pyridine and 34.5 ml of acetic acid (pH 4); 3), 73.5 ml of pyridine and 3.6 ml of acetic acid (pH 6.5).

RESULTS AND DISCUSSION

Digestion of *Salmonella typhi* and *Escherichia coli* mureins with lysozyme was performed as described in Methods, in dialysis tubing to ensure rapid removal of the released low-molecular fragments and thus to avoid transglycosylation cata-

lysed by lysozyme (Sharon, 1967), which may obscure the results (Weidel & Pelzer, 1964).

Under the applied conditions, both investigated mureins yielded on average 90% of soluble products; the diffusible fractions corresponded roughly to 80% of murein weight. The insoluble residue accounted for 2 - 5% but it did not contain diaminopimelic acid, as shown by chromatography according to Rhuland, Work, Denman & Hoare (1955), after hydrolysis in 4 N-HCl for 17 h at 103°C, so it probably represented a contamination from the other cell wall structures.

Since separation of the diffusible and non-diffusible soluble fractions was not reproducible, both fractions were combined for the analysis of muropeptides resulting from digestion of mureins by lysozyme.

Separation of muropeptides by thin-layer chromatography on cellulose with pyridine - water - *n*-amyl alcohol - *n*-propanol - isobutanol (twice) in one dimension, and electrophoresis at pH 1.9 (20 V/cm) in the second dimension did not give unequivocal results as to the identity of the obtained pattern. Therefore the muropeptides were separated by preparative paper chromatography as described by Primosigh, Pelzer, Maass & Weidel (1961) on MN 2214 FF paper in *n*-butanol - - acetic acid - water (4:1:5, by vol.). Muropeptides (10 mg) were applied on 14 cm length of the paper strip. To make sure of the correspondence in separated fractions, the muropeptides derived from both mureins were separated on two parts of the same paper sheet, conditioned for 2 h in vapours of the lower phase of the solvent system and then developed for 7 days in the upper phase. Fig. 1 shows a guiding strip of the chromatogram. The fractions were denoted according to the nomenclature of Primosigh *et al.* (1961). The separated muropeptides were eluted from chromatograms and dried in siliconized microbeakers. Each fraction of the 10 mg muropeptide mixture was dissolved in 300 µl of 10% isopropanol in water. For paper electrophoresis 3 µl of each fraction was applied to Whatman no. 1 paper and the separation was performed in buffers of pH 1.9; 4.0; and 6.5. In each case the corresponding fractions from *Salmonella typhi* and *Escherichia coli* showed the same muropeptide pattern. The best separation, shown in Fig. 2, was achieved at pH 1.9 (20 V/cm, 90 min). It appeared from the control sample that the faint, fast spots were accidental contaminations, probably from the dialysing tubing and chromatographic paper. The results of the electrophoresis convinced us that mureins from *Salmonella typhi* and *Escherichia coli* gave identical muropeptides on lysozyme treatment. Nevertheless, it should be mentioned that in preliminary experiments, when several batches of murein were used, an additional muropeptide in *Salmonella typhi* digest was sometimes found similarly as in the experiments of Weidel *et al.* (1963). This muropeptide was isolated and hydrolysed in 4 N-HCl (17 h, 103°C) and found to contain, in addition to five characteristic murein components, glycine, aspartic acid and lysine together with lesser amounts of other unidentified amino acids. The appearance of this additional peptide may be connected with the result obtained recently by Braun & Sieglin (1970) who showed that in *E. coli* the murein is covalently bound with other heteropolymers of bacterial cell wall. The connecting link was composed of lysine and lysyl-arginine, which might

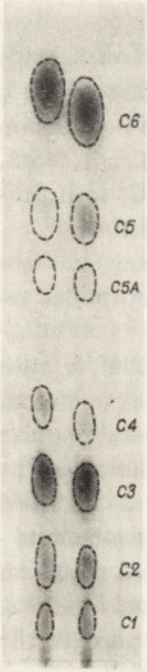

Fig. 1. A guiding strip of a preparative chromatogram developed in the upper phase of *n*-butanol - acetic acid - water (4:1:5, by vol.) for 7 days (details in the text). *Salmonella typhi* muropeptides are given on the left side, and those of *E. coli*, on the right side.

remain bound with every tenth diaminopimelic acid residue of the murein after trypsin and pronase digestion. Okuda & Weinbaum (1968) isolated several glycoproteins from cytoplasmic membrane of *Escherichia coli* and showed that at least one of them contains a high proportion of aspartic acid and *N*-acetylglucosamine. The authors assume that this glycoprotein may represent an initiation site of murein synthesis, which would justify the presence of small amounts of aspartic acid in the

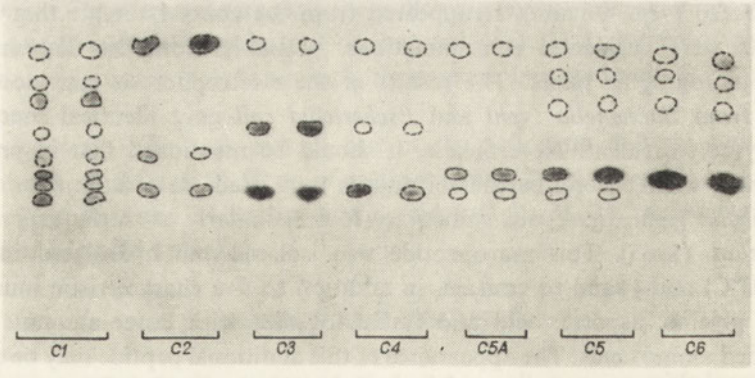

Fig. 2. Electrophoretic separation of muropeptide fractions (*C1* - *C6*) from *Salmonella typhi* and *Escherichia coli* B. The muropeptides from *S. typhi* are on the left side of each pair, and those from *E. coli* on the right side. Electrophoresis was carried out at pH 1.9, 20 V/cm, for 90 min.

purified murein preparations. It seems then that an additional muropeptide appearing sometimes in the *Salmonella typhi* murein digests originates from the other layers of bacterial envelope.

The main fractions of lysozyme digests of the mureins from *Salmonella typhi* and *Escherichia coli* were muropeptides *C6* and *C3*. *C6* was homogeneous under all conditions of electrophoresis that were tested, but from *C3* a faster moving component separated at pH 1.9. The structure of muropeptides *C3* and *C6* from *Escherichia coli* shown in Fig. 3 was established by Primosigh *et al.* (1961), Pelzer (1962),

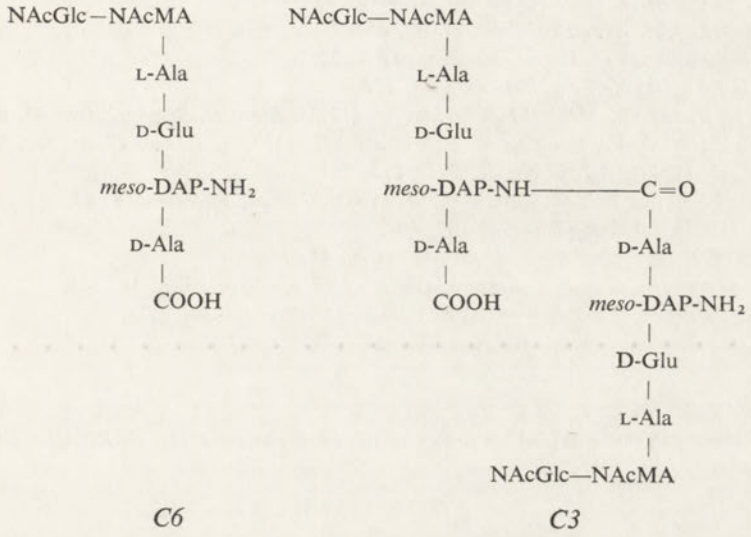

Fig. 3. The structure of muropeptide *C6* and *C3* (Weidel & Pelzer, 1964).

Heijenoort *et al.* (1969) and Dezélée & Bricas (1970). The homogeneity of *C6* and electrophoretically purified *C3* muropeptides from both mureins was confirmed by thin-layer chromatography in pyridine - water - *n*-propanol - isobutanol - *n*-amyl alcohol (3:3:1:1:1, by vol.). The corresponding muropeptides from *S. typhi* and *E. coli* appeared at the same position on the chromatograms.

Additional evidence for the identity of the *C3* muropeptides from both mureins comes from our previous work on the specificity of Vi-phage II endopeptidase (Taylor, 1970). The muropeptide *C3* from *S. typhi* was split by this enzyme into *C6* units. It was proved by dinitrophenylation that a free amino group of diaminopimelic acid appeared in the reaction, which means that in the *Salmonella typhi* murein, as in *Escherichia coli*, there occurs the same kind of cross-linkage: through the amino group of diaminopimelic acid.

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PODOBIEŃSTWO MUREINY *SALMONELLA TYPHI* I *ESCHERICHIA COLI*

Streszczenie

Porównano budowę mureiny *Salmonella typhi* ze znaną mureiną *Escherichia coli*. Obie mureiny trawiono lizozymem i stwierdzono, że powstające produkty są jednakowe. Identyczność wiązań peptydowych stanowiących poprzeczne usieciowanie mureiny przedyskutowano na podstawie wcześniejszych doświadczeń nad działaniem endopeptydazy bakteriofaga Vi II.

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SPECIFICITY OF ACID PHOSPHATASE FROM HUMAN PROSTATE

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1. The rate of dephosphorylation of oligonucleotides by the purified preparation of acid phosphatase decreases with increasing chain length of the nucleotide. 2. Mono- and oligonucleotides containing C or A are hydrolysed more readily than those containing U or G. 3. The rate of hydrolysis of nucleoside 3′-phosphates is greater than that of the 5′- and 2′-isomers, irrespective of the type of base and sugar moiety of the nucleotide. The hydrolysis of nucleoside 3′- and 5′-phosphates and 3′,5′--diphosphates showed that the free 5′-OH group of the sugar plays an important role in formation of the enzyme-substrate complex. 4. Deoxynucleotides are dephosphorylated more readily than the ribose-containing nucleotides; some nucleotides as derivatives of D-arabinose and D-β-glucopyranose are hydrolysed at a lower rate than the corresponding ribonucleotides.

Acid phosphatase (EC 3.1.3.2) from human prostate was applied for splitting of the terminal phosphate from oligonucleotides to improve their separation on chromatographic columns, and for determination of nucleotide sequence (Markham & Smith, 1952a; Staehelin, 1961; Roberts, Dekker, Rushizky & Knight, 1962; Becker & Hurwitz, 1967; Richards & Laskowski, 1969). Heppel, Harkness & Hilmoe (1962) and Petersen & Burton (1964) reported that the results were dependent on the type of the nucleotides being dephosphorylated, and Staehelin (1964) observed the effect of chain length and base composition on the dephosphorylation rate.

In the present work we have undertaken an examination of the specificity of acid phosphatase from prostate toward various mono- and oligonucleotides, taking into account chain length, type of sugar moiety, base composition and position of the terminal phosphate.

MATERIALS AND METHODS

The enzyme termed acid phosphomonoesterase I (Dziembor, Gryszkiewicz & Ostrowski, 1970) was prepared and purified from human prostate as described by Ostrowski (1968). The obtained preparation had an activity toward *p*-nitrophenylphosphate of 1.7 mmol/mg of protein/min at 37°C, and was homogeneous

on ultracentrifugation and disc electrophoresis, whereas on isoelectric focusing it gave four fractions, all having the same specific activity (Ostrowski, Wasyl, Weber, Gumińska & Luchter, 1970). The preparation was devoid of the activities of phosphodiesterase, ribonuclease and deoxyribonuclease.

As substrates, the following commercial nucleotide preparations were used: 5′-AMP and 5′-dAMP (Koch-Light, Colnbrook, Bucks., England); 5′-IMP and 5′-dTMP, disodium salts (Biochem. Research Co., Calif., U.S.A.); 3′-dGMP and 5′-dGMP (Pabst Labs., Milwaukee, Wis., U.S.A.); dpGp, dGpGp, dTpGp and dApGp were prepared by the method of Sulkowski & Laskowski (1962) and were a kind gift of Dr. M. Laskowski, Sr.; 2′-, 3′- and 5′-D-arabinofuranosylcytosine phosphates were products of Upjohn Comp. (Kalamazoo, Mich., U.S.A.), obtained through the courtesy of Dr. J. Wechter; 2′-, 4′- and 6′-1-β-D-glucopyranosyluracil phosphates were a kind gift of Dr. D. Shugar; *p*-nitrophenylphosphate, disodium salt (Sigma Biochem. Co., St. Louis, Mo., U.S.A.); poly A (Miles-Seravac, Ltd., Maidenhead, England); yeast RNA (Merck, Darmstadt, West Germany) was additionally purified according to Kado (1968).

Ribo-3′-mononucleotides were prepared from yeast RNA by alkaline hydrolysis after Schmidt (1957) and separated by chromatography on DEAE-cellulose column as described by Staehelin (1961). The obtained 3′-mononucleotides were contaminated by small amounts of 2′-isomers.

The "core" of yeast RNA was prepared according to Markham & Smith (1952b); the freeze-dried "core" preparation was separated into fractions by DEAE-cellulose column chromatography in acetate buffer of pH 7.6, containing 7 M-urea, as described by Rushizky, Skavenski & Sober (1965). A typical fractionation is presented in Fig. 1. The fractions corresponding to the particular peaks were pooled, freeze-dried and then desalted according to Zamir, Holley & Marquisee (1965).

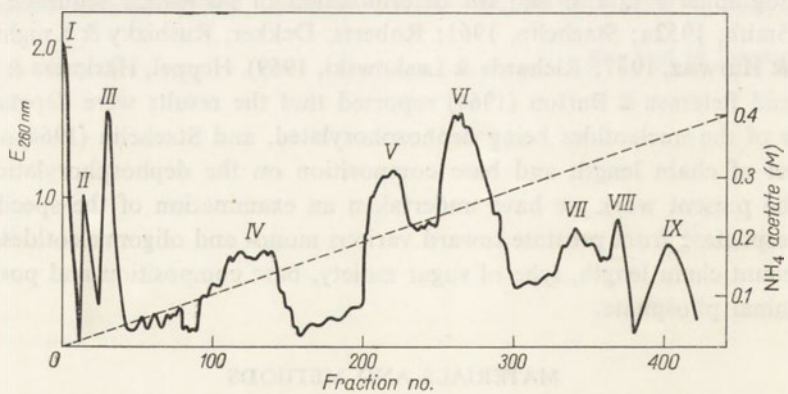


Fig. 1. Chromatographic separation of yeast RNA "core" fraction on DEAE-cellulose column. A 100 mg sample of "core" in 2 ml of the starting buffer was applied to the column (47 × 1 cm) equilibrated with 1 mM-acetate buffer, pH 7.6, containing 7 M-urea. The fractions were eluted with a linear concentration gradient from 1 mM to 400 mM-ammonium acetate buffer, pH 7.6, in 7 M-urea. Fractions of 5 ml were collected at a rate of 20 ml/h at room temperature.

Poly A oligomers[1] with a chain length of 1 to 9 nucleotides, were obtained as follows: 40 mg of the commercial preparation of poly A was incubated at 37°C in 40 ml of 2.5 mM-$CaCl_2$ - 0.05 M-triethylamine-acetate buffer, pH 9.0, with the addition of 0.075 unit per 1 ml of micrococcal nuclease prepared according to Sulkowski & Laskowski (1966). After 2 h of incubation, 30 ml of 0.01 M-EDTA solution, pH 7.0, was added and then water to bring the volume to 300 ml. Then the mixture was adsorbed on DEAE(Cl)-cellulose column (25×1 cm), washed with water, and the individual oligomers were eluted with a linear concentration gradient of NaCl prepared with 0.02 M-sodium acetate, pH 4.8 (1.5 litre) and 0.6 M-NaCl - 0.02 M--sodium acetate, pH 4.8 (1.5 litre). The course of the elution is presented in Fig. 2. The obtained oligonucleotide fractions were diluted each with three volumes of water, adjusted to pH 8.0 with NaOH and adsorbed on a DEAE-cellulose column (5×1 cm) equilibrated with 0.02 M-NH_4HCO_3, pH 8.0 (Tomlinson & Tener, 1963). The column was washed with the same buffer solution until the reaction for chloride ion had disappeared, then the oligonucleotide was eluted with 0.5 - 1.0 M-NH_4HCO_3 solution (Rushizky & Sober, 1962) and freeze-dried. The average yield was 70%.

The activity of acid phosphatase was determined with 0.02 M-*p*-nitrophenylphosphate as substrate, in 0.1 M-citrate buffer of pH 5.5 (Ostrowski & Tsugita, 1961) as in this buffer solution the enzyme exhibits the highest activity (Anagnostopoulos, 1953).

In the experiments with the nucleotides as substrates, if not otherwise indicated, the incubation mixture contained in a volume of 1 ml: 0.15 - 0.2 μmole of substrate

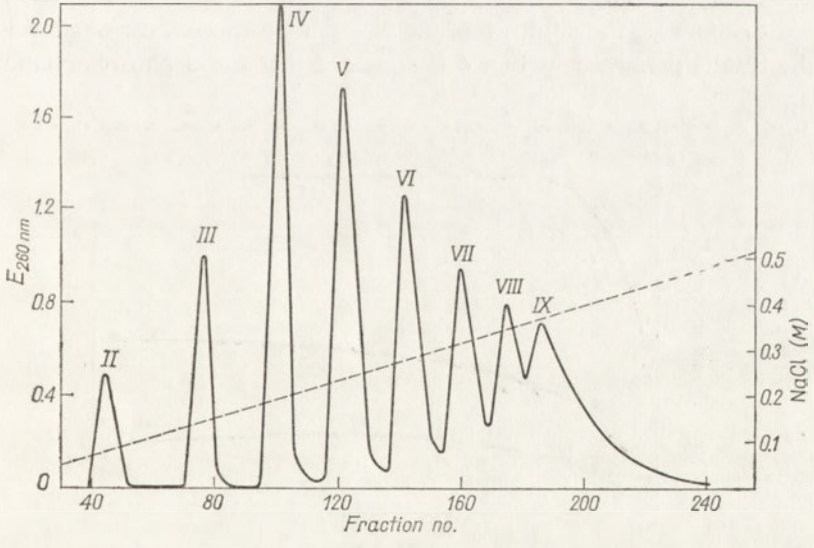


Fig. 2. Chromatographic separation on DEAE-cellulose column of the oligomers obtained from poly A by treatment with micrococcal nuclease. For details see Methods.

[1] We are grateful to Dr. E. Sulkowski, Laboratory of Enzymology, Roswell Park Memorial Institute, Buffalo, N.Y., for this preparation.

expressed as terminal phosphate, 100 μmoles of citrate buffer, pH 5.5, and 2.2 μl (0.4 μg protein) of the acid phosphatase preparation. The samples were incubated in a water bath at 37°C and at determined time intervals 200-μl portions of the solution were withdrawn and the liberated inorganic phosphate was determined by the method of Fiske-Subbarow as modified by Martland & Robison (1926).

The concentration of mononucleotides and some oligonucleotides was determined from molar extinction coefficients cited in the literature (Dawson, Elliott, Elliott & Jones, 1959; Wenkstern & Bayev, 1967). The concentration of those oligonucleotides for which the molar extinction coefficients are not known, was calculated on the basis of the content of terminal phosphate, which was calculated from the total content of phosphate and a known chain length (Bendich, 1957).

RESULTS

Effect of chain length. The course of dephosphorylation of mononucleotide, oligonucleotide mixture ("core") and commercial yeast RNA, is presented in Fig. 3. Complete dephosphorylation of the mononucleotide was achieved within about 2.5 h, the dephosphorylation of the mixture of oligonucleotides forming the "core" attained during this time only 13%, and the dephosphorylation of RNA was as low as 6%. It should be noted that, as described in Methods, the concentration of terminal phosphate and the ratio of enzyme to substrate were practically the same with all three substrates used.

In kinetic studies on the effect of chain length on the dephosphorylation rate, oligomers composed only of A, separated on DEAE-cellulose column (cf. Fig. 2) were used to eliminate the influence of the base composition of the oligonucleotide. From the results presented in Fig. 4 it appeared that the dephosphorylation rate

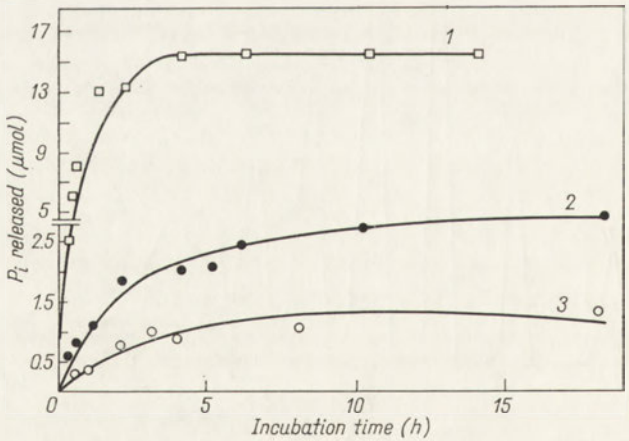


Fig. 3. Time-course of enzymic dephosphorylation of: (*1*), 3′-CMP; (*2*), RNA "core"; (*3*), RNA. Solutions of substrates, 5 mM (calculated per terminal phosphate) in 0.7 ml of 0.05 M-citrate buffer, pH 5.5, were incubated with 10 μl of enzyme solution at 37°C. At indicated time intervals, 60 μl portions were withdrawn and P_i determined.

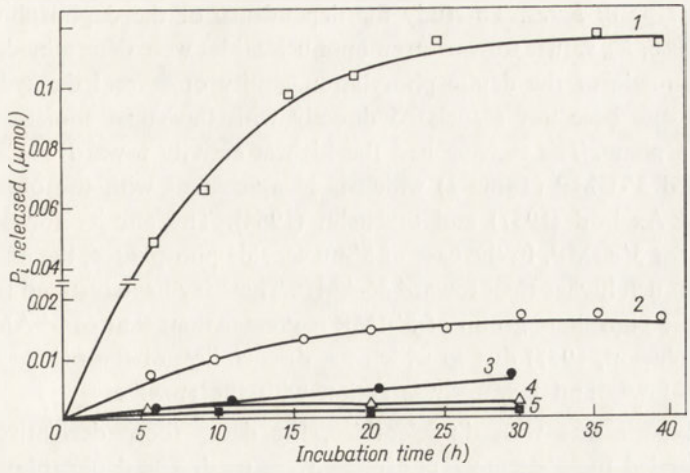


Fig. 4. Time-course of enzymic dephosphorylation of oligomers of poly A (see Fig. 2). The curves 1 - 5 represent, successively: Ap, $(Ap)_2$, $(Ap)_4$, $(Ap)_6$, and $(Ap)_8$.

decreased with increasing chain length of the oligonucleotide. The plot of logarithm of the relative reaction rate against chain length (Fig. 5) demonstrates the steady decrease in the reaction rate with increasing chain length, the plot being linear from $(Ap)_2$ to $(Ap)_8$ whereas there is a sharp inflection between Ap and $(Ap)_2$. This may indicate that the enzyme-substrate complex is readily formed in the case of the mononucleotide whereas beginning with the dinucleotide it is thermodynamically much less favoured.

Similar experiments were carried out with the separated "core" fractions (see Fig. 1). The differences in the dephosphorylation rate of different chain length fractions were less regular than with oligonucleotides composed of one type of base. This could be due to the less ordered structure of the "core" oligonucleotides resulting from their various base composition (Ts'o, Kondo, Schweizer & Hollis, 1969).

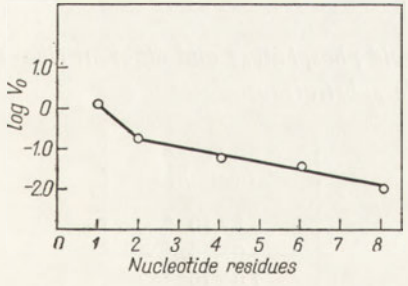


Fig. 5

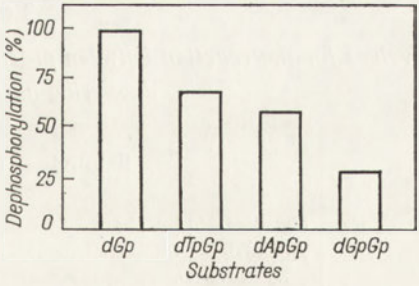


Fig. 6

Fig. 5. The rate of enzymic dephosphorylation of adenine oligonucleotides as a function of the number of nucleotide residues in the chain.

Fig. 6. Enzymic dephosphorylation of deoxyribonucleotides. The incubation time was 10 min.

Effect of type of bases. To study the dependence of the dephosphorylation on the type of base, K_m values for various mononucleotides were determined, and a comparison was made of the dephosphorylation ability of several deoxydinucleotides differing by one base not associated directly with the sugar moiety bearing the terminal phosphate. The enzyme had the highest activity toward 3′-CMP and the lowest toward 3′-UMP (Table 1) which is in agreement with the observations of Kilsheimer & Axelrod (1957) and Staehelin (1964). The affinity for 3′-AMP was higher than for 3′-GMP. In the case of 5′-nucleoside phosphates, the affinity toward 5′-IMP was much higher than toward 5′-AMP. This may have resulted from the fact that pK of the phosphate group of 5′-IMP is greater than that of 5′-AMP (Beaven, Holiday & Johnson, 1955) due to which the dissociation of the primary OH group at pH 5.5 is lower and favours interaction with the enzyme.

In the experiments with dinucleotides, the deoxyribose derivatives in which guanosine carried the 3′-terminal phosphate,were used. The dephosphorylation rate was doubled when the second base was not guanine but adenine or thymine (Fig. 6). In similar experiments Staehelin (1964) has found that cytidine dinucleotides, e.g. ApCp and GpCp, are dephosphorylated more rapidly than the uridine ones, ApUp and GpUp. Thus it appears that both with mono- and dinucleotides containing U or G the dephosphorylation is slower than with those containing C or A. The rate of phosphate splitting of dGp was as much as four times greater than that of dGpGp, similarly as it was observed with Ap and ApAp (cf. Fig. 4).

Effect of position of the phosphate residue. Kilsheimer & Axelrod (1957) reported that 2′-nucleoside phosphates were hydrolysed at a slower rate than 3′-isomers, and the 5′-phosphates still more slowly. This was confirmed in our experiments for the isomers of AMP and dGMP, as shown in Figs. 7 and 8. It is of interest that the isomers of D-arabinosylcytosine phosphate behave in somewhat different manner than the isomers of adenylic acid (Table 2); the 3′-isomer was hydrolysed at the greatest rate (K_m 2.5 mM), the 5′-isomer more slowly (K_m 4.1 mM), and the2′-isomer the most slowly (K_m having a very high value). The very low affinity of the enzyme

Table 1

K_m *values for the reaction between prostatic acid phosphatase and different ribo- and deoxyribo-nucleotide substrates*

Substrate	K_m values (moles/l)
Cytidine-3′-phosphate	6.8×10^{-4}
Adenosine-3′-phosphate	9.0×10^{-4}
Guanosine-3′-phosphate	1.0×10^{-3}
Uridine-3′-phosphate	3.3×10^{-3}
Adenosine-5′-phosphate	2.2×10^{-3}
Inosine-5′-phosphate	7.7×10^{-4}
Deoxythymidine-5′-phosphate	2.2×10^{-4}
Deoxyguanosine-5′-phosphate	1.6×10^{-3}

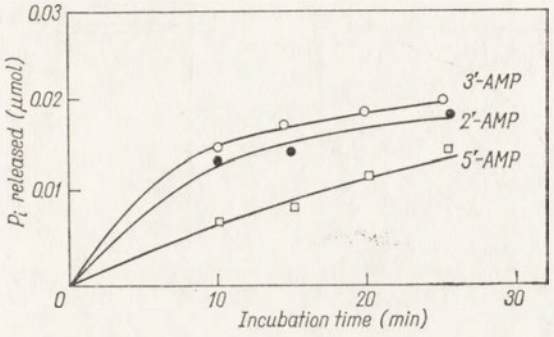


Fig. 7. Time-course of enzymic dephosphorylation of AMP isomers.

toward arabinosylcytosine 2′-phosphate could be due to its conformation in which the phosphate is located as if in a cleft of the furanose ring of the sugar and the base, whereas in the 3′- and 5′-isomers the phosphate is located more to the outside of the sugar ring (Wechter, 1967; Inoue & Satoh, 1969). It seems of interest that *E. coli* alkaline phosphatase dephosphorylated arabinose nucleotides at a rate decreasing in a different order, i.e. 2′-, 3′-, and 5′-, respectively (Wechter, 1967), whereas snake venom phosphomonoesterase hydrolysed only the 5′-derivative.

Also the phosphate derivatives of D-β-glucopyranosyluracil (Table 2) were hydrolysed at a rate decreasing in the following order: 4′-. 6′-, 2′-phosphates (cf. Fig. 10); this could be due to the same causes as in the case of arabinosyl nucleotides.

Of the isomers of dGMP (Fig. 8), the 3′-phosphate was dephosphorylated only slightly more rapidly than the 5′-isomer. The 1:1 mixture of 3′- and 5′-dGMP was dephosphorylated more slowly than the monomers separately, and nucleoside diphosphate, dpGp, was hydrolysed even five times slower. The above experiment seems to indicate that the free hydroxyl group at position 5′ is essential for the binding of the enzyme with substrate, as the enzyme has lower affinity toward both 5′-isomers of nucleotides and nucleoside diphosphate than toward nucleoside 3′-phosphates.

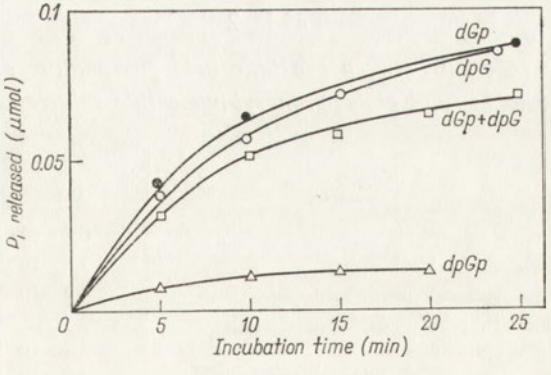


Fig. 8. Time-course of enzymic dephosphorylation of 3′- and 5′-deoxyguanosine mono- and diphosphates.

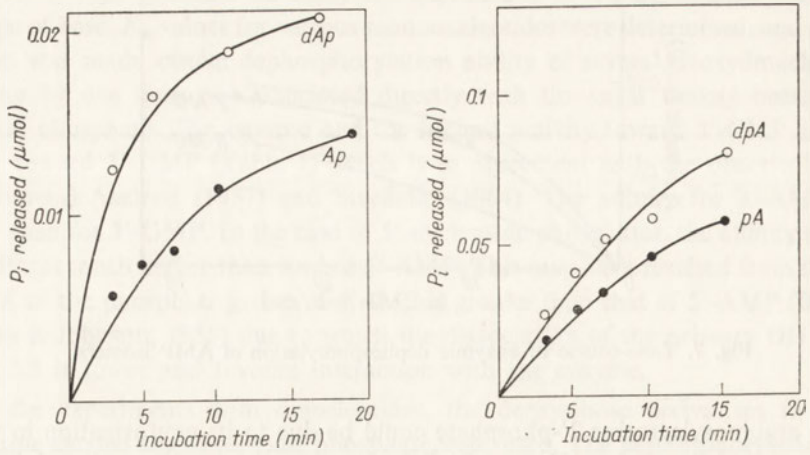


Fig. 9. Time-course of enzymic dephosphorylation of 3′- and 5′- ribo- and deoxyadenosine phosphates.

Effect of type of sugar residue. Nucleotides containing ribose, deoxyribose, arabinose and glucose were compared; 3′- and 5′-dAMP were hydrolysed more rapidly than the analogues of AMP (Fig. 9). This was probably due to the 2′-OH group in ribonucleotides forming an internal hydrogen bridge with the phosphate oxygen at carbon 3′ (Brahms, Maurizot & Michelson, 1967; Maurizot, Brahms & Eckstein, 1969), which in turn could result in lower affinity of enzyme to the substrates.

Replacement of ribose by arabinose or glucose in the nucleotide led to a further decrease in the dephosphorylation rate, which was also influenced by the position of the phosphate (Table 2, Fig. 10).

From Figs. 11 and 12 it appears that although K_m values for the arabinose and glucose nucleotides are lower than those for the corresponding ribonucleotides the V_m values are the same, which points to a definitely lower affinity of the enzyme studied toward the unnatural nucleotides.

Table 2

K_m *values for the reaction between prostatic acid phosphatase and different ribo-, arabino- and glucopyranosyl-nucleotide substrates*

Substrate	K_m values (moles/l)
Cytidine-3′-phosphate	6.8×10^{-4}
Arabinocytidine-3′-phosphate	2.5×10^{-3}
Arabinocytidine-5′-phosphate	4.2×10^{-3}
Arabinocytidine-2′-phosphate	very large value
Uridine-3′-phosphate	3.3×10^{-3}
Glucopyranosyluridine-4′-phosphate	4.0×10^{-3}
Glucopyranosyluridine-6′-phosphate	very large value
Glucopyranosyluridine-2′-phosphate	very large value

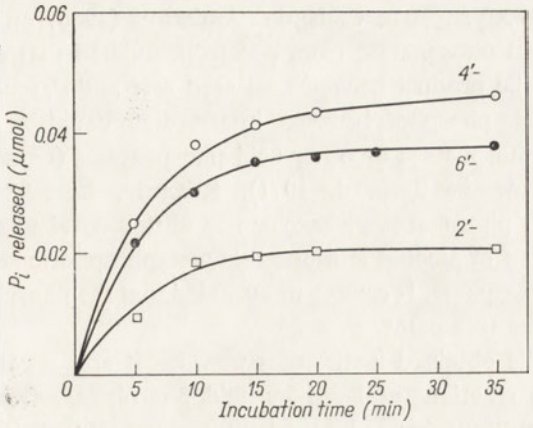


Fig. 10. Time-course of enzymic dephosphorylation of 2'-, 4'- and 6'-glucopyranosyluridine phosphates.

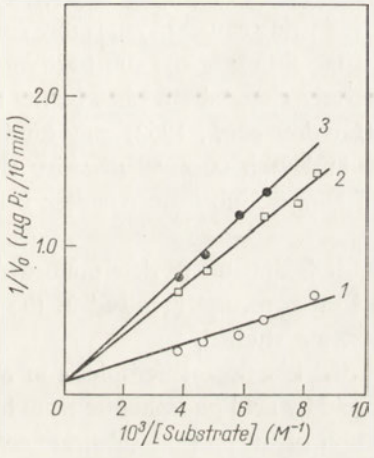
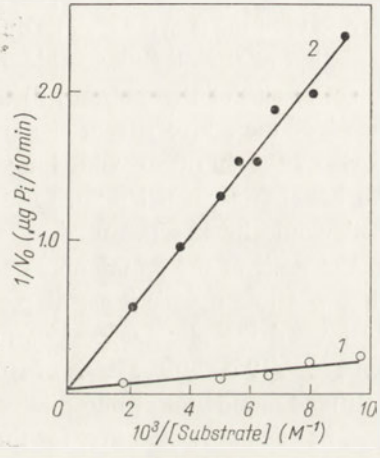


Fig. 11



Fig, 12

Fig. 11. Lineweaver-Burk plots for enzymic dephosphorylation of: (*1*), 3'-cytidine phosphate; (*2*), 3'-arabinosylcytidine phosphate; (*3*), 5'-arabinosylcytidine phosphate.

Fig. 12. Lineweaver-Burk plots for enzymic dephosphorylation of: (*1*), 3'-uridine phosphate; (*2*), 4'-glucopyranosyluridine phosphate.

DISCUSSION

In contrast to alkaline phosphatases, the mechanism of action of acid phosphatases so far has not been elucidated. From the kinetic studies of London, McHugh & Hudson (1958, 1962) who used anionic inhibitors, it appears that the active site of the acid phosphatase from human prostate consists of a coiled fragment of polypeptide chain with positive and negative charged groups. The authors postulate

"four critical points: two positive sites and two hydrogen-bonding sites" situated at a distance of 2.5 - 3.0 Å. Hsu, Cleland & Anderson (1966) on the basis of their experiments on acid phosphatase from potato postulated that during the enzymic reaction an alcoholic product becomes released first and P_i second, but so far no evidence for a stable phosphoryl-enzyme intermediate could be found either in the case of prostatic phosphatase or other acid phosphatases (Greenberg & Nachmansohn, 1965; Ostrowski & Barnard, 1971). Recently, Boer & Steyn-Parvé (1970) suggested for some phosphatases a mechanism of non-covalent single displacement leading to formation of alcohol and unstable metaphosphoric acid ion, which with water gives orthophosphate. It cannot be excluded that the phosphatase from human prostate also shares this pathway.

From the results obtained in the present work it appears that the rate of the dephosphorylation reaction catalysed by acid phosphatase from human prostate is affected to a significant degree by the length of the oligonucleotide chain, nature of sugar and base, and especially by the position of the phosphate group. The marked difference in the affinity of the enzyme for the mononucleotides and oligonucleotides results in the low efficiency of dephosphorylation even of dinucleotides (cf. Fig. 5). This property of the enzyme may be due to the following causes: 1) the more complex conformation of the oligonucleotide may limit the fitting of the substrate molecule to the active site of the enzyme; 2) the negatively charged sub-sites in the neighbourhood of the active site of the enzyme (London *et al.*, 1962) may hinder the approach of the substrate with longer chain and with strongly negative charge; 3) with increasing chain length, formation of enzyme-substrate complex becomes thermodynamically less favoured.

On the basis of the so far obtained results it is difficult to determine which of the three possible mechanisms, or perhaps all of them, are involved in the lower affinity toward the oligonucleotides of the enzyme studied.

Neumann (1968) on the basis of studies on the kinetics of hydrolysis of *S*- and *O*-substituted monoester of phosphorothionic acid by acid phosphatase from human prostate, has demonstrated that for the formation of the enzyme-substrate complex at least one of the hydroxyl groups of orthophosphate must be present in the undissociated form. It is known that at pH 5.0, i.e. within the range of optimum activity and the greatest stability of acid phosphatase from prostate (London, Wigler & Hudson, 1954), all monoesters of phosphoric acid occur in the monoanionic form (Inoue & Satoh, 1969) and thus fulfil the essential requirement for formation of the enzyme-substrate complex.

As it appears from our experiments and from observations of other authors (cf. Schmidt, 1961), the nucleotides possessing 3′-terminal phosphate group are hydrolysed at a much greater rate than the 5′- and 2′-isomers. This is probably due to the conformation of nucleoside 3′-phosphates in which the phosphate group is located more to the outside of the sugar and the base moiety (Inoue & Satoh, 1969). Moreover, it seems that the free hydroxyl group at position 5′ plays an essential role in enzymic hydrolysis of the O-P bond at carbon 3′, possibly by forming a hydrogen bond with the appropriate sub-sites of the enzyme, postulated by London *et al.*

(1958, 1962). The distance between the O-P bond at carbon 3′ and the 5′-hydroxyl group in the nucleotide is about 2.5 Å (Furberg, 1950; Wilkins & Arnott, 1965), which is the distance between the positive point and the electron pair of the hydrogen--bonding site, in the scheme of the active centre proposed by London *et al.* Also the course of hydrolysis of nucleoside diphosphates (dpGp) points to the role of the primary alcohol group in position 5′ in the process of cleavage of phosphate in position 3′; dpGp was hydrolysed at a rate one-sixth that for dGp, and about one-fifth that for an equimolar mixture of dpG and dGp (Fig. 8). Thus it is probable that both the undissociated hydroxyl group of the phosphate and 5′-hydroxyl group of the nucleotide are essential for formation of the enzyme-substrate complex, possibly by binding with the hydrogen-bonding sub-sites (Nath, 1969).

The dependence of the dephosphorylation rate on the nature of base and sugar of a nucleotide may indicate that the overall conformation of substrate molecule also plays a role; as demonstrated by Ts'o *et al.* (1969), the conformation of guanine nucleotides and nucleotides containing arabinose or glucose instead of ribose or deoxyribose, is less ordered. It may be that this is the reason why dGpGp was hydrolysed much more slowly than dTpGp or dApGp (cf. Fig. 6), and the nucleotides containing arabinose or glucose slower than the corresponding ribonucleotides (cf. Table 2). It seems, however, that the base and sugar moieties of the nucleotide have a smaller effect on the affinity of the enzyme for the substrate than the position of the phosphate residue and the conformation of the oligonucleotide.

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SPECYFICZNOŚĆ KWAŚNEJ FOSFATAZY STERCZU LUDZKIEGO

Streszczenie

1. Szybkość defosforylacji oligonukleotydów przez kwaśną fosfatazę sterczu maleje wraz ze wzrostem długości łańcucha nukleotydu.

2. Mono- i oligonukleotydy zawierające C lub A hydrolizowane są szybciej niż zawierające U lub G.

3. Fosfonukleozydy 3′ są defosforylowane szybciej niż 5′ i 2′-izomery, niezależnie od rodzaju zasady i cukrowca w nukleotydzie. Przebieg hydrolizy 3′- i 5′-fosfonukleozydów oraz 3′,5′-dwufosfonukleotydów wskazuje, że wolna grupa OH w pozycji 5′ cukrowca spełnia istotną rolę w tworzeniu kompleksu enzym-substrat.

4. Dezoksyrybonukleotydy defosforylowane są łatwiej niż rybonukleotydy; nukleotydy pochodne D-arabinozy i D-β-glukopyranozy hydrolizowane są wolniej niż odpowiadające im rybonukleotydy.

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THE ROLE OF PROTEIN IN THE STRUCTURE OF GLYCOGEN

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1. It was found that glycogen can be determined by the turbidimetric tannin micromethod of Mejbaum-Katzenellenbogen (1955) elaborated for protein determination. 2. Acid hydrolysis of liver glycogen leads to an increase in its reactivity with tannin. This increase is the greatest in the case of glycogen obtained by extraction with cold water, and the lowest with that extracted with trichloroacetic acid. 3. Digestion of glycogen with amylase, as well as with pronase, causes a decrease in the amount of the material which can be determined with tannin. 4. Pronase has a greater effect on glycogen preparations partially hydrolysed with acid than on "native" preparations. 5. On the basis of the obtained results and data from the literature, a model of glycogen structure is proposed in which polysaccharide subunits are bound together through the glycoprotein moiety.

Lazarow (1942) isolated from guinea pig liver, by extraction with cold water, a glycogen preparation (referred to as "particulate" glycogen) the molecular weight of which was much higher than that of the preparation obtained by the method of Pflueger (extraction with boiling KOH) or by extraction with cold trichloroacetic acid. Lazarow demonstrated the presence in the obtained preparation of about 1% of protein, to which he ascribed a role in binding of glycogen molecule.

Drochmans (1962, 1963) demonstrated that particulate glycogen is composed of α, β and γ particles. Particles α represent the native glycogen of average dimension 105 nm. They are composed of morphologically distinct particles β of average dimension 37 nm. The action of weak acid leads to dissociation of particles α into β which at pH below 3 dissociate into subunits γ of dimensions 15 - 22 nm and a molecular weight close to the mean molecular weight (2×10^6) of the glycogen obtained by the method of Pflueger (Drochmans & Dantan, 1968). Mejbaum-Katzenellenbogen, Łomako & Maskos (1971) have demonstrated that, from glycogen, after adequate digestion of the polysaccharide moiety, a glycoprotein fraction can be isolated by the tannin-caffeine method.

The aim of the present work was to study the formation of glycogen-tannin complexes under various conditions, which may give some information concerning the structure of native glycogen.

MATERIALS AND METHODS

Glycogen was obtained from rabbit liver using four different extraction procedures: 1, with hot concentrated KOH (Somogyi, 1957); 2, cold solution of trichloroacetic acid[1] (Ostern & Hübl, 1939); 3, $HgCl_2$ solution (Mordoh, Krisman & Leloir, 1966) and 4, cold water (Bueding & Orrell, 1964).

Determination of glycogen was carried out by the turbidimetric method of Mejbaum-Katzenellenbogen (1955) designed for protein determination (in this method, tannin is added as 10% solution in 1 N-HCl containing 2% of phenol, and the turbidity formed is measured at 610 nm). The standard curve was constructed for glycogen concentrations ranging from 50 to 500 µg/ml.

Acid hydrolysis of glycogen preparations was carried out in 4 N-HCl at 30°C (in a Hoeppler ultrathermostat). Glycogen, 40 mg, was dissolved in 2 ml of water and, after temperature equilibration, 2 ml of 8 N-HCl heated to 30°C was added, then samples of 0.02 ml were withdrawn for analysis at intervals of 15 min. To obtain partially hydrolysed glycogen, after 105 min of hydrolysis cold ethanol was added to a final concentration of 75%. The sediment was collected by centrifugation, washed with ethanol, ether, and dried in vacuum over P_2O_5.

Gel filtration of glycogen was carried out on Sepharose 2B column (2.4×68 cm) equilibrated with 0.05 M-tris-HCl buffer, pH 7.65, in 0.01% sodium azide. The applied glycogen was eluted with the same buffer, fractions of about 2 ml being collected, and sugar was determined by the phenol method (Hodge & Hofreiter, 1962).

Reagents. Crystalline salivary amylase was prepared by the method of Fischer & Stein (1961); thymus lysine-rich histones were prepared by K. Maskos according to Kinkade & Cole (1966). Tannin was a U.S.S.R. product (series 75066) and was purified by extraction to ethyl acetate according to Armitage *et al.* (1961); Sepharose 2B was from Pharmacia Fine Chemicals (Uppsala, Sweden), bovine serum albumin from British Drug Houses (Poole, Dorset, England), pronase from Koch-Light Lab. (Colnbrook, Bucks., England). Other chemicals were reagent grade products of Polish origin.

RESULTS

Determination of glycogen. It was found that the turbidimetric tannin micromethod of Mejbaum-Katzenellenbogen (1955) may be applied also for quantitative determination of glycogen; however, its sensitivity in the case of glycogen is about one-fifth that for protein determination. The turbidity (extinction at 610 nm) increased with glycogen concentration and was reproducible over the range 50 - 500 µg/ml (Fig. 1). With the $HgCl_2$-extracted glycogen, the turbidity was linear with glycogen concentration, whereas with other glycogen preparations the values at higher concentrations deviated from linearity. At higher concentrations, the

[1] Abbreviation: TCA, trichloroacetic acid.

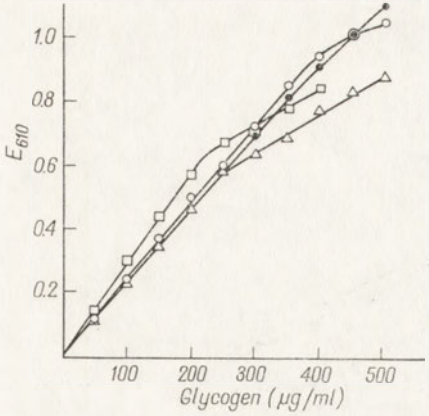

Fig. 1. The effect of glycogen concentration on the extinction of glycogen-tannin complexes. Preparations of glycogen extracted with: △, cold water; □, hot concentrated KOH; ○, trichloroacetic acid solution; ●, $HgCl_2$ solution.

water-extracted glycogen showed the lowest turbidity, and that extracted with $HgCl_2$, the highest. Thus it appeared that the tannin method may be applied for determination of glycogen provided that for constructing adequate standard curves, the glycogen obtained by the same method of extraction should be used.

Effect of acid and enzymic hydrolysis of glycogen on its reaction with tannin. Hydrolysis of glycogen preparations with 4 N-HCl at 30°C led initially to an increase in their reactivity with tannin, which was apparent as an increase in the turbidity (Fig. 2). The highest, fourfold increase in extinction reaching a maximum after 75 min of hydrolysis, was observed with the water-extracted glycogen. Within the next 15 min, the extinction decreased rapidly, then a slower steady decrease of reactivity of glycogen with tannin was observed. For $HgCl_2$-extracted glycogen the maximum increase in extinction, almost threefold, appeared after 60 min of hydrolysis. On the other hand, the effect of acid hydrolysis of the TCA-extracted glycogen on the glycogen-tannin complex formation was smaller, and the time-dependence curve had a different shape.

Unlike acid hydrolysis, enzymic hydrolysis of glycogen with salivary amylase (0.13 unit/40 mg of glycogen) resulted in a gradual decrease in the amount of material reacting with tannin.

The reaction of glycogen with tannin during acid hydrolysis was estimated in the whole incubation mixture. To eliminate the effect of the medium, the water-extracted glycogen after partial hydrolysis (105 min) was isolated and its reaction with tannin estimated (Fig. 3). The extinction obtained with 100 μg of the isolated partially hydrolysed glycogen was about 2.5 times greater than that with the native water-extracted glycogen (Fig. 1); a similar increase in extinction was observed in the whole incubation mixture between the initial value and that after 105 min of incubation (Fig. 2).

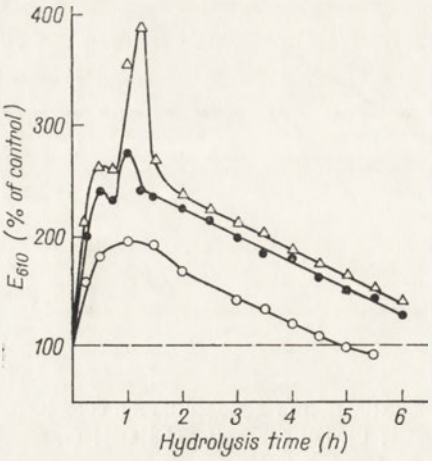

Fig. 2

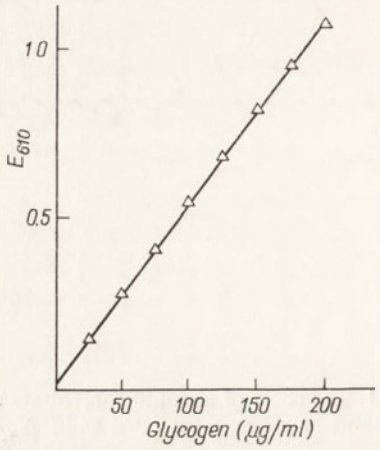

Fig. 3

Fig. 2. The effect of hydrolysis of glycogen with 4 N-HCl at 30°C, on the extinction of glycogen-tannin complexes. The results are expressed as percentages of the zero time value taken as 100. Preparations of glycogen extracted with: △, cold water; ●, $HgCl_2$ solution; ○, trichloroacetic acid solution.

Fig. 3. The effect of concentration of the partially hydrolysed glycogen on the extinction of glycogen-tannin complexes. The cold water-extracted glycogen after hydrolysis for 105 min was used.

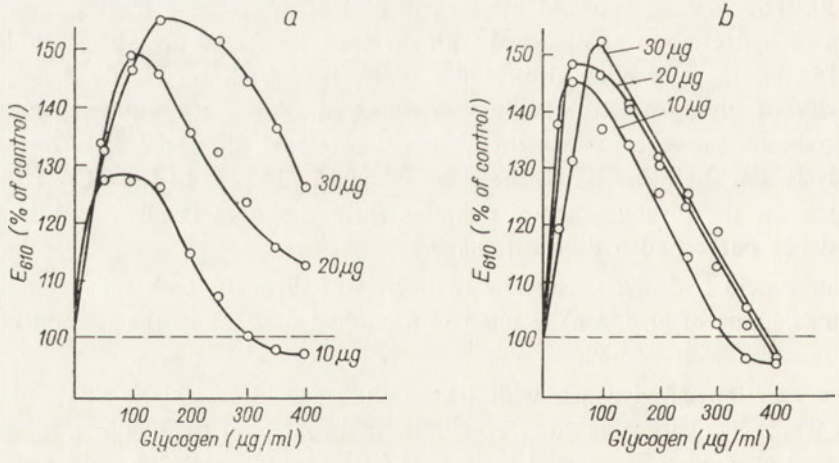

Fig. 4. The effect of *a*, lysine-rich thymus histones, and *b*, bovine serum albumin, on the extinction of glycogen-tannin complexes. To 0.9 ml solutions containing from 50 to 400 μg of TCA-extracted glycogen, the indicated amounts of protein in 0.1 ml of water were added. The extinction was measured under conditions of the tannin method. As control (100%) was taken the sum of extinctions determined separately for the applied concentrations of protein and glycogen.

Effect of protein on the determination of glycogen with tannin. To increasing concentrations of the TCA-extracted glycogen, 10, 20 and 30 μg of lysine-rich histone from thymus or crystalline bovine albumin were added, and formation of turbidity with tannin determined. It was found that the turbidity of the glycogen-protein mixture was distinctly higher than the sum of turbidities obtained with glycogen and protein alone. The increase was the greatest at the histone to glycogen ratio of 1:5 (Fig. 4a). The curves for different histone concentrations had a similar course. The addition of bovine serum albumin had a similar effect on the turbidity (Fig. 4b) but the maximum increase was somewhat higher than in the presence of histones. The addition of 10 μg of albumin caused a maximum increase by 45%, and 20 μg by 55% in relation to the sum of extinctions determined separately for the respective concentrations of glycogen and albumin.

Sepharose gel filtration. The TCA-extracted glycogen applied to the Sepharose 2B column emerged after 80 ml of the eluent had passed; at 220 ml 90% and at 280 ml total recovery of the applied glycogen was obtained (Fig. 5). The 89 - 116 ml and 190 - 200 ml fractions were pooled and the respective *A* and *B* fractions were dialysed against cold water for 48 h. The glycogen was precipitated with two volumes of ethanol in the presence of 0.05% LiBr, washed with ethanol, acetone and dried in vacuum over P_2O_5.

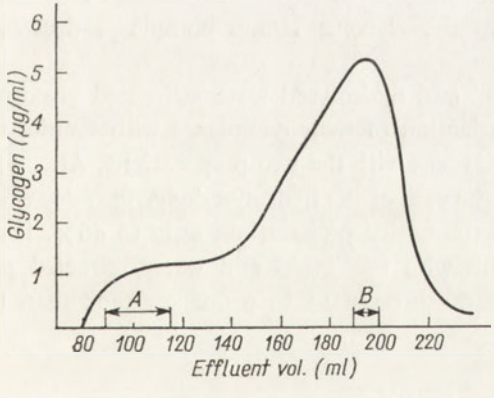

Fig. 5

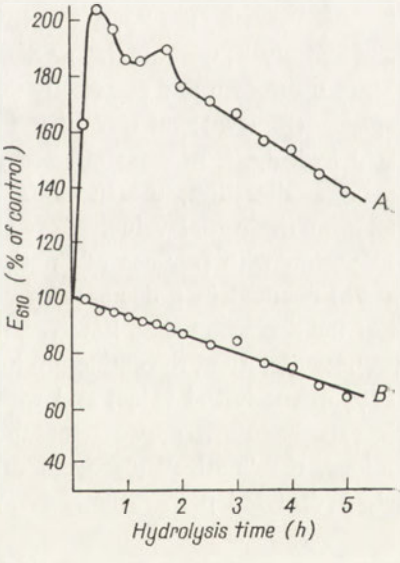

Fig. 6

Fig. 5. Gel filtration of TCA-extracted glycogen on Sepharose 2B column. Five ml of 8% glycogen solution was applied. For details see Methods.

Fig. 6. The effect of acid hydrolysis of glycogen fractions *A* and *B* (see Fig. 5) on the extinction of glycogen-tannin complexes. The results are expressed as percentages of the zero time value taken as 100.

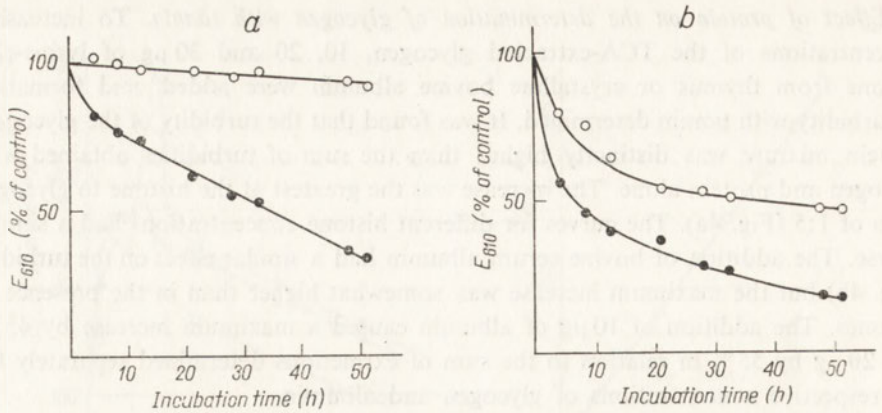

Fig. 7. The effect of pronase digestion of *a*, TCA-extracted glycogen, and *b*, water-extracted glycogen, on their reaction with tannin; ○, "native" glycogen; ●, glycogen isolated after 105 min hydrolysis with 4 N-HCl. The extinction of the complex of tannin with the glycogen preparations at zero time was taken as 100.

During acid hydrolysis of fraction *A*, the turbidity of the glycogen-tannin complexes at first increased rapidly (Fig. 6) reaching a maximum after 30 min, then gradually declined. Fraction *B*, in contrast to fraction *A* and the whole, non-separated preparation, showed a steady decrease in turbidity with hydrolysis time.

The effect of digestion of glycogen with pronase on the glycogen-tannin reaction. In the experiments, the "native" glycogen and the glycogen isolated after 105 min of acid hydrolysis, were used. The digestion with pronase of "native" TCA-extracted glycogen up to 51 h had practically no effect on the turbidity of the glycogen-tannin complexes (Fig. 7a); on the other hand, when the glycogen had been submitted to acid hydrolysis, pronase affected distinctly its reaction with tannin. After 51 h of pronase digestion, the turbidity of the glycogen-tannin complexes decreased to 32% of the initial value.

Digestion with pronase of "native" and hydrolysed water-extracted glycogens (Fig. 7b) resulted in a decrease of extinction of their complexes with tannin, the rate of the decrease being, however, different with the two preparations. After 51 h of pronase digestion, the extinction in the case of the hydrolysed glycogen decreased to 17% of the initial value, and with the native preparation, only to 46%. From these experiments it appears that both with the TCA- and water-extracted preparations, the "native" glycogens are more resistant to pronase attack than the partly hydrolysed preparations.

DISCUSSION

Orrell & Buending (1964) have demonstrated that dodecyl sulphate, tween 80, triton X-100, urea, guanidine, changes in pH values from 5 to 12, freezing and thawing, do not affect the molecular weight of water-extracted glycogen measured by sedimentation coefficient distribution. Nor was the molecular weight of the glycogen changed on treatment with chloroform and octanol which resulted in

a decrease in the content of protein from 16% to about 0.01%. On this basis the authors inferred that protein does not participate in aggregation of glycogen subunits.

Mordoh, Krisman & Leloir (1966) and Parodi, Krisman, Leloir & Mordoh (1967) studied the effect of acid hydrolysis on highly polymerized "native" $HgCl_2$-extracted glycogen and a synthetic preparation obtained from glucose-1-phosphate using purified muscle phosphorylase and liver branching enzyme; they have found that the "native" glycogen, unlike the synthetic one, at the beginning of hydrolysis dissociates forming particles of about 8 000 000 molecular weight. They concluded that in the native glycogen there are some bonds more labile to the action of the acid, spaced every 50 000 glucose residues. Mejbaum-Katzenellenbogen, Łomako & Maskos (1968, 1971) isolated a glycoprotein fraction, both from muscle and liver glycogen, after digestion of the sugar moiety with salivary amylase. It may be supposed that these glycoproteins are bound to the glucose residues through the more labile bonds. If it is assumed that the highly polymerized glycogen is composed of polysaccharide fragments bound together through protein fragments, then the turbidity of the glycogen-tannin complexes would be dependent on the number of protein fragments accessible to tannin as well as the size of the polysaccharide fragments bound to these proteins. When glycogen is treated with acid, at first the more labile bonds are broken and consequently there is an increase in the number of sites accessible to tannin. These changes in glycogen structure are manifested by an increase in the turbidity with tannin, and when glycogen is broken into subunits, the turbidity decreases. This supposition is supported by the effect of lysine-rich histones and bovine serum albumin on the reaction of glycogen with tannin, as in their presence a more than additive increase of turbidity was observed. This

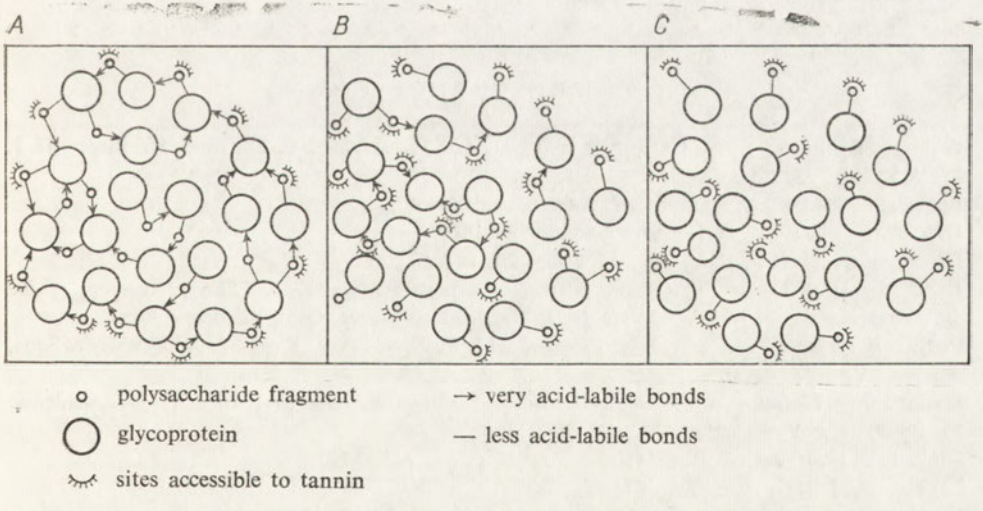

Fig. 8. Scheme of the proposed structure of the glycogen molecule. The sites accessible to tannin are indicated. *A*, Native glycogen; *B*, acid-treated glycogen at the maximum reactivity with tannin; *C*, acid-treated glycogen decomposed into subunits.

phenomenon may be due to the effect of binding of polysaccharide fragments to the added protein.

The effect of pronase on the reactivity of glycogen with tannin also supports the participation of protein in binding of glycogen subunits.

The differences in the maximum increase in turbidity of glycogen-tannin complexes during acid hydrolysis of the glycogen preparations obtained by different methods, may be explained in the following way. The number of subunits which determine the molecular weight of glycogen would determine the maximum increase in the turbidity. The greater the number of aggregated subunits in a glycogen molecule, the greater would be the maximum increase in turbidity during hydrolysis because more sites accessible to tannin would become liberated. As found in the present experiments, the greatest relative increase in turbidity was observed with the cold water-extracted glycogen, which according to Bueding & Orrell (1964) has a molecular weight of 100 to 500 million; the increase was the lowest with the TCA-extracted glycogen, which according to Stetten, Katzen & Stetten (1956) has a molecular weight of 11 to 80 million.

The differences in the turbidity formed with tannin in the course of acid hydrolysis of the glycogen fraction separated by Sepharose gel filtration, suggest that the first emerging fraction *A* is composed of aggregated polysaccharide units, and the second fraction *B*, of dissociated subunits.

Taking into account the occurrence of a glycoprotein fraction in glycogen, and the above presented data, a model of glycogen structure is proposed in which the polysaccharide units are bound together by protein fragments (Fig. 8).

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ROLA BIAŁKA W BUDOWIE GLIKOGENU

Streszczenie

1. Stwierdzono, że glikogen daje się oznaczać turbidymetryczną mikrometodą taninową wg Mejbaum-Katzenellenbogen (1955) opracowaną do oznaczania białka.

2. Kwaśna hydroliza glikogenu wątroby powoduje zwiększenie jego reaktywności z taniną. Największe zmętnienie dają preparaty glikogenu ekstrahowane zimną wodą, a najmniejsze — ekstrahowane kwasem trójchlorooctowym.

3. Trawienie glikogenu amylazą, jak również pronazą, zmniejsza ilość materiału dającego oznaczać się taniną.

4. Pronaza jest aktywniejsza wobec preparatów glikogenu częściowo zhydrolizowanych kwasem niż wobec preparatów „natywnych".

5. Na podstawie otrzymanych wyników i danych z piśmiennictwa zaproponowano model budowy glikogenu, w którym polisacharydowe podjednostki łączą się ze sobą za pośrednictwem fragmentów glikoproteidowych.

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A C T A B I O C H I M I C A P O L O N I C A
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WANDA MEJBAUM-KATZENELLENBOGEN, J. ŁOMAKO and CH. MASKOS

GLYCOGEN AS SOURCE OF GLYCOGEN PHOSPHORYLASE INHIBITOR

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1. The presence in the glycogen molecule of a glycoprotein fraction was demonstrated. 2. The isolated glycoprotein acts as inhibitor of glycogen phosphorylase; its possible role in glycogen metabolism is discussed.

It is well known since the investigation of Cori & Cori (1945) that glycogen phosphorylase occurs in two forms, the inactive form *b* and the active form *a*. Later it has been demonstrated and well documented (Posner, Stern & Krebs, 1965; Kilpatrick & Major, 1970) that the conversion of phosphorylase *b* into *a* is subjected to hormonal regulation mediated by cyclic 5′,3′-AMP. However, there is no information in the literature concerning regulation of the activity of phosphorylase *a*.

The occurrence of a glycoprotein fraction in the sulphosalicylic acid-soluble proteins of rabbit skeletal muscle has been demonstrated by Mejbaum-Katzenellenbogen, Łomako & Maskos (1968). On the assumption that it originated from glycogen, in the present work an attempt was made to isolate this fraction and to study its possible role.

MATERIALS AND METHODS

Glycogen was extracted from rabbit skeletal muscle and liver with 10% trichloroacetic acid according to Ostern & Hübl (1939). Glycogen phosphorylase was prepared by the method of Cori, Illingworth & Keller (1955), and crystalline salivary amylase according to Fischer & Stein (1961).

Phosphorylase activity was determined according to Cori *et al.* (1955) by measuring the release of phosphate from glucose-1-phosphate in the presence of a small amount of glycogen serving as "primer".

Paper electrophoresis was performed in 0.1 M-citrate-phosphate buffer, pH 4.4, for 8 h at 220 V. The material studied, about 400 µg, was applied on Whatman no. 1 paper (38×4 cm) at a distance of 2 cm from the middle of the strip toward the anode. The electrophoretogram was stained with Amido Black and basic fuchsin.

Protein was determined by the turbidimetric tannin micromethod of Mejbaum-Katzenellenbogen (1955), total sugar by the phenol method of Hodge & Hofreiter (1962), reducing sugar according to Park & Johnson (1949), and hexosamines according to Elson & Morgan (1933).

Reagents. Tannin was a U.S.S.R. product (series no. 75066) and was purified by extraction to ethyl acetate; basic fuchsin was from British Drug Houses (Poole, Dorset, England), sodium β-glycerophosphate and cysteine hydrochloride from Reanal (Budapest, Hungary), 5′-AMP from Sigma Chem. Co. (St. Louis, Mo., U.S.A); glucose-1-phosphate was prepared according to Summer & Somers (1944). Other chemicals were reagent grade products of Polish origin.

RESULTS

Isolation of the glycoprotein fraction from glycogen. The muscle glycogen preparation was subjected to amylolysis. For this purpose, to 50 ml of 10% glycogen solution in 6.7 mM-NaCl - 0.02 M-Na,K-phosphate buffer, pH 6.9, crystalline salivary amylase was added in such an amount (0.2 - 1 ml of enzyme suspension) as to obtain maximum digestion of glycogen within about 48 h. The incubation was carried out at room temperature under toluene. The course of digestion was followed by measuring reducing sugars and total sugar. On completion of amylolysis (as ascertained by maximum reduction) the enzyme was precipitated with cold sulphosalicylic acid at 0.15 M final concentration and after 2 h removed by centrifugation for 20 min at 3000 rev./min at 0°C. Then tannin, dissolved in a small volume of water, was added (1 g/100 ml), left overnight, and the formed sediment was separated by centrifugation at 16 000 rev./min for 30 min and washed three times with 0.9% NaCl solution. To the precipitate a small volume of water was added and from the protein-tannin complex protein was released with caffeine according to Mejbaum-Katzenellenbogen (1959).

Depending on the preparation, from 5 g of glycogen 1 - 5 mg of glycoprotein was obtained. On paper electrophoresis this protein gave a single fraction stained both with Amido Black and basic fuchsin, which indicates its glycoprotein character (Fig. 1). A similar glycoprotein fraction was obtained when liver glycogen was used as starting material. If a larger amount, about 80 g, of muscle glycogen was used for preparation, on electrophoresis four bands staining with Amido Black were obtained, and only one band, that with the slowest cathodic mobility, staining with fuchsin; the electrophoretic pattern was similar to that of 0.15 M-sulphosalicylic acid-soluble protein isolated from rabbit skeletal muscle (Mejbaum-Katzenellenbogen *et al.*, 1968).

The effect of the isolated glycoprotein on phosphorylase activity. The occurrence of a glycoprotein fraction both in muscle and liver glycogen suggested that it may play a role in the metabolism of glycogen. On this assumption, the effect of the glycoprotein isolated from glycogen on the activity of purified active glycogen phosphorylase was studied. It was found that the glycoprotein inhibited the enzyme both in the presence and absence of adenylic acid. An excess of adenylic acid

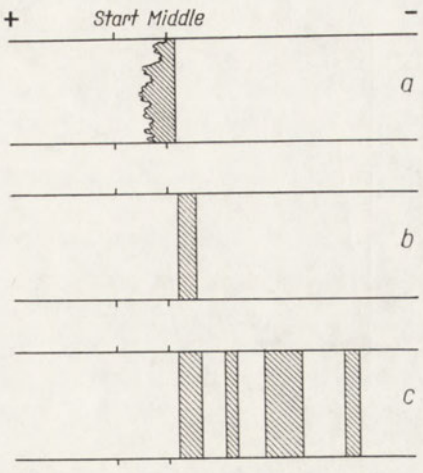

Fig. 1. Paper electrophoresis of *a*, glycogen and *b*, protein fraction isolated from 5 g of glycogen by digestion with salivary amylase; *c*, protein fraction from 80 g of glycogen. Staining with Amido Black.

and glucose-1-phosphate had no effect on the inhibition. The inhibitory activity was dependent on the degree of glycogen digestion. The fraction isolated before the maximum digestion by amylase occurred, showed smaller inhibitory activity. With some of the preparations, half-inhibition was achieved by 3 μg of glycoprotein per 0.5 enzyme unit, whereas with other it was necessary to take as much as 50 - 80 μg (Table 1 and Fig. 2).

Devor, O'Brien Barichievich & Sidelgui (1966) have demonstrated that glycogen extracted with trichloroacetic acid contains about 0.26% of hexosamines. In the present work it was found that the content of hexosamines in the isolated glycoprotein was severalfold higher; preparation no. 2 (see Table 1) contained 4.8% of the whole amount of hexoses determined by the phenol method. With the in-

Table 1

The effect of the degree of glycogen amylolysis on the phosphorylase inhibitor activity of the isolated glycoprotein

The degree of digestion of glycogen by salivary amylase was judged by the amount of material determined by the turbidimetric tannin method after removal of amylase with sulphosalicylic acid.

Preparation no.	Glycoprotein from 100 g of glycogen (g)	50% inhibition (μg/0.5 enzyme unit)
1	2	no inhibition
2	0.6	32
3	0.3	3

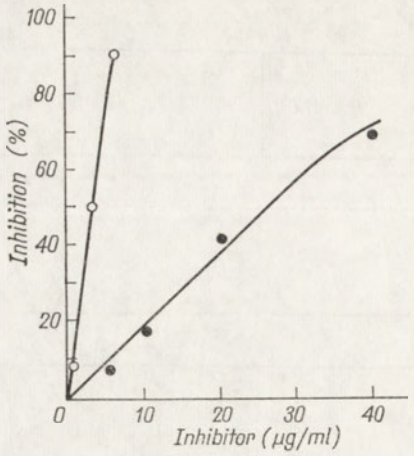

Fig. 2. The effect of the glycoprotein isolated from glycogen on the activity of crystalline muscle glycogen phosphorylase. The incubation mixture contained: 0.2 ml of 4% glycogen solution, 0.1 ml of 0.1% gelatin (as protection against the possible presence of traces of tannin in the glycoprotein preparation; it has been checked that gelatin does not affect phosphorylase activity); 0.1 ml of a solution of the glycoprotein, 0.4 ml of enzyme solution (0.5 unit); 0.2 ml of 64 mM-glucose-1--phosphate in 4 mM-adenosine-5′-phosphate; the incubation was carried out for 20 min at 30°C. Glycoprotein preparation: ○, no. 3; ●, no. 2 (see Table 1).

creasing degree of digestion of glycogen by amylase, there occurs a "concentration" of hexosamines in the glycoprotein fraction.

As the glycoprotein fraction is soluble in 0.15 M-sulphosalicylic acid, attempts were made to obtain the phosphorylase inhibitor directly from autolysed muscles, from muscle phosphate extract autolysed for 48 h, or from aqueous extracts from muscles submitted to amylase digestion; all these attempts proved unsuccessful.

DISCUSSION

Lazarow (1942) and Drochmans (1962) have demonstrated that glycogen occurs in the organism in the form of particles composed of smaller subunits. On the basis of the experiments of Łomako (1971) it appears that these subunits are held together by glycoprotein fragments. Madsen & Cori (1958) isolated glycogen from sweet corn and found that one molecule of 20×10^6 molecular weight can bind 33 molecules of phosphorylase *a*. Taking into account these data and the results of the present experiments, a simple mechanism of control of phosphorylase *a* activity could be proposed. The phosphorolysis of glycogen would proceed up to the liberation of the glycoprotein fragments which act as phosphorylase inhibitors. The action of n molecules of the phosphorylase on the glycogen composed of subunits bound by x glycoprotein fragments, in the final step of phosphorolysis would lead to formation of enzyme-glycoprotein complexes and free enzyme molecules in a number lower by $n-x$ than the initial number participating in the reaction.

For the synthesis of glycogen from UDP-glucose catalysed by glycogen synthetase, the presence of a polysaccharide "primer" is required. Thus phosphorylase inactivation may serve as a mechanism for preservation of the "primer" for the resynthesis of glycogen.

The stable binding of the phosphorylase with the glycoprotein is indicated by the fact that when the time of extraction with water of minced muscle exceeds two hours, the yield of the crystalline phosphorylase is very small, probably due to formation of an inactive complex of the phosphorylase with the glycoprotein inhibitor released in the process of phosphorolysis. Likewise, the active inhibitor cannot be demonstrated either in autolysed muscles or in muscle extracts. The incomplete amylolysis of glycogen with the salivary enzyme, even if carried out to a considerable degree, gives a glycoprotein preparation which exhibits no inhibitory activity toward glycogen phosphorylase. This indicates that the binding sites of the glycoprotein become liberated only on exhaustive amylolysis of glycogen. At present, it is possible to demonstrate and isolate the inhibitor only by digestion of the isolated glycogen with salivary amylase; an alternative method could be the isolation of the phosphorylase-inhibitor complex and its subsequent dissociation.

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GLIKOGEN JAKO ŹRÓDŁO AKTYWNOŚCI ANTYFOSFORYLAZOWEJ

Streszczenie

1. Wykazano w glikogenie obecność frakcji glikoproteidowej.

2. Stwierdzono, że izolowany glikoproteid wykazuje aktywność inhibitora fosforylazy glikogenowej.

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PURIFICATION OF HYDROXYPROLINE-CONTAINING PROTEIN FROM HUMAN SERUM

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1. A method is described for isolation and purification from human blood serum of a protein with a high content of hydroxyproline. 2. The purified preparation was immunoelectrophoretically homogeneous and contained about 1.2% of hydroxyproline.

The presence in human blood serum of a hydroxyproline-containing protein has been first demonstrated by Keiser, LeRoy, Udenfriend & Sjoerdsma (1963). This protein, referred to as hypro-protein, appeared to be heterogeneous, and on Sephadex G-200 gel filtration of serum three hydroxyproline-containing fractions were obtained (LeRoy, Kaplan, Udenfriend & Sjoerdsma, 1964).

By immunoelectrophoresis with anti-serum of rabbits immunized with soluble human skin collagen, at least three components precipitating with anti-collagen antibodies were found to be present (Sea, Neff & Block, 1968). These results, and the fact that hydroxyproline is the characteristic amino acid of collagen, suggested a relationship between collagen metabolism and the hydroxyproline-containing protein. To confirm this supposition, it is necessary to isolate these proteins from serum and to determine their properties. In the present work an attempt has been made to isolate from human serum and purify the main hypro-protein fraction.

MATERIALS AND METHODS

Serum obtained from blood-donors was kept at 4°C and any sediment formed was removed by centrifugation. Purification of the main hypro-protein fraction was carried out as follows:

1. Dialysis: 50 ml of the serum was dialysed for 36 h against 20 vol. of 1 mM-acetate buffer, pH 4.7, at 4°C; the formed sediment of euglobulins was collected by centrifugation for 45 min at 4000 *g*, washed twice with the same buffer and dissolved in 20 ml of 0.45 M-NaCl.

2. Gel filtration: The solution, containing about 150 mg of protein, was applied to the Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (4×75 cm) equili-

brated with 0.15 M-NaCl. The protein was eluted with 0.45 M-NaCl, extinction at 280 nm was measured in a recording spectrophotometer (Japan-JEOL Optical Detection System) and fractions of 5 ml were collected. The hydroxyproline-containing fractions were pooled.

3. Concentration to 1/10 volume by dialysis against anhydrous dextran (Polfa, Kutno, Poland), mol. wt. 40 000.

4. Second gel filtration: The concentrated fraction was submitted to Sephadex gel filtration under the above described conditions.

5. Concentration dialysis against dextran.

6. Electrophoresis: This was performed as described by Hollmén & Kulonen (1966). The lower part of the column was filled with 0.5% agar in 0.25 M-phosphate buffer of pH 7.4 up to the height of 6 cm. The hypro-protein fraction (after step 5) was mixed with an equal volume of 1% agar solution at a temp. of about 30°C and applied on the solidified gel in the column. After cooling, another 2 cm layer of 0.5% agar was applied. The electrode vessels were filled with 0.25 M-phosphate buffer, pH 7.4. The electrophoresis was carried out for 10 h at 120 V with constant cooling of the column, and the flow of the buffer through the elution chamber cell was maintained at a rate of 0.25 ml/min. On completion of the electrophoresis, the gel was removed from the column and cut into sections about 0.8 cm thick, which were eluted with 5 ml portions of 0.45 M-NaCl.

Analytical methods. The content of hydroxyproline in the protein was determined by the method of Prockop & Udenfriend (1960), with some modifications. The protein solution, 0.5 ml, was treated with 0.5 ml of concentrated HCl and hydrolysed in sealed tubes for 16 h at 110°C. The hydrolysate was filtered through a 1 cm layer of mixture of Dowex 2 X8 (Dow Chem. Co., U.S.A.) and active charcoal, washed with 1 ml of 3 N-HCl, and the filtrate was neutralized with 10 N- and 1 N-KOH in the presence of phenolphthalein. The volume of the samples was adjusted to 4 ml with water, and hydroxyproline determined by the method of Kivirikko, Laitinen & Prockop (1967). The modified procedure was checked by comparing the absorption spectra of the investigated protein hydrolysate, with those of standard hydroxyproline (Fig. 1).

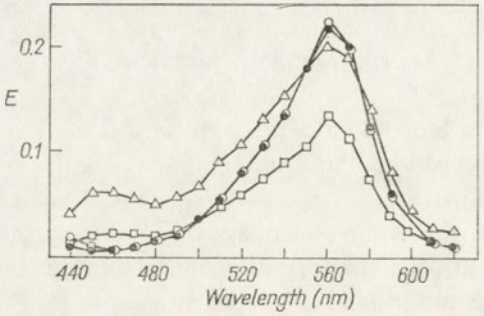

Fig. 1. Absorption spectra of: ○, standard hydroxyproline; ●, mixture of standard hydroxyproline and proline; △, total protein-bound serum hydroxyproline; □, purified hypro-protein fraction. For details see Methods.

Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Immunoelectrophoresis was carried out by the micromethod of Scheidegger (1955) using horse polyvalent anti-human serum.

Paper electrophoresis was carried out in 0.05 M-barbiturate buffer, pH 8.6, for 16 h at 160 V. The strips were stained with Amido Black 10B (Gurr, London, England).

RESULTS

The euglobulins which under the described conditions sedimented on dialysis of blood serum, were soluble in 0.45 M-NaCl; the preparation obtained from 100 ml of serum contained 140 - 170 mg of proteins and 365 - 440 µg of hydroxyproline. On paper electrophoresis, four fractions were found to be present, and on immunoelectrophoresis the components with properties of γ, β_1 and α_2-macroglobulins prevailed. On Sephadex G-200 gel filtration (Fig. 2) the hydroxyproline-containing macromolecular fraction emerged at the void volume of the column. The proteins

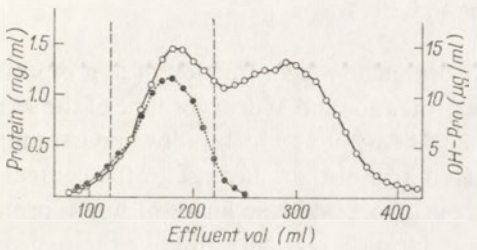

Fig. 2

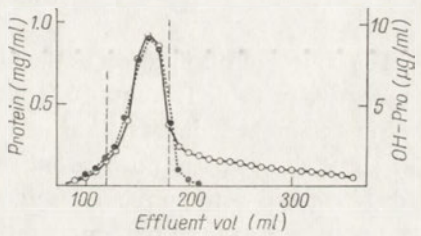

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Fig. 2. Sephadex G-200 gel filtration of the protein sedimented on dialysis. ●, Hydroxyproline; ○, protein. The effluents collected for further purification are indicated in the diagram.

Fig. 3. Second filtration on Sephadex G-200 of the concentrated hypro-protein fraction. Symbols as in Fig. 2.

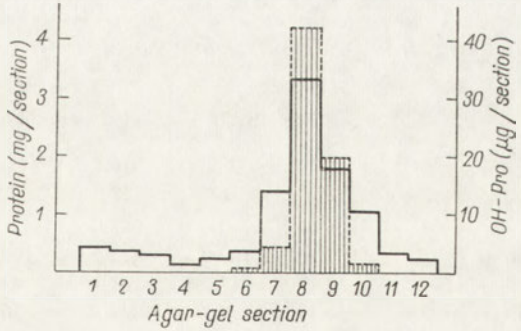

Fig. 4. Agar gel column electrophoresis pattern of the concentrated hypro-protein fraction after second gel filtration. The gel was cut into 0.8 cm sections and protein was eluted with 0.45 M-NaCl. Shaded areas, hydroxyproline; outlined areas, protein.

emerging at higher elution volumes contained no hydroxyproline and they constituted about 60% of the applied protein. In the pooled peak of hypro-protein, the ratio of hydroxyproline to protein amounted on the average to 6.57 μg/mg protein. Concentration of this fraction by dialysis against dextran resulted in some losses of protein and hydroxyproline.

The second gel filtration (Fig. 3) permitted to remove small amounts of contaminating proteins. The elution volume of the hydroxyproline-containing fraction was unchanged and the content of hydroxyproline increased on the average to 9.55 μg/mg protein. This purified fraction exhibited on immunoelectrophoresis one main precipitin band near the place of application (see Fig. 5c).

On agar-gel electrophoresis, by continuous elution only small amounts of protein with anodic mobility were removed. Almost the whole hydroxyproline-containing protein was found at the site of application (sections 8, 9) and the neighbouring fractions (sections 7, 10) (Fig. 4). In the protein eluted with 0.45 M-NaCl from sections 8 and 9, the content of hydroxyproline amounted to 12.68 μg/mg protein.

DISCUSSION

The course of purification of the high-molecular-weight fraction of hypro-protein is presented in Table 1. About 50% of the protein-bound hydroxyproline of the serum was present in the sediment obtained on dialysis against a buffer of low ionic strength. The successive steps of purification resulted in a distinct increase in the content of hydroxyproline in the protein. Simultaneously, considerable losses of hypro-protein were observed so that after preparative electrophoresis only 10% of the hydroxyproline present in the sediment after dialysis was recovered. This is probably related to considerable lability of the protein and partial denaturation during the purification procedure.

It seems that the euglobulins precipitated by dialysis correspond to fraction I of hyproprotein obtained by LeRoy *et al.* (1964). Arnold, Hvidberg & Rasmussen

Table 1

Purification of the hydroxyproline-containing protein from human serum

Purification step no.	No. of expts.	Protein (mg)	Protein (%)	Hydroxyproline (μg)	Hydroxyproline (%)	OH-Pro:Protein ratio (μg/mg)
Serum (100 ml)	5	7167.00		812.0		0.11
1. Sediment after dialysis	5	155.16	100.0	412.2	100.0	2.66
2. Gel filtration	3	39.57	25.5	261.1	63.3	6.57
3. Concentration dialysis	3	34.39	22.2	232.4	56.5	6.76
4. Second gel filtration	3	17.75	11.4	169.2	41.1	9.55
5. Concentration dialysis	3	13.49	8.7	133.8	32.5	9.89
6. Electrophoresis	2	3.32	2.1	42.1	10.2	12.68

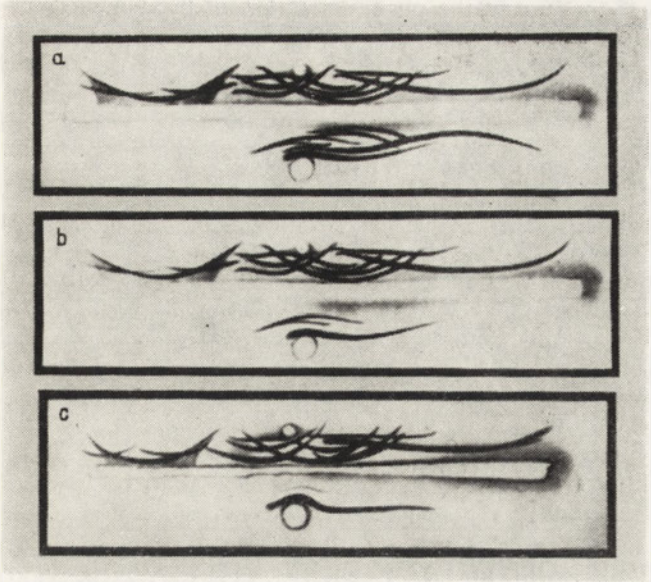

Fig. 5. Immunoelectrophoretic patterns of the hypro-protein preparations obtained on successive steps of purification. Upper wells, normal human serum; central trough, anti-human horse serum; lower well: *a*, protein sedimented on dialysis; *b*, concentrated hypro-protein fraction after step 3; *c*, hypro-protein fraction after step 6.

(1969) obtained a similar fraction from sera of normal human subjects by Sephadex G-200 gel filtration. The high-molecular fraction contained 42% of the total hydroxyproline present in the protein. On refiltration, this fraction emerged at the same elution volume and was not further separated.

The immunoelectrophoretic examinations showed that in the course of purification low-molecular proteins having properties similar to those of IgG-γ and β-globulin were removed. The purified preparation gave one precipitin band which could be supposed to be IgM γ-globulin (Fig. 5c). The additional weak band visible on the immunoelectrophoretogram corresponds probably to β-lipoproteins or α_2-macroglobulin.

Sea, Neff & Block (1968), by Sephadex gel filtration and sucrose-gradient centrifugation, obtained serum protein fractions containing high-molecular hypro-proteins. These fractions reacted with the anti-(skin collagen) antibodies and on immunoelectrophoresis the precipitin bands were located symmetrically to the place of application. This would support the supposition that α_2-macroglobulins possess antigenic properties toward anti-(soluble collagen) antibodies. On the other hand, the preparation of hydroxyproline-rich protein obtained in the present work was contaminated only by a small amount of protein showing different immunoelectrophoretic properties (Fig. 5c). It should be noted that, as demonstrated by Mandema, Schaaf & Huisman (1955), α_2-macroglobulin isolated from human serum does not contain hydroxyproline.

Although the nature of the purified fraction of hypro-proteins requires further study, the results so far obtained support the presence of hydroxyproline in distinct serum proteins which are not involved in collagen metabolism.

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OCZYSZCZANIE BIAŁKA ZAWIERAJĄCEGO HYDROKSYPROLINĘ Z SUROWICY LUDZKIEJ

Streszczenie

1. Opisano metodę wydzielania i oczyszczania z surowicy krwi ludzkiej białka o znacznej zawartości hydroksyproliny.

2. Przy pomocy dializy, dwukrotnej filtracji na żelu Sephadex G-200 i elektroforezy kolumnowej, otrzymano białko jednorodne immunoelektroforetycznie, o zawartości około 1.2% hydroksyproliny.

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RADIATION-INDUCED CHANGES IN SUBUNIT COMPOSITION OF ACID-SOLUBLE RAT SKIN COLLAGEN *IN VITRO*

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1. Rat skin acid-soluble collagen irradiated in deaerated solution up to 80 krad was subjected to disc electrophoresis, column chromatography on carboxymethylcellulose and sedimentation analysis. 2. Subunit composition of irradiated, heat-denatured collagen showed changes evidencing formation of intra- and inter-molecular cross-links, the latter being more pronounced at higher range of radiation dose.

Cross-linking has been considered to be the main radiation effect in gelatin (Tomoda & Tsuda, 1961) and collagen (Bailey, 1967) in deaerated aqueous solutions. It is manifested by increased intrinsic viscosity and gelation of irradiated solutions. The analysis of collagen subunit composition described in the present paper adds a more direct evidence for covalent bond formation in collagen irradiated in the absence of oxygen.

MATERIALS AND METHODS

Preparation of collagen. Acid-soluble collagen was extracted from the skin of mature Wistar male rats according to Bornstein & Piez (1964). Solutions containing 1.5 mg of collagen per 1 ml, in 0.15 M-acetic acid were used if not otherwise stated. Before separation, the collagen solutions were heat-denatured for 30 min at 45°C.

Irradiation procedure. Samples to be irradiated were deaerated for 6 min at 3 mm Hg. The radiation was delivered from Stabilipan-250 X-ray apparatus (Siemens, G.F.R.) at 200 kV, 20 mA and filtered through 0.5 mm Cu. The dose-rate estimated with the Fricke-Fe dose-meter was 410 rad/min.

Disc electrophoresis was carried out on polyacrylamide gel according to Nagai, Gross & Piez (1964). A 7.5% gel was used as a separator gel and the separation time was 90 min. The developed gels were analysed with Zeiss (Jena, G.D.R.) microdensitometer, model ER I, type 10 adapted to accept the gel columns.

Column chromatography on CM-cellulose. The chromatography was carried out according to the method of Piez, Eigner & Lewis (1963) as modified by Cooper

& Davidson (1968) in a 2×20 cm column of Whatman CM-32 carboxymethylcellulose, thermostated at 40°C. The fractions were eluted using a linear gradient of NaCl from 0 to 0.1 M in 0.06 M-acetate buffer, pH 4.8 (900 ml) and then with 0.2 M-NaCl in the same buffer. The effluent was continuously monitored in a 1 cm flow cell at 230 nm using MOM (Budapest, Hungary) UV spectrophotometer and recorder.

Sedimentation analysis. The sedimentation velocities of denatured collagen of different concentrations, 0.3 - 0.8 mg/ml in 0.1 M-NaCl - 0.15 M-acetic acid solution, pH 2.8, were measured in analytical Spinco Model E Beckman (München, G.F.R.) ultracentrifuge at 40°C and 59 780 r.p.m. The sedimentation coefficients were obtained by extrapolation to zero concentration.

Protein concentration was determined by the Kjeldahl method.

RESULTS AND DISCUSSION

The patterns of disc electrophoretic separation of irradiated collagen presented in Fig. 1 show that both the amount of γ component and the fraction of protein remaining at the top of the spacer gel increase with the increasing irradiation dose. The content of α and β subunits decreases simultaneously. The quantitative assessment of these changes made by integration of densitometric tracings of polyacrylamide-gel columns shows a linear dose-dependence (Fig. 2).

For calculation of the number of interchain cross-links, the equation of Tomoda & Tsuda (1961) was applied:

$$Cr = M_0 N_0 \left(\frac{1}{M_0} - \frac{1}{M} \right) \tag{1}$$

where Cr is the number of cross-links; M, the mean molecular weight after irradiation; M_0, the mean molecular weight before irradiation, and N_0 the average number of individual subunits present in 1 ml of thermally denatured collagen solution before irradiation.

Using data from Fig. 2 and assuming that no subunit larger than γ was formed, one gets 1.26×10^{15} cross-links/ml formed in collagen at a concentration of 1.5 mg/ml irradiated with 45 krad. Accordingly, the G value was about 0.044. This radiation yield seems to be underestimated because the number of cross-links formed in the insoluble fraction of protein remaining at the top of the spacer gel was not assessed.

According to Piez *et al.* (1963) chromatography on CM-cellulose column allows to separate denatured collagen into six fractions. There is no agreement as to the identity of fractions number *V* and *VI* (Tristram, Worrall & Steer, 1965; Davidson & Cooper, 1967) but they may represent the γ subunit (fraction *V*) and a component of a higher molecular weight (fraction *VI*). The relative amount of these fractions increases with the irradiation dose (Fig. 3).

When the respective amounts are used for calculation of radiation yield of cross-link formation, the G value increases to about 0.060 but is still much lower than the corresponding value for cross-link formation in gelatin solutions reported

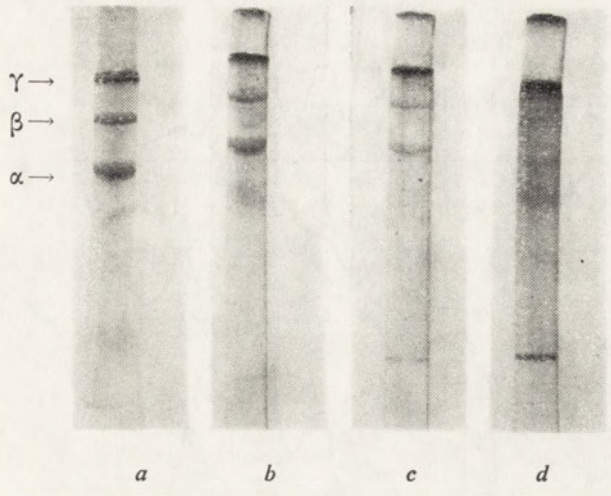

Fig. 1. Polyacrylamide-gel electrophoresis of the acid-soluble collagen irradiated in deaerated solutions. *α*, *β*, and *γ*, bands of monomeric, dimeric and trimeric subunits of collagen. *a*, Control, non-irradiated; *b*, *c* and *d*, irradiated with 15, 60 and 80 krad, respectively.

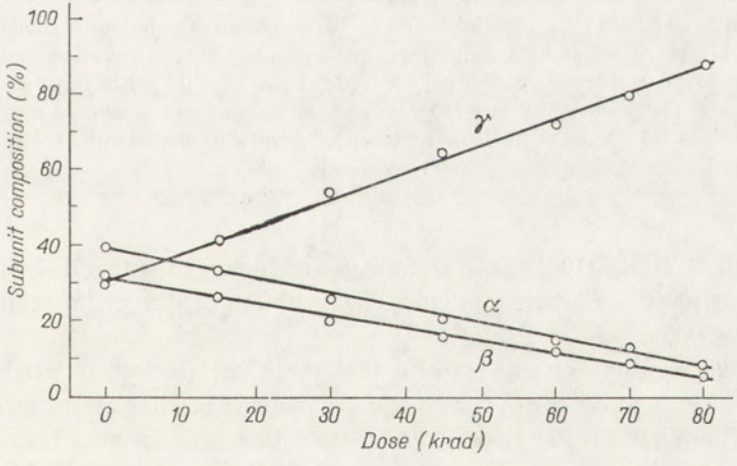

Fig. 2. Dose-dependent changes in the subunit composition of acid-soluble collagen irradiated in deaerated solutions. The content of the subunits was estimated by integration of the densitometric tracings of disc electrophoretograms.

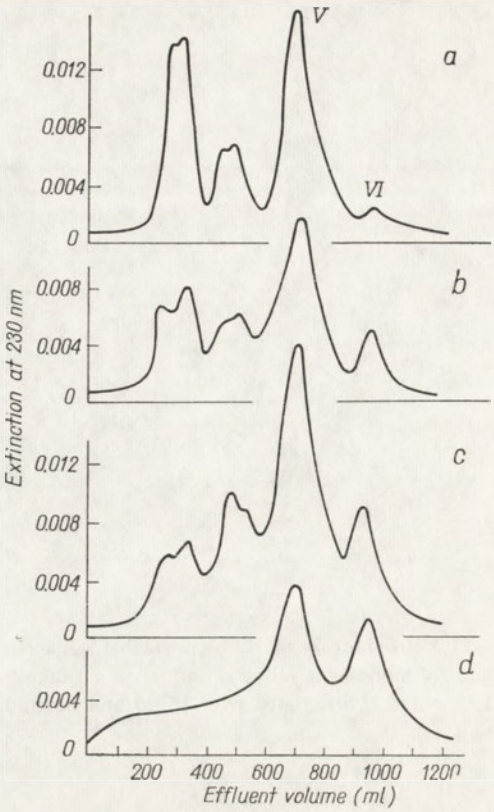

Fig. 3. Elution profiles from column chromatography of irradiated acid-soluble collagen. Thermally denatured collagen (about 35 mg) was loaded on 2×20 cm column of carboxymethylcellulose (Whatman CM-32) thermostated at 40°C. Elution was made with a linear concentration gradient from 0 to 0.1 M-NaCl in 0.06 M-acetate buffer, pH 4.8 (900 ml) and with 0.2 M-NaCl in the same buffer (about 300 ml). The elution rate was 150 ml/h and the effluent was monitored continuously in a 1 cm flow cell at 230 nm. *a*, Control, non-irradiated; *b*, *c*, and *d*, irradiated with 15, 45, and 70 krad, respectively.

by Tomoda & Tsuda (1961). The main cause accounting for this difference seems to be about 100 times higher molecular concentration of the gelatin solutions used by these workers.

Although chromatography revealed that the higher molecular weight subunit is formed already at doses at which α and β components still exist in the irradiated solution, the ultracentrifugation studies showed that a component heavier than γ appears in appreciable amount only at irradiation doses exceeding 45 krad. On applying the irradiation up to 45 krad the sedimentation velocities at 59 780 r.p.m., referring to the α, β and γ subunits were: 2.8, 3.7 and 4.63, respectively. When a higher irradiation dose was used only one sedimentation peak was observed with the constant of 5.7 corresponding to a still heavier, non-identified component. The approximate molecular weight amounted to 120 000 for the α subunit and 203 500

for the β subunit as calculated by combining the obtained sedimentation coefficients and diffusion coefficients reported by Orekhovich & Shpikiter (1958) and using for the first approximation the equation:

$$M = \frac{S_0}{D_0} \cdot \frac{RT}{(1-\rho\bar{v})} \qquad (2)$$

where S_0 is the sedimentation coefficient; D_0, diffusion coefficient; $\bar{v}$, partial specific volume assumed as 0.73, and ρ, solution density as 1.0.

Assuming the proportionality between sedimentation constants and the molecular weight, the heavier component appearing after irradiation with 45 krad would constitute a species of about 500 000 daltons. Thus, at higher doses of irradiation, formation of γ-β or γ-γ species could be anticipated.

The results presented in this paper indicate that the formation of intramolecular cross-links in collagen irradiated in deaerated solutions precedes the induction of intermolecular cross-links, which in turn leads mainly to the formation of heavier aggregates.

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WPŁYW NAPROMIENIOWANIA *IN VITRO* NA SKŁAD PODJEDNOSTKOWY ROZPUSZCZALNEGO W KWASACH KOLAGENU ZE SKÓRY SZCZURA

Streszczenie

1. Kolagen ze skóry szczura, rozpuszczalny w kwasach, napromieniano w roztworach dawkami do 80 krad, a następnie analizowano metodami elektroforezy dyskowej, chromatografii kolumnowej na karboksymetylocelulozie i sedymentacji w ultrawirówce.

2. W napromienionym, zdenaturowanym cieplnie kolagenie stwierdzono zmiany składu podjednostkowego świadczące o tworzeniu się wewnątrz- i międzycząsteczkowych wiązań poprzecznych. Tych ostatnich było więcej przy wyższych dawkach promieniowania.

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STRUCTURAL REQUIREMENTS FOR INHIBITION OF YEAST IMIDAZOLEGLYCEROL PHOSPHATE DEHYDRATASE BY TRIAZOLE AND ANION INHIBITORS

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1. The effect of two groups of inhibitors, i.e. triazole and anionic compounds, on yeast imidazoleglycerol phosphate (IGP) dehydratase (EC 4.2.1.19) was studied. 3-Amino-1,2,4-triazole was found to be a more effective inhibitor than 1,2,4-triazole, 3-β-aminoethyl-1,2,4-triazole and DL-1,2,4-triazole-3-alanine. None of the disubstituted triazole derivatives affected this enzymic activity. 2. Inhibition of IGP dehydratase by triazoles and divalent anions was competitive with substrate. 3. Measurements of interaction constants a provided evidence that triazole and anion inhibitors showed affinity to the two distinctly different sites within the active centre of IGP dehydratase. Binding of one inhibitor favoured the binding of the inhibitor of the other group to its respective site. The two sites are separated by a distance corresponding to the size of the substrate molecule. 4. Structural requirements for inhibition of IGP dehydratase by triazole compounds differed from those reported for inactivation of catalase.

It has been found that the growth inhibitory effect of aminotriazole on yeast (Kłopotowski & Wiater, 1965) and bacteria (Hilton, Kearney & Ames, 1965) results from inhibition of IGP [1] dehydratase (EC 4.2.1.19), an enzyme of histidine biosynthesis (Ames & Horecker, 1956; Ames, 1957). It has been also reported that aminotriazole shows a synergistic inhibitory effect with phosphate or sulphate (Kłopotowski & Wiater, 1965). There are only few reported examples of this kind of synergistic inhibition of enzyme activity. Slater & Bonner (1952) observed that fluoride and inorganic phosphate exhibit such an effect on succinate dehydrogenase complex from heart muscle and Fridovich (1964, 1965) analysed synergistic inhi-

[1] Abbreviations: IGP, imidazoleglycerol phosphate; aminotriazole, 3-amino-1,2,4-triazole; triazolealanine, DL-1,2,4-triazole-3-alanine. The triazole compounds used are all derivatives of 1,2,4-triazole, therefore the numerals indicating positions of nitrogen atoms in the ring are omitted further in the text. Numbering of triazole ring atoms is that used by Potts (1961). Aminotriazole is known as herbicide under the name of amitrole.

bition of milk xanthine oxidase. Several methods of evaluating inhibition caused by pairs of inhibitors have been described (Yagi & Ozawa, 1960; Anderson & Reynolds, 1965; see also Webb, 1963).

A multiple inhibition of liver alcohol dehydrogenase by compounds which interact with the binding sites for nicotinamide-adenine dinucleotides was reported by Yonetani & Theorell (1964), and a procedure based on Dixon's plot (1953) applied by the same authors allowed to distinguish the individual binding sites.

This paper presents evidence that the affinity of triazole compounds to the active centre of yeast IGP dehydratase depends on the presence of unsubstituted carbon and two associated nitrogen atoms. It has been also concluded that two negative charges are essential for the inhibition of the enzyme by phosphate and other anion inhibitors. Interaction constants of both groups of inhibitors indicate that triazole and anion inhibitors of IGP dehydratase are bound to distinctly different sites. Binding of one inhibitor favours the binding of the inhibitor of the other type, which explains their synergistic interaction.

MATERIALS AND METHODS

Methods for growing *Saccharomyces cerevisiae*, preparing cell-free yeast extracts and purifying IGP dehydratase were described previously (Kłopotowski & Wiater, 1965). Since the enzyme preparations purified by thermal precipitation of inactive proteins were inactivated by freezing and thawing, ammonium sulphate fractionation was used in the present experiments.

IGP dehydratase was assayed by the modified method of Ames (1957). The activity was determined by measuring absorption of imidazoleacetol phosphate in alkaline solution at 290 nm. One enzyme unit was defined as the amount of enzyme that forms 1 μmole of imidazoleacetol phosphate per hour under the experimental conditions. The enzyme was activated immediately before assay by a 20 min preincubation at 37°C in 0.02 M-triethanolamine-HCl buffer, pH 8.0, containing 85 mM-2-mercaptoethanol and 0.4 mM-manganous chloride. The incubation mixtures contained in a total volume of 0.40 ml: 0.15 M-triethanolamine-HCl buffer, pH 7.2, 70 mM-2--mercaptoethanol, 0.32 mM-manganous chloride, and 0.1 - 0.3 unit of the preincubated enzyme. Substrate and inhibitor concentrations are given in the text. The reaction mixture was incubated at 37°C for 60 min and the reaction was stopped by adding 2.0 ml of 1.2 N-sodium hydroxide. After another 60 min the tubes were cooled at room temperature and the extinction was measured at 290 nm. Blanks for UV-absorbing contaminations of IGP and protein were subtracted in calculating the net extinction due to imidazoleacetol phosphate formed. Ten nmoles of the product has extinction at 290 nm equal to 0.051. Reaction velocity V was expressed in μmoles of the product formed per hour.

Chemicals. Imidazoleglycerol phosphoric acid, disodium salt, was synthesized by the Ames procedure as described previously (Kłopotowski & Wiater, 1965). 3-Amino-1,2,4-triazole and 1-amino-1,2,4-triazole were products of Fluka (Buchs,

Switzerland). All the other triazole compounds were obtained through the courtesy of Dr. R. G. Jones (Lilly Research Laboratories, Indianapolis, Indiana, U.S.A.): 1,2,4-triazole, 3,5-diamino-1,2,4-triazole, 3,5-dihydroxy-1,2,4-triazole, 3-β-aminoethyl-1,2,4-triazole, 3,5-dimethyl-1,2,4-triazole, 1,2,4-triazole-5-thiol and DL-1,2,4-triazole-3-alanine. The other chemicals used were: L-histidine (Chemapol, Prague, Czechoslovakia), D-histidine (Hofmann-La Roche, Paris, France), histidinol and histidinol phosphoric acid (Cyclo Chemical, Los Angeles, Calif., U.S.A.). Non-listed chemicals were products of FOCH (Gliwice, Poland).

RESULTS

Inhibition of yeast IGP dehydratase by triazole compounds

It appeared in the preliminary experiments that only aminotriazole, triazole, aminoethyltriazole and triazolealanine inhibited the purified yeast IGP dehydratase at low, 0.03 mM, concentration of the substrate. The 3,5-disubstituted triazoles: 3,5-diaminotriazole, 3,5-dihydroxytriazole, 3,5-dimethyltriazole, triazole-5-thiol, as well as imidazole, D- and L-histidine, histidinol and histidinol phosphate did not affect IGP dehydratase activity.

It has been found that the inhibition of IGP dehydratase by triazole, aminoethyltriazole and triazolealanine is competitive with the substrate, as it has been shown for aminotriazole (Kłopotowski & Wiater, 1965). Table 1 presents the calculated inhibitor constants, K_i. Aminotriazole and triazole had K_i value lower than that of Michaelis constant (0.3 mM). This indicates that the affinity of 3-monoamino derivative and of unsubstituted triazole to the active centre of IGP dehydratase is higher than that of the substrate itself.

The fact that out of the following pairs of compounds: imidazole and triazole, 1-aminotriazole and 3-aminotriazole, 3,5-diaminotriazole and 3-aminotriazole, L- or D-histidine and DL-triazolealanine, only the second compound listed is inhibitory to IGP dehydratase, indicates that structural requirements for the inhibition of IGP dehydratase are very specific.

Table 1

Inhibitor constants of triazole inhibitors

K_i values were calculated from Lineweaver-Burk plots (cf. Webb, 1963).

Inhibitor	K_i (mM)
3-Aminotriazole	0.03
Triazole	0.12
3-β-Aminoethyltriazole	1.20
Triazole-3-alanine	2.00

Inhibition of yeast IGP dehydratase by multivalent anions

The previous experiments (Kłopotowski & Wiater, 1965) showed that inorganic phosphate and sulphate, as well as α- and β-glycerophosphates and 3-phosphoglycerate were weak inhibitors of yeast IGP dehydratase. In this study oxalate and arsenate were found to inhibit the enzyme. Chloride and fluoride ions had only very slight inhibitory effect detectable at concentrations exceeding 50 mM; this effect was not competitive with IGP.

Hence it was supposed that divalent anion is responsible for competitive inhibition caused by this group of compounds. A way to prove or disprove this point was to measure K_i values at different relative concentrations of $H_2PO_4^{1-}$ and HPO_4^{2-} anions. This could be done by determining the apparent K_i values at two hydrogen ion concentrations. However, these values are calculated on the basis of Michaelis constant, which changes depending on ionization of substrate and that of active groups in catalytic centre. A more practical approach was therefore used. The apparent K_i values were determined for sulphate which is almost completely dissociated to SO_4^{2-} at pH values above 5 (pK_2 1.92). The ratio of K_i value for sulphate at pH 7.4 to that at pH 6.8 was found to be 2.6. In the case of phosphate the concentrations of ionic forms, i.e. $H_2PO_4^{1-}$ or HPO_4^{2-}, were used alternatively in calculation of K_i for phosphate at these two pH values. It appeared that the ratios $K_{i(7.4)}/K_{i(6.8)}$ were 0.7 and 2.9 for the concentration of $H_2PO_4^{1-}$ and HPO_4^{2-}, respectively. The latter value was close enough to that of SO_4^{2-} to conclude that phosphate ion with two negative charges is the active form of the inhibitor.

Table 2 presents values of K_i for anionic inhibitors of IGP dehydratase corrected for the active forms.

Interaction of triazole and anion inhibitors of IGP dehydratase

Calculation of interaction constants α for pairs of inhibitors enables to decide whether the two inhibitors used interact with the same site or with two distinct sites. For this purpose the graphic procedure devised by Yonetani & Theorell (1964)

Table 2

Inhibitor constants of anionic inhibitors

K_i values were calculated from Lineweaver-Burk plots (cf. Webb, 1963). The apparent values obtained for phosphate and arsenate were corrected for actual concentrations of divalent anions (see text). pK_2 values used were 7.21 and 6.77, respectively (Weast *et al.*, 1965 - 1966).

Compound used	Inhibitory anion	K_i (mM)
Potassium phosphate, dibasic	HPO_4^{2-}	5.0
Sodium oxalate	$(COO)_2^{2-}$	20.0
Sodium sulphate	SO_4^{2-}	24.0
Sodium arsenate, dibasic	$HAsO_4^{2-}$	33.0

based on Dixon's (1953) plot was adapted. The results are exemplified in Figs. 1, 2 and 3. Reciprocal values of reaction velocity were plotted against concentration of one inhibitor. Three lines in each Figure represent three series of IGP dehydratase assays. In series *A* and *B* (Dixon's plot), IGP concentration was 0.75 and 2.0 mM, respectively. In series *C* the reaction was run at 2.0 mM-IGP in the presence of both inhibitors: the second one was present at constant concentration. Series *B* and *C* constitute the Yonetani-Theorell plot. In such plots intersection of lines *A* and *B*

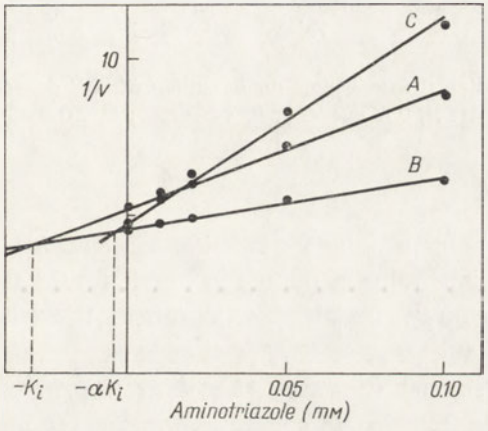

Fig. 1. Dixon and Yonetani-Theorell plots for inhibition of IGP dehydratase by aminotriazole and phosphate. Curves: *A*, 0.75 mM-IGP; *B*, 2.0 mM-IGP; *C*, 2.0 mM-IGP plus 5 mM-potassium phosphate. The enzyme assay as in Methods.

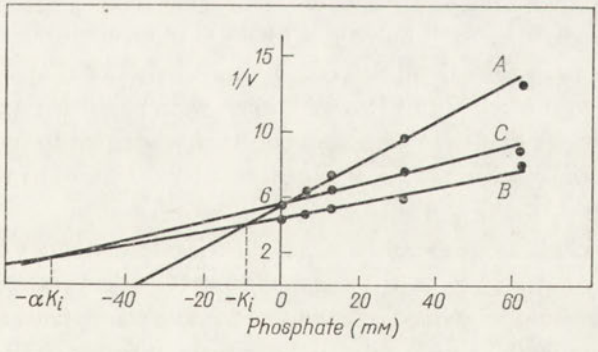

Fig. 2. Dixon and Yonetani-Theorell plots for inhibition of IGP dehydratase by phosphate and triazolealanine. Curves: *A*, 0.75 mM-IGP; *B*, 2 mM-IGP; *C*, 2.0 mM-IGP plus 2.5 mM-triazolealanine.

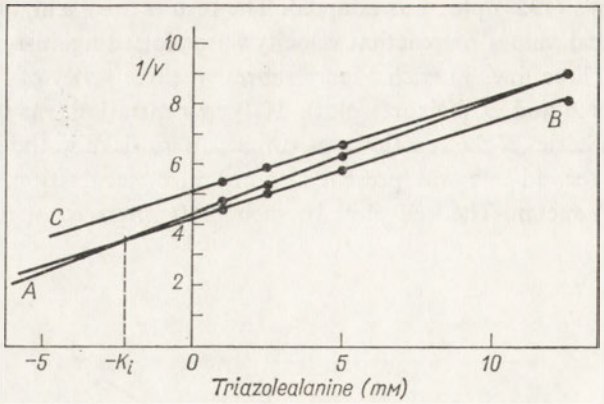

Fig. 3. Dixon and Yonetani-Theorell plots for inhibition of IGP dehydratase by triazolealanine and aminotriazole. Curves: *A*, 0.75 mM-IGP; *B*, 2 mM-IGP; *C*, 2.0 mM IGP plus 0.05 mM-aminotriazole.

indicates on abscissa a minus value for inhibitor constant, K_i. Intersection of lines *B* and *C* points to a minus value of the product of K_i for the same inhibitor and the interaction constant α for two inhibitors, αK_i. From the two values found on abscissa, the interaction constant α can be calculated.

The obtained interaction constants α for different pairs of inhibitors are given in Table 3. It may be seen that all constants, with the exception of those referring to triazolealanine, had values lower than 1. This means that there was a positive interaction between these triazole and anionic inhibitors. It indicates also that the inhibitors are bound to their specific but distinctly different sites on enzyme molecule and that the attachment of one inhibitor favours binding of the other one to its respective site. This kind of interaction is shown in Fig. 1.

The interaction constant for triazolealanine and phosphate was found to be 7.5 (see Fig. 2). Values higher than 1 but lower than infinity indicate that there is a negative interaction between inhibitors which may result from steric hindrance.

When triazolealanine and aminotriazole were used lines *B* and *C*, representing series of assays with a single and two inhibitors, were parallel (Fig. 3). The lack of intersection indicates that the interaction constant was equal to infinity. It follows therefrom that triazolealanine and aminotriazole have affinity to the same site on the enzyme molecule.

One can conclude from these data that all triazole inhibitors tested, including triazolealanine, have affinity to the same site on IGP dehydratase molecule. The anionic inhibitors bind to another site. Out of triazole compounds studied, triazolealanine is structurally the most similar to the enzyme substrate, IGP. This compound only was able to interact negatively with the inhibitors of the other group. This may suggest that the two sites, one for triazole inhibitors and the other one for anionic inhibitors, are within a distance of triazolealanine (or IGP) from each other.

Table 3

Interaction constants of the triazole and anion inhibitors

The values are calculated according to Yonetani & Theorell (1964). Conditions and concentrations as in Methods.

Inhibitor present at varying concentrations	Inhibitor present at constant concentration	Interaction constant α
Aminotriazole	Phosphate	0.17
	Oxalate	0.21
	Sulphate	0.26
	Arsenate	0.25
	Triazolealanine	∞
Phosphate	Aminotriazole	0.17
	Triazole	0.15
	β-Aminoethyltriazole	0.55
	Triazolealanine	7.5
Sulphate	Triazole	0.35

DISCUSSION

Structural requirements for inhibition of IGP dehydratase activity by triazole compounds were found to be very specific. Aminotriazole and triazole were more effective than aminoethyltriazole and triazolealanine. The 3,5-disubstituted triazole derivatives including diaminotriazole did not affect IGP dehydratase activity and none of imidazole analogues of IGP had any detectable effect. Thus one unsubstituted carbon and two associated nitrogen atoms constitute two obligatory elements for interaction of triazole ring with the site in the active centre of IGP dehydratase. This interaction is enhanced by substitution of C-3 with amino group.

To the other group of inhibitors of IGP dehydratase belong inorganic anions and some of their esters with glycerol or glycerate (Kłopotowski & Wiater, 1965). Phosphate ion with the two dissociated acidic groups was found to be the actual form of this inhibitor. All divalent anions were found to inhibit the enzyme in contrast to monovalent chloride and fluoride ions. Therefore, it is concluded that divalent dissociated anion is the actual inhibitory form of this group.

Interaction constants α of the triazole and anion inhibitors indicated that they interact with distinctly different sites of IGP dehydratase molecule. Since compounds of both groups inhibited the enzyme competitively with the substrate, on can assume that both respective sites are located in the enzyme active centre. The values of interaction constants of triazolealanine with aminotriazole or phosphate indicated that this triazole inhibitor interacts with the same site as aminotriazole and abolishes the interaction of phosphate with another site of the active centre. It follows therefrom that the two sites are within a distance of triazolealanine or IGP molecule from each other. Thus it seems justified to suppose that triazoles have affinity to

the site which binds imidazole moiety of IGP, and anionic inhibitors to that which binds phosphate group of the substrate.

The synergistic action of the triazole and anion inhibitors was also found with *Salmonella typhimurium* IGP dehydratase. The interaction constant of triazole and phosphate was equal to 0.13 (Wiater & Kłopotowski, unpublished).

The obtained results show that the most effective inhibition of IGP dehydratase can be achieved by using two compounds which have affinity to two different sites within the active centre, both engaged in binding of the substrate. Yonetani & Theorell (1964) have described a similar situation with inhibitors of liver alcohol dehydrogenase. α values found by these authors for interaction of *o*-phenanthroline with AMP and ADP were 0.3 and 0.5, respectively. The interaction constant for two competitive inhibitors of D-amino acid oxidase : riboflavin-5′-sulphate and adenosine-5′-sulphate was 0.2 (Yagi & Ozawa, 1960). The lowest value of the interaction constant, 0.0034, was found by Slater & Bonner (1952) for fluoride and phosphate, the competitive inhibitors of succinate dehydrogenase.

Similarly to our results, Feinstein, Seaholm & Balonoff (1964) have found that, out of the same triazole compounds, aminotriazole was the most effective inhibitor of liver catalase. However, 4-aminotriazole and triazole-5-thiol, which do not inhibit IGP dehydratase, were effective inhibitors of catalase. Evidently, structural requirements for inhibition of liver catalase are different from those for inhibition of yeast IGP dehydratase. It should be mentioned that the mode of action of aminotriazole on these two enzymes is different. Aminotriazole reacts with catalase - hydrogen peroxide (complex I) and inactivates the enzyme irreversibly (Margoliash, Novogrodsky & Schejter, 1960) whereas its inhibitory effect on IGP dehydratase is reversible by IGP. Besides, the latter enzyme is more sensitive to low concentrations of aminotriazole. In the presence of phosphate, yeast IGP dehydratase was inhibited at low substrate concentration to more than 90% when 0.05 mM-aminotriazole was used, whereas aminotriazole at 5 mM concentration inhibited liver catalase by only 50%.

Although structural requirements of triazole compounds for inhibiting IGP dehydratase were shown to be stringent, these compounds have also affinity to other enzymes. In addition to the two enzymes aforementioned, an enzyme of aminoimidazole ribotide synthesis was found to be sensitive to aminotriazole and triazole (Hulanicka, Kłopotowski & Bagdasarian, 1969). Triazole, but not aminotriazole was reported to interfere with cysteine biosynthesis (Bogusławski, Walczak & Kłopotowski, 1967; Hulanicka, Kłopotowski & Smith, in press).

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CZYNNIKI STRUKTURALNE WARUNKUJĄCE HAMOWANIE DEHYDRATAZY IGP DROŻDŻY PRZEZ TRIAZOLOWE I ANIONOWE INHIBITORY

Streszczenie

1. Badano wpływ dwóch grup inhibitorów, związków triazolowych i anionów, na dehydratazę IGP (EC 4.2.1.19). Stwierdzono, że 3-amino-1,2,4-triazol jest silniejszym inhibitorem niż 1,2,4-triazol, 3-β-aminoetylo-1,2,4-triazol i DL-1,2,4-triazolo-3-alanina. Dwu-podstawione związki triazolowe nie wpływały na aktywność enzymu.

2. Dwuwartościowe aniony, podobnie jak związki triazolowe, wykazują hamowanie typu kompetycyjnego w stosunku do substratu.

3. Pomiary stałych współdziałania α udowodniły, że inhibitory anionowe i triazolowe wykazują powinowactwo do dwu odrębnych miejsc w obrębie aktywnego centrum dehydratazy IGP. Miejsca te są od siebie oddalone o odległość równą wielkości cząsteczki substratu.

4. Układ strukturalny inhibitorów warunkujący działanie hamujące w stosunku do dehydratazy IGP różni się od analogicznych wymogów dla inaktywacji katalazy.

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HISTIDINE BIOSYNTHESIS AND ITS REGULATION IN HIGHER PLANTS

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1. The activities of three enzymes of histidine biosynthesis: phosphoribosyl-ATP synthetase (PR-ATP synthetase), imidazoleglycerol phosphate (IGP) dehydratase (EC 4.2.1.19) and histidinol phosphate phosphatase (EC 3.1.3.15) have been found in shoot extracts of barley, oats and pea. 2. PR-ATP synthetase from oats and barley shoots is sensitive to feedback inhibition by L-histidine. 3. No evidence for regulation of IGP dehydratase synthesis has been found in barley and oats shoots starved for histidine in the aminotriazole-containing germination medium.

Histidine biosynthesis has been most extensively studied in *Salmonella typhimurium* (Brenner & Ames, 1971). This metabolic pathway consists of ten reactions catalysed by nine distinct enzymes coded for by a single gene cluster. The first enzyme, PR-ATP synthetase, is sensitive to feedback inhibition by L-histidine. PR-ATP undergoes a sequence of reactions which eventually yield IGP[1] and phosphoribosyl--aminoimidazole carboxamide, a precursor of purine ring. IGP is converted by IGP dehydratase to imidazoleacetol phosphate which is transaminated by a specific aminotransferase to L-histidinol phosphate. This compound is dephosphorylated by the same enzyme which dehydrates IGP. Histidinol is oxidized to L-histidine by a two-step reaction catalysed by histidinol dehydrogenase. Biosynthesis of all enzymes of the histidine pathway is coordinately regulated by histidyl-tRNA (Ames, Goldberger, Hartman, Martin & Roth, 1967). In other bacteria, *Escherichia coli* (Garrick-Silversmith & Hartman, 1970), *Bacillus subtilis* (Chapman & Nester, 1969), *Staphylococcus aureus* (Kloos & Patte 1965) and in fungi *Streptomyces coelicolor* (Russi, Carere, Fratello & Khoudokormoff, 1966), *Saccharomyces lactis* (Tingle, Herman & Halvorson, 1968), *Saccharomyces cerevisiae* (Fink, 1964, 1966), *Asper-*

[1] Abbreviations: Aminotriazole, 3-Amino-1,2,4-triazole (amitrole); IGP, D-*erythro*-imidazoleglycerol-3-phosphate; PR-ATP, *N*-1-(5′-phospho-D-ribosyl)-adenosine-5′-triphosphate; PRPP, 5--phosphoribosyl 1-pyrophosphate; BBM II, *N*′-(5′-phospho-D-ribosylformimino)-5-amino-1-(5′--phosphoribosyl)-4-imidazolecarboxamide.

gillus nidulans (Berlyn, 1967) and *Neurospora crassa* (Ames & Mitchell, 1955; Ahmed, Case & Giles, 1964) histidine is synthesized by the same pathway.

However, some differences in enzymes of histidine biosynthesis among microorganisms were reported: e.g. the activities of IGP dehydratase and histidinol phosphate phosphatase are associated with a single enzymic entity in *S. typhimurium* (Whitfield, Smith & Martin, 1964), whereas in fungi (*N. crassa*, Ames, 1957a, 1957b; Ahmed *et al.*, 1964; *S. cerevisiae*, Fink, 1964), these two reactions are catalysed by two separate enzymes. In some organisms, genetic and biochemical evidence for existence of an enzyme responsible for isomerization of the BBM II intermediate is still lacking (e.g. *B. subtilis*, Chapman & Nester, 1969; *S. cerevisiae*, Fink, 1964; *N. crassa*, Ahmed *et al.*, 1964).

Information on histidine biosynthesis in higher organisms is very scarce. There are no reports on histidine biosynthesis in plants, except for demonstration of an ability to synthesize and excrete IGP by algae *Prototheca zopfi* and *Chlorella vulgaris* (Casselton, 1966; Siegel & Gentile, 1966). Animals were reported to be unable to synthesize histidine *de novo*, but could utilize imidazole pyruvate or D-histidine as a source of L-histidine (Meister, 1965).

In this paper we report on the presence of PR-ATP synthetase, IGP dehydratase and histidinol phosphate phosphatase in young shoots of higher plants.

MATERIALS AND METHODS

Organisms. Seeds of barley (*Hordeum vulgare* L), oats (*Avena sativa* L) and pea (*Pisum sativum* L) were used. *S. typhimurium* LT-2 (wild type) and its *his-1302* mutant were obtained from Dr. B. N. Ames. Mutant *his-1302* used instead of *his G70* has a deletion which results in complete absence of PR-ATP synthetase activity and eightfold derepression of all other enzymes of histidine biosynthesis (Ames, Hartman & Jacob, 1963).

Enzyme preparations. Plant extracts. Seeds were germinated on a thick layer of gauze soaked with tap water. Four to six day-old shoots were cut off, frozen in dry ice and ground in a mortar. PR-ATP synthetase was extracted by grinding with an equal weight of 0.1 M-tris-HCl buffer, pH 7.5, containing 1.4 mM-2-mercaptoethanol. For extraction of other enzymes, 0.2 M-triethanolamine-HCl buffer, pH 7.2, was used. The extracts were filtered through a double layer of gauze and centrifuged at 16 000 rev./min for one hour. The centrifuged extracts were dialysed overnight against 0.02 M buffer or desalted on Sephadex G-50 columns. Only about two-thirds of initial volume of an extract introduced to a column was collected in order to discard low-molecular metabolites extracted from plants.

Extracts of S. typhimurium *mutant* his-1302. The mutant was grown on minimal medium C of Vogel & Bonner (1956), containing 0.5% glucose and 0.1 mM-L-histidine. The cells from stationary phase of growth were harvested and washed three times with 0.9% sodium chloride by centrifugation and resuspension. The washed cells were suspended in 0.1 M-tris-HCl buffer, pH 7.5, and disrupted in a French press under a 6 ton pressure. The thawed homogenate was suspended in an equal volume

of tris buffer and passed through Sephadex G-50 column as described for the plant extracts. The *S. typhimurium* extract was kept at -20°C for about a month without appreciable loss of the enzymic activity for conversion of PR-ATP to BBM II.

Assay of PR-ATP synthetase. The procedure of Voll, Apella & Martin (1967) was followed with some minor modifications to increase sensitivity of the assay (R. G. Martin, personal communication) or to decrease interference due to high protein concentration. The reaction mixture in quartz spectrophotometer cuvettes contained in a total volume of 0.44 ml: 0.20 ml of "G enzyme buffer" (0.2 M-tris, free base, 0.02 M-magnesium chloride, 0.3 M-potassium chloride and diluted hydrochloric acid to bring pH to pH 8.5), 0.1 M-ATP 0.02 ml, enzyme protein (0.1-1.0 mg) and water, 0.20 ml. The reaction was started by the addition of 0.02 ml of 0.01 M--PRPP and was run at room temperature (24 - 26°C) or 37°C. Changes in extinction at 290 or 310 nm were followed. Molar absorbancy coefficients of BBM II, 4270 cm^2 $mole^{-1}$ at 290 nm and 2850 cm^2 $mole^{-1}$ at 310 nm, were calculated from published data (Smith & Ames, 1964) and from the ratio E_{290}/E_{310} of BBM II equal to 1.5 found in these experiments. The activity of PR-ATP synthetase was expressed in nmoles of product/min/mg protein.

Assay of IGP dehydratase. The activity was determined after Ames (1957b). The enzyme was activated by a 20 min preincubation in the buffer containing 0.02 M--triethanolamine-HCl, pH 8.0, 85 mM-2-mercaptoethanol and 0.4 mM-manganous chloride. The incubation mixture contained 85 mM-triethanolamine-HCl buffer, pH 7.5, 60 mM-mercaptoethanol, 0.28 mM-manganous chloride, 3.0 mM-IGP and the enzymic protein (0.1 - 1.0 mg) in a final volume of 0.33 ml. The reaction was started by addition of the enzyme and stopped after a 60 or 120 min incubation at 37°C with 0.70 ml of 1.43 N-sodium hydroxide. The tubes were shaken and kept for one more hour at the same temperature to convert imidazoleacetol phosphate formed into its UV-absorbing derivative. Extinction at 290 nm was read against appropriate blanks (enzyme or substrate omitted). The net increase in extinction was converted into nmoles of the reaction product using absorbancy coefficient (Ames, Garry & Herzenberg, 1960). The enzyme activity is expressed in nmoles/h/mg protein.

Assay of histidinol phosphate phosphatase. The assay was based on determination of inorganic phosphate released in the reaction, according to Ames *et al.* (1963). The incubation mixture contained in a total volume of 0.41 ml: 0.20 ml of 0.1 M--triethanolamine-HCl buffer, pH 7.5, 0.01 ml of 0.5 M-magnesium chloride, 0.10 ml of 0.25 M-histidinol phosphate neutralized with diluted sodium hydroxide to pH 7.5, and 0.10 ml of plant extract (0.05 - 2.0 mg protein). The reaction was started by adding the enzyme and was run at 37°C. After 15 min the reaction was stopped by adding 1.0 ml of 10% (w/v) trichloroacetic acid. Insoluble material was discarded by centrifugation. The 0.3 ml samples were incubated at 37°C for 1 h with 0.70 ml of ascorbic acid - ammonium molybdate reagent and the extinction measured at 820 nm. The enzyme activity is expressed in nmoles/h/mg protein.

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

Chemicals. Imidazoleglycerol phosphate, sodium salt, was prepared after Ames, as described previously (Kłopotowski & Wiater, 1965). All other chemicals were commercial products of reagent grade: 3-amino-1,2,4-triazole, Fluka (Buchs, Switzerland); ATP, Sigma (St. Louis, Mo., U.S.A.); L-histidine, Chemapol (Prague, Czechoslovakia); L-histidinol phosphate, Cyclo Chemical Comp. (Los Angeles, Calif., U.S.A.); and PRPP, Mann Research Laboratories (New York, U.S.A.).

RESULTS

PR-ATP synthetases and their sensitivity to feedback inhibition by L-*histidine*

It has been found that specific activity of PR-ATP synthetase in crude extracts of barley, oats and pea shoots at 24 - 26°C were 0.5, 1.5 and 1.6, respectively. In wild-type strain of *S. typhimurium* this specific activity was 5.7.

The activity of PR-ATP synthetase in *S. typhimurium* and pea was twice as high at 37°C as at 25°C, but barley and oats enzymes showed higher activity at lower temperature. Prolonged incubation for 30 and 60 min at 37°C resulted in a gradual decrease of the activity of pea enzyme and complete inhibition of barley and oats synthetases. Thermolability of barley and oats synthetases distinguished these enzymes from those from *S. typhimurium* and pea.

L-Histidine is a feedback inhibitor of the first step in histidine biosynthesis in *S. typhimurium* (Martin, 1963) and *S. cerevisiae* (Kłopotowski, Luzzati & Słonimski, 1960). Therefore, its effect on two plant PR-ATP synthetases has been examined. The results are presented in Table 1.

Pea and oats PR-ATP synthetases from fresh extracts were inhibited by about 50% by 0.01 mM-L-histidine, while the aged pea PR-ATP synthetase preparation was inhibited by 80%. On the contrary, PR-ATP synthetase in fresh extracts from *S. typhimurium* was inhibited by 50% at 0.06 mM-L-histidine concentration and the overnight storage of the bacterial enzyme resulted in a decreased sensitivity to feedback inhibition. Thus, upon identical treatment the pea PR-ATP synthetase

Table 1

Feedback inhibition of plant PR-ATP synthetases by L-*histidine*

The extract from pea shoots was incubated at 37°C and that from oats at 24°C. Aged extract stands for the preparation stored overnight at 4°C. NT, not tested. The enzymic activity is expressed in nmoles/min/mg protein; inhibition is in percent.

Histidine (mM)	Pea extract				Oats extract	
	fresh		aged		fresh	
	Activity	Inhibition	Activity	Inhibition	Activity	Inhibition
0	4.45	—	2.26	—	0.86	—
0.001	NT	—	1.97	13	0.73	15
0.01	2.06	54	0.40	82	0.39	54

sensitivity to L-histidine changed in the opposite way. The presented results indicate that plant PR-ATP synthetases are subject to feedback inhibition by L-histidine; their sensitivity is at least equal if not greater than that of the bacterial enzyme.

IGP dehydratase

Specific activity of IGP dehydratase in barley and oats was found to be very low, namely 22 and 37 nmoles of imidazoleacetol phosphate formed/hour/mg protein, respectively, i.e. almost ten times lower than that of *S. cerevisiae* (Kłopotowski & Wiater, 1965).

IGP dehydratases from barley and oats extracts were purified about tenfold by using two steps of the purification procedure described for the yeast enzyme (Kłopotowski & Wiater, 1965). Protamine-precipitable proteins were removed and most of IGP dehydratase activity was precipitated from pH 5.5 solution with 4 M-ammonium sulphate added to a final concentration of 2.2 M. The precipitate was dissolved in 0.02 mM-triethanolamine-HCl buffer, pH 8, and dialysed against the same buffer, or passed through Sephadex G-25 and G-200 column.

The K_m values found by the Lineweaver-Burk procedure for barley and oats IGP dehydratases, were 0.6 and 1.7 mM, respectively (Fig. 1), whereas this value for yeast enzyme was 0.3 mM (Wiater, Hulanicka & Kłopotowski, 1971a) and for bacterial IGP dehydratase 0.4 mM (Hilton, Kearney & Ames, 1965).

Sensitivity of plant IGP dehydratases to the inhibition by triazole and anion inhibitors is reported in the accompanying paper (Wiater, Kłopotowski & Bagdasarian, 1971b).

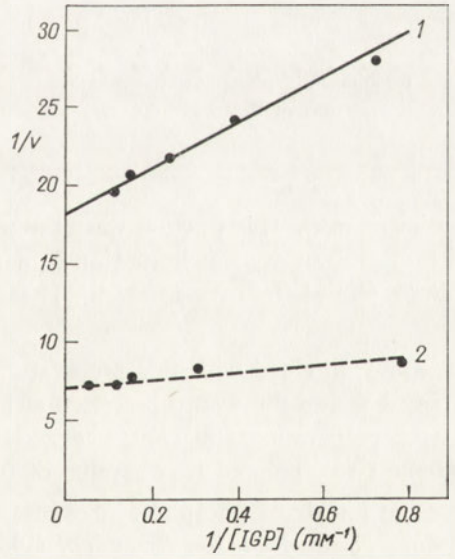

Fig. 1. Activity of IGP dehydratase from oats and barley. Lineweaver-Burk plots: *1*, for enzyme from oats, K_m 1.7 mM; *2*, for enzyme from barley, K_m 0.6 mM.

Histidinol phosphate phosphatase

Incubation of crude dialysed plant extracts with histidinol phosphate resulted in substrate-dependent release of inorganic phosphate. The Lineweaver-Burk plots showed curved lines indicating participation of more than one enzyme in the reaction. It was assumed that the extracts contained several phosphatases; only one of them could be specific for histidinol phosphate.

To demonstrate and characterize plant histidinol phosphate phosphatase, the pea enzyme was purified about 20-fold. Proteins were precipitated by adding solid ammonium sulphate to 0.75 saturation. The precipitate was dissolved in a small volume of 0.02 M-triethanolamine-HCl buffer, pH 8.0. The solution was passed through Sephadex G-25 column. Total protein was recovered in four fractions. The third fraction contained most of the histidinol phosphate hydrolysing activity at

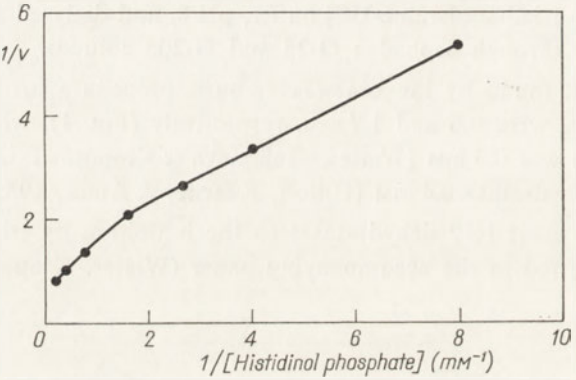

Fig. 2. Lineweaver-Burk plot for histidinol phosphate phosphatase from pea. K_m for the straight portion of the curve 0.4 mM.

low substrate concentration (1 mM). This fraction was applied on Sephadex G-200 column. Small fractions of effluent were collected and phosphatase activity was assayed at 1 mM-histidinol phosphate concentration. The active fractions were combined.

The Lineweaver-Burk plot obtained with this preparation is shown in Fig. 2. A straight line was obtained with a downward inflection at high substrate concentrations. It means that the preparation still contained at least two phosphatases. The straight portion of the curve pointed to K_m value of 0.4 mM.

K_m of *N. crassa* specific histidinol phosphate phosphatase is 4.2 mM (Ames, 1957a) and that of *S. typhimurium* assayed after Ames *et al.* (1963), 0.075 mM (T. Kłopotowski, unpublished). Thus the K_m value for the pea histidinol phosphate phosphatase is intermediate between those for enzymes of fungal and bacterial origin.

Effect of histidine shortage on the activity of histidine biosynthetic enzymes

Shortage of a final product of biosynthetic pathway leads to derepression of enzymes of this sequence. Specific activity of histidine biosynthetic enzymes was increased up to 20-fold in *S. typhimurium* histidine auxotrophs grown at limiting supply of histidine, e.g. in the presence of *N*-formylhistidine (Ames & Garry, 1959) or histidinol (Smith & Ames, 1964). The same result can be achieved by using inhibitors of histidine biosynthesis like 2-thiazolealanine or aminotriazole (Hilton *et al.*, 1965; Kovach, Berberich, Venetianer & Goldberger, 1969). This latter compound inhibits plant IGP dehydratase (Wiater *et al.*, 1971b) and therefore was used to induce histidine shortage in barley and oats shoots in the experiments on derepression mechanism of histidine biosynthesis in plants.

Barley and oats seeds were germinated in tap water with aminotriazole at concentrations varying from zero to 1 mM. At concentration above 0.4 mM-aminotriazole, barley shoots were completely chlorotic and those of oats showed chlorosis only at concentrations as high as 1 mM-aminotriazole. Shoot crops after 5 and 11 days of germination at these two concentrations of aminotriazole were reduced by 50% with barley and 30% with oats. This result was in agreement with the reported greater sensitivity of barley to aminotriazole (Hilton, Jansen & Hull, 1963).

Crude shoot extracts were subsequently prepared and the activity of IGP dehydratase determined therein (Table 2). Barley shoots grown at 0.8 mM-aminotriazole showed about threefold higher activity than those grown in the absence of aminotriazole. In oats shoots IGP dehydratase activity was not dependent on aminotriazole concentration.

Histidine added to the aminotriazole-containing germination solution was unable to bring down the elevated IGP dehydratase activity in barley shoots. Hence, the apparent increase in specific activity was not due to histidine shortage, and thereby cannot be considered as evidence for IGP dehydratase derepression.

Table 2

IGP dehydratase in plants grown in the presence of aminotriazole

Extracts were prepared from 5-day-old barley shoots, and 11-day-old oats shoots. Crude dialysed extracts were used. NT stands for not tested.

Aminotriazole in growth medium (mM)	Specific activity (nmoles/h/mg protein)	
	Barley	Oats
0	10	28
0.4	22	26
0.8	32	40
1.0	NT	34

DISCUSSION

Activities of PR-ATP synthetase, IGP dehydratase and histidinol phosphate phosphatase have been demonstrated in crude extracts from young shoots of higher plants. The activities correspond to the first, seventh and ninth steps of histidine biosynthesis in *S. typhimurium* (Brenner & Ames, 1971). Thus it can be assumed that the sequence of reactions leading to biosynthesis of L-histidine is virtually the same in bacteria and higher plants.

The other common feature of these enzymes was sensitivity of PR-ATP synthetase to feedback inhibition by L-histidine. The plant enzyme was at least equally sensitive to this physiological regulator as reported in bacteria (Martin, 1963).

The occurrence of the other mechanism regulating histidine biosynthesis, i.e. derepression, was not evidenced in our studies on IGP dehydratase. Coordinate derepression of histidine enzymes in *S. typhimurium* results from the fact that their genes constitute a unit of regulation. In *S. cerevisiae* in which genes for histidine enzymes are located on different chromosomes, enzymes of only one gene cluster can be derepressed in certain mutants (Shaffer, Rytka & Fink, 1969). Probably, a similar situation should be taken into account with plants, but no further attempts to demonstrate the derepression were undertaken in this laboratory.

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BIOSYNTEZA HISTYDYNY I JEJ REGULACJA U ROŚLIN WYŻSZYCH

Streszczenie

1. Stwierdzono aktywność trzech enzymów biosyntezy histydyny, syntetazy PR-ATP, dehydratazy IGP i fosfatazy histydynylofosforanu w ekstraktach kiełków jęczmienia, owsa i grochu.

2. Syntetaza PR-ATP owsa i grochu była wrażliwa na inhibicję wsteczną przez L-histydynę.

3. Nie znaleziono dowodów regulacji syntezy dehydratazy IGP w kiełkach jęczmienia i owsa, w których ograniczono biosyntezę histydyny przez dodanie aminotriazolu do pożywki.

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SYNERGISTIC INHIBITION OF PLANT IMIDAZOLEGLYCEROL PHOSPHATE DEHYDRATASE BY AMINOTRIAZOLE AND PHOSPHATE

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1. It has been found that imidazoleglycerol (IGP) dehydratase (EC 4.2.1.19) — an enzyme of histidine biosynthesis — from higher plants such as barley and oats shoots is more sensitive to the inhibitory action of 3-amino-1,2,4-triazole and other inhibiting triazole derivatives than the same enzyme of microbial origin. 2. The inhibitory effect of aminotriazole on barley (but not oats) IGP dehydratase is potentiated by phosphate anions. 3. The possible significance of these findings is discussed in relation to the mechanism of aminotriazole action on the growth of higher plants.

We have previously reported that yeast IGP[1] dehydratase, an enzyme of histidine biosynthesis (Ames, Goldberger, Hartman, Martin & Roth, 1967) was strongly inhibited by aminotriazole; inorganic phosphate and some phosphate esters potentiated this inhibitory effect (Kłopotowski & Wiater, 1965). Two groups of compounds: triazoles and divalent anions were found to be competitive inhibitors of the yeast enzyme, the latter being less effective. Evaluation of interaction constants a measured according to Yonetani & Theorell (1964) indicated that binding of an inhibitor of one group favoured the attachment of inhibitors of the other group (Wiater, Hulanicka & Kłopotowski, 1971a).

Aminotriazole, the most effective of the triazole inhibitors of yeast IGP dehydratase, is known under the name of amitrole as a potent herbicide (Hilton, Jansen & Hull, 1963; Hilton, 1969). On the basis of experiments with microorganisms, histidine and purine biosynthetic enzymes were considered to be possible targets of herbicidal action of aminotriazole (Hilton, 1960; Hilton, Kearney & Ames, 1965; Hulanicka, Kłopotowski & Bagdasarian, 1969). Inability of exogenous histidine

[1] Abbreviations: IGP, D-*erythro*-imidazoleglycerol phosphate; aminotriazole, 3-amino-1,2,4, -triazole; triazole, 1,2,4-triazole; triazolealanine, DL-1,2,4-triazole-3-alanine; thiotriazole, 1,2,4--triazole-3-thiol; 3-β-aminoethyltriazole, 3-β-aminoethyl-1,2,4-triazole; diaminotriazole, 3,5-diamino--1,2,4-triazole; dimethyltriazole, 3,5-dimethyl-1,2,4-triazole.

to reverse the effect of aminotriazole on plant growth argued against the idea that histidine deficiency is responsible for the herbicidal action of this compound (Hilton *et al.*, 1963; Hilton, 1969).

In this paper we report on the inhibition of IGP dehydratase from higher plants by aminotriazole. This effect was potentiated by phosphate. The relation of this finding to the problem of identifying a primary target of herbicidal action of aminotriazole is discussed.

MATERIALS AND METHODS

Organisms. Seeds of barley (*Hordeum vulgare* L) and oats (*Avena sativa* L) were used. *Saccharomyces cerevisiae* wild-type strain YF was obtained from Dr. P. P. Słonimski. *Salmonella typhimurium* mutant *hisT1505* was supplied by Dr. B. N. Ames; the specific activities of all histidine biosynthetic enzymes were tenfold higher in the mutant than in the wild-type strain (Roth, Anton & Hartman, 1966).

Enzyme preparations. Plant shoot extracts were prepared as described previously (Wiater, Krajewska-Grynkiewicz & Kłopotowski, 1971b). The about 10 - 20 fold purified preparations of plant IGP dehydratases were obtained by clupeine treatment and ammonium sulphate precipitation (Kłopotowski & Wiater, 1965) and were desalted by passing through Sephadex G-50 column. Yeast IGP dehydratase was purified by the same procedure. The bacterial crude desalted extract was prepared as described by Smith & Ames (1964).

Assay of IGP dehydratase activity. The enzyme was assayed by the procedure of Ames as described by Wiater *et al.* (1971b).

Chemicals. D-*Erythro*-imidazoleglycerol phosphate was synthesized by the method of Ames (Kłopotowski & Wiater, 1965); 3-amino-1,2,4-triazole was purchased from Fluka (Buchs, Switzerland). Other triazole compounds were kindly supplied by Dr. R. G. Jones from Lilly Research Laboratories (Indianapolis, Ind. U.S.A.)

RESULTS

Competitive inhibition of IGP dehydratase by aminotriazole

IGP dehydratase could be detected in the crude extracts of barley and oats shoots after Sephadex gel filtration. The specific activity of barley and oats extracts was 20 and 40 nmoles imidazoleacetol phosphate formed/h/mg protein, respectively. Crude yeast extracts had a specific activity of about 200. Therefore, for determining the Michaelis and inhibitor constants, IGP dehydratase from barley and oats shoots was purified and concentrated by clupeine treatment and ammonium sulphate precipitation.

It was found from the Lineweaver-Burk plots that K_m values for barley and oats IGP dehydratases were 0.52 and 1.6 mM, respectively (Figs. 1, 2). In each case curves representing IGP dehydratase activity in the presence of aminotriazole were steeper,

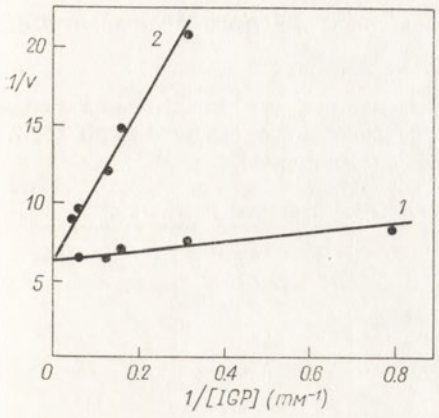

Fig. 1

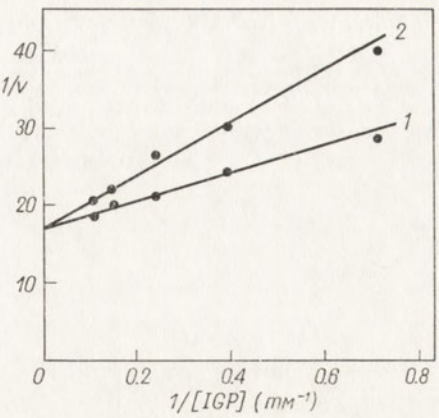

Fig. 2

Fig. 1. Competitive inhibition of barley IGP dehydratase by aminotriazole. The Lineweaver-Burk plot in the absence (*1*) and in the presence of 0.025 мм-aminotriazole (*2*). K_m, 0.4 мм; K_i, 0.01 мм.

Fig. 2. Competitive inhibition of oats IGP dehydratase by aminotriazole. The Lineweaver-Burk plot in the absence (*1*) and in the presence of 0.014 мм-aminotriazole (*2*). K_m, 1.0 мм; K_i, 0.01 мм.

which indicates that aminotriazole inhibited this enzymic activity at all substrate concentrations. The two lines had intersection points on $1/v$ axis, which proves that the inhibitory effect of aminotriazole was competitive with IGP.

Inhibitor constants, K_i, calculated from the data given in Fig. 1 and 2 were 0.01 мм both with barley or oats enzymes. This value is lower than that of 0.03 мм found for yeast (Wiater *et al.*, 1971a) or bacterium (Hilton *et al.*, 1965). Since microbial IGP dehydratases had K_m values of 0.3 - 0.4 мм it should be concluded that plant IGP dehydratases are more sensitive to aminotriazole inhibition than those of microbial origin.

Inhibitory effect of other triazole compounds on IGP dehydratases

The inhibitory effect of several triazole compounds on yeast IGP dehydratase activity was previously reported (Wiater *et al.*, 1971a) and it seemed of interest to compare plant IGP dehydratases with those of microbial origin in this respect. The purified preparations of barley, oats and yeast enzymes and the crude extract of *S. typhimurium* mutant *hisT1504* after Sephadex gel filtration were used. As it can be seen from Table 1, the two examined plant IGP dehydratases were more sensitive not only to aminotriazole but also to triazole and triazolealanine as compared with the microbial enzymes; β-aminoethyltriazole inhibited the yeast enzyme less than the other ones. Thiotriazole inhibited plant IGP dehydratases, but was

Table 1

Inhibition of IGP dehydratases from plants and microorganisms by aminotriazole and its analogues

The purified and concentrated plant and yeast IGP dehydratases (see Methods) and the crude desalted extract of constitutive strain *hisT1504* of *S. typhimurium* were incubated at pH 7.5, 7.2 and 8.0, respectively. NT stands for not tested.

Inhibitor	Inhibition of IGP dehydratase activity (%)			
	Barley	Oats	*Saccharomyces cerevisiae*	*Salmonella typhimurium*
Aminotriazole, 0.06 mM	53	43	17	21
Triazole, 0.6 mM	71	68	27	28
Triazolealanine, 0.4 mM	58	44	3	NT
Thiotriazole, 3.0 mM	47	22	0	0
β-Aminoethyltriazole, 2.5 mM	43	34	13	43
Diaminotriazole, 2.5 mM	0	0	0	NT
Dimethyltriazole, 2.5 mM	0	0	0	0

ineffective toward the microbial enzymes. It may be concluded that plant IGP dehydratases are more sensitive to the inhibitory effect of all triazole inhibitors tested than the enzymes of microbial origin.

Synergistic inhibitory action of aminotriazole and phosphate on barley IGP dehydratase

It was reported that phosphate and several divalent anions are weak competitive inhibitors of yeast IGP dehydratase (Kłopotowski & Wiater, 1965); the anions synergistically potentiated inhibition of the enzyme by triazole compounds (Wiater *et al.*, 1971a).

In preliminary experiments phosphate was found to inhibit competitively barley and oats IGP dehydratases; K_i value for phosphate was about 10 mM. The combined action of aminotriazole and phosphate was examined with purified barley and oats IGP dehydratase preparations (Fig. 3). The K_m value found with the barley enzyme was 0.3 mM, and the respective K_i values for aminotriazole and phosphate were 0.01 and 11 mM. The steepest line represents enzyme activities in the presence of both aminotriazole and phosphate. The slope increase due to simultaneous presence of both inhibitors exceeds 6 times the sum of the effects produced separately by aminotriazole and phosphate. This is in agreement with the results observed with yeast enzyme (Kłopotowski & Wiater, 1965), i.e. barley IGP dehydratase is inhibited synergistically by aminotriazole and phosphate. No evidence for such synergism was found with oats IGP dehydratase.

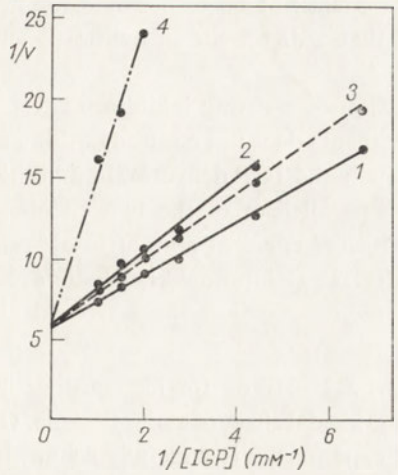

Fig. 3. Synergistic effect of aminotriazole and phosphate on barley IGP dehydratase activity. The Lineweaver-Burk plot in the absence of an inhibitor (*1*), and in the presence of: 0.004 mM-aminotriazole (*2*), 4.4 mM-potassium phosphate (*3*), and 0.004 mM-aminotriazole and 4.4 mM-potassium phosphate (*4*). K_m, 0.3 mM; K_i of aminotriazole, 0.01 mM; K_i of phosphate, 11 mM.

DISCUSSION

The presented results demonstrate that plant IGP dehydratases are more sensitive to aminotriazole and its analogues than the enzymes of microbial origin, although some differences among individual plant dehydratases were also observed.

Phosphate enhanced the inhibitory effect of triazole compounds on barley IGP dehydratase similarly as on yeast (Wiater *et al.*, 1971a) and *S. typhimurium* enzymes (A. Wiater, unpublished). The effect of phosphate and aminotriazole on oats enzyme was additive.

These results may be interesting from two points of view: heterogeneity of the enzymes and their role in the herbicidal action of aminotriazole. IGP dehydratases of different origin may constitute a set of isoenzymes with the same catalytic activity but with modified structure of active centre. In this connection the most prominent differences are: lack of synergistic action of aminotriazole and phosphate on oats IGP dehydratase activity and, secondly, susceptibility of plant, but not microbial, isoenzymes to thiotriazole. Apparently, the negative charge of thiol group enables an effective interaction of only some IGP dehydratases with triazole-specific site. On the other hand, these results may be connected with the herbicidal effect of aminotriazole. This compound is at least as effective as an inhibitor of barley IGP dehydratase as it is with the yeast enzyme. However, growth inhibitory effect of aminotriazole is easily reversible by exogenous histidine with yeast (Hilton, 1960; Kłopotowski, 1963) but not with plants (Hilton, 1969).

In a recent review on inhibitory effects of aminotriazole on histidine biosynthesis, purine, one-carbon and riboflavin metabolism, Hilton (1969) concluded that inhi-

bition of these pathways could not be responsible for herbicidal effect of aminotriazole. He emphasized that a direct site of aminotriazole action in higher plants was still to be detected.

Our direct demonstration of a strong inhibitory effect of aminotriazole on IGP dehydratases from higher plants does not contradict this statement. Our unpublished experiments on plant growth and IGP dehydratase inhibition by triazole derivatives indicate that there is no parallelism of the two effects. It means that structural requirements for the herbicidal effect of aminotriazole are different than those for inhibition of IGP dehydratase (Kłopotowski, Wiater & Kwiek, unpublished).

We wish to thank Dr. J. L. Hilton for his constant interest and valuable suggestions concerning the herbicidal effect of aminotriazole. Generous supply of triazole derivatives used in these experiments is gratefully acknowledged to Dr. R. G. Jones from Lilly Research Laboratories, Indianapolis, Ind., U.S.A. This work was supported in part by grant FC-Po-191 from the U.S. Dept. of Agriculture.

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SYNERGETYCZNE HAMOWANIE DEHYDRATAZY IMIDAZOLOGLICEROLOFOSFORANU PRZEZ AMINOTRIAZOL I FOSFORAN

Streszczenie

1. Stwierdzono, że dehydrataza IGP (EC 4.2.1.19) — enzym biosyntezy histydyny — z kiełków wyższych roślin, jęczmienia i owsa, jest bardziej wrażliwa na hamujące działanie 3-amino-1,2,4-triazolu oraz innych badanych pochodnych triazolu niż analogiczny enzym u drobnoustrojów.

2. Hamujący wpływ aminotriazolu na dehydratazę IGP z jęczmienia (ale nie z owsa) jest potęgowany przez aniony fosforanowe.

3. Omówiono znaczenie tych wyników dla ustalenia mechanizmu działania aminotriazolu na wzrost roślin wyższych.

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FRACTIONATION OF PROTEINS FROM 80 s RIBOSOMES OF RAT LIVER

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Preparative fractionation of proteins from 80 s ribosomes of rat liver, consisting of CM-cellulose chromatography and Sephadex G-100 filtration is described. This method permits isolation of three electrophoretically homogeneous components. Further nine components can be easily isolated in homogeneous state by direct extraction from electrophoretograms.

High heterogeneity of ribosomal proteins is the main difficulty in isolation of individual components in a pure state. So far the best results in the fractionation of these proteins were obtained by successive application of ion-exchange chromatography, gel filtration and preparative polyacrylamide-gel electrophoresis (Moore, Traut, Noller, Pearson & Delius, 1968; Wittmann, 1969). These experiments were carried out mainly with proteins from microbial ribosomes.

Recently, however, Westermann, Bielka & Böttger (1969) separated proteins from 80 s ribosomes of rat liver into 11 fractions by CM-cellulose chromatography and Biogel P 10 filtration. Two fractions were practically homogeneous on electrophoresis but the remaining ones were highly heterogeneous and presented difficulties in further separation.

In the present paper an alternative method of the preparative fractionation of proteins from 80 s ribosomes of rat liver is described. The method is based on the combined use of CM-cellulose chromatography and Sephadex G-100 filtration.

MATERIALS AND METHODS

Albino male Wistar rats (200 - 300 g) fasted for 24 hours were killed by decapitation. Livers were removed within 1 - 2 min after, washed with ice-cold 0.25 M-sucrose, dried with filter-paper and immediately used for preparation of ribosomes.

Preparation of ribosomes. Ribosomes were prepared by the modified procedure of Cohn & Simson (1963). For one experiment 10 livers were used.

The postmitochondrial supernatant (obtained by centrifugation of homogenate for 10 min at 14 000 *g*) was treated with sodium deoxycholate (final concentration

1%). The ribosomes were sedimented in a VAC-60 (H. Janetzki, Leipzig, G.D.R.) centrifuge for 90 min at 105 000 *g*, washed with buffered sucrose and recentrifuged.

Isolation of ribosomal proteins. Proteins were obtained according to Cohn & Simson (1963). A sample of ribosomes was diluted with 0.25 N-HCl to contain about 3 mg protein per 1 ml, and the mixture was incubated for 30 min at 0°C. The insoluble material was sedimented for 15 min at 15 000 *g* and reextracted under the same conditions with one-half previous volume of 0.25 N-HCl. The combined supernatants were dialysed overnight against 500 vol. of distilled water and lyophilized.

Disc electrophoresis. Polyacrylamide-gel electrophoresis was carried out by the method of Möller & Chrambach (1967) modified by the use of 6 mM-potassium-formate buffer, pH 3.5, containing 6 M-urea instead of 60 mM buffer used originally.

Gel rods were stained with 1% Amido Black in 7% acetic acid for 16 h and destained according to Johns (1967). Densitometric measurements were made by means of the ERI 65 densitograph (Carl Zeiss, Jena, G.D.R.), adapted for gel scanning.

CM-cellulose chromatography. This was carried out on a CM-cellulose column (17×90 mm) by the modified method of Waller (1964) and Moore *et al.* (1968) used for fractionation of *E. coli* ribosomal proteins. A sample containing 80 - 100 mg protein in 5 ml of 6 M-urea - 5 mM-sodium acetate buffer, pH 5.6, was applied to a column equilibrated with the same buffer. The protein was eluted by a linear gradient of this buffer, ranging from 5 to 500 mM. Concentration of the buffer increased at a rate of 1.3 µmol/ml. Samples of about 3.5 ml were collected at a flow rate of 4.5 - 6.0 ml/h/cm^2.

The separated proteins were precipitated with trichloroacetic acid (final concentration 20%) and transformed to their respective hydrochlorides according to Cohn & Simson (1963).

Sephadex G-100 filtration. This was performed on a 1.3×50 cm Sephadex G-100 column. A sample of 0.5 - 1.0 ml containing 5 - 15 mg protein in 1 M-acetic acid was applied to a column equilibrated with the same solution. The column was eluted with 1 M-acetic acid at a flow rate of 12 ml/h. The collected peak fractions were pooled and evaporated in vacuum over KOH. The CM-cellulose and Sephadex fractions were characterized by electrophoresis; the respective components were identified by comparing the electrophoretic profile of the separated fraction with that of the initial preparation enriched with this particular fraction.

Designation of the chromatographic fractions and electrophoretic bands. Electrophoretic bands were numbered with Arabic numerals according to their electrophoretic mobility. The successive CM-cellulose fractions were designated with Roman numerals and the subfractions obtained therefrom on Sephadex were lettered correspondingly. The components of the same electrophoretic mobility recovered in different chromatographic fractions were denoted by additional lettering.

Protein was estimated either by the method of Lowry *et al.* (1951) or by measuring the extinction at 280 nm.

Reagents. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, sodium persulphate and CM-cellulose were purchased from Serva

(Heidelberg, West Germany); Sephadex G-100, from Pharmacia (Uppsala, Sweden); Dowex 1 X8 (200 - 400 mesh) and Dowex 50 WX4 (200 - 400 mesh), from Calbiochem Inc. (Los Angeles, Calif., U.S.A.). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland). Urea solutions used for electrophoresis and chromatography were previously deionized on Dowex 1 X8 (OH form) and Dowex WX4 (H form) columns.

RESULTS AND DISCUSSION

The electrophoretogram and the respective densitogram of proteins from 80 s ribosomes of rat liver show 15 distinct bands, which are numbered according to the order of their electrophoretic mobility (Fig. 1). CM-cellulose chromatography was the first step in preparative separation of ribosomal proteins. By this procedure 97% of protein isolated from rat liver ribosomes was eluted from CM-cellulose column with the increasing concentration (5 - 500 mM) of sodium acetate buffer, pH 5.6, containing 6 M-urea, at a gradient rate of 1.3 μmol/ml.

The obtained elution profile is shown in Fig. 2. As it may be seen, the total proteins isolated from 80 s ribosomes of rat liver were separated into nine distinct fractions, designated *I* - *IX*. Distribution of protein in the isolated fractions, expressed as percentage of total protein, is presented in Table 1.

The densitograms of the electrophoretic patterns of proteins from chromatographic fractions *II* - *IX* are shown in Fig 3; fraction *I* gave on the densitogram

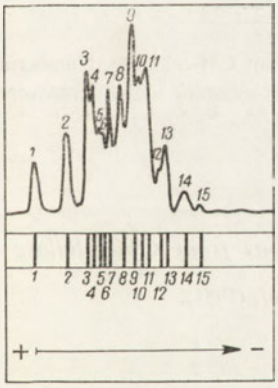

Fig. 1

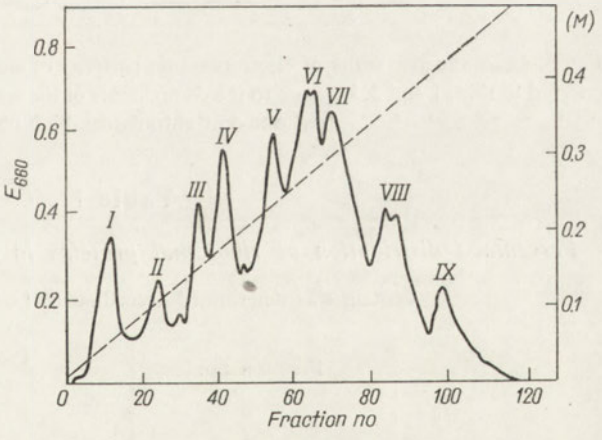

Fig. 2

Fig. 1. Electrophoretic pattern and densitometric scan of total proteins of 80 s ribosomes from rat liver. The electrophoresis was performed at 125 V for 2.5 h. The gels were stained with Amido Black.

Fig. 2. Elution diagram of proteins from 80 s ribosomes of rat liver on CM-cellulose. ——, E_{660}; - - -, concentration of sodium acetate buffer, pH 5.6, containing 6 M-urea. Flow rate 4.5 - 6.0 ml/h/cm² of column. Fraction volume 3.5 ml.

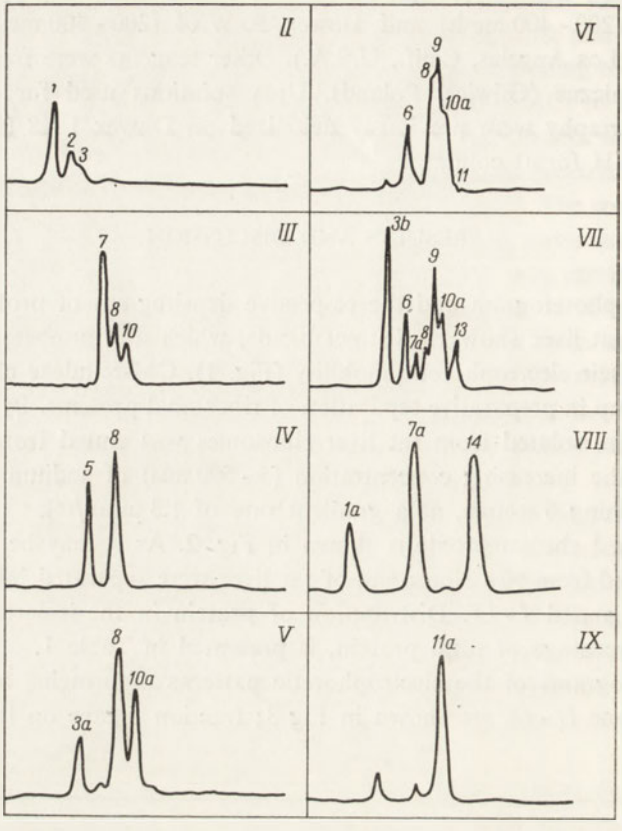

Fig. 3. Densitometric scans of electrophoretic patterns of eluates from CM-cellulose. Numbering referred to Figs. 1 and 2, lettering to the components of the same electrophoretic mobility recovered in different chromatographic fractions.

Table 1

Percentage distribution of ribosomal proteins in fractions from CM-cellulose

Protein was determined according to Lowry *et al.* (1951).

Fraction no.	Protein (%)
I	9.7
II	5.1
III	6.3
IV	9.2
V	15.6
VI	24.2
VII	17.3
VIII	9.3
IX	3.3

diffuse bands, difficult to identify, probably due to RNA contamination. Further chromatographic fractions were practically devoid of RNA. The unfractionated, initial ribosomal protein preparation contained up to 2.5% RNA. Fraction *IX* showed practically one (*11a*) band; fraction *IV*, two (*5* and *8*) and fractions *II*, *III*, *V* and *VIII*, three bands each. The most heterogeneous were fractions *VI* and *VII*.

It is conspicuous from the above that there is no correlation between the sequence of elution and electrophoretic mobility of proteins, thus the proteins with the same electrophoretic mobility were separated by CM-cellulose chromatography. For instance proteins of band *3* were found in fractions *II (3)*, *V (3a)* and *VII (3b)*, and band *7* in fractions *III (7)* and *VIII (7a)*.

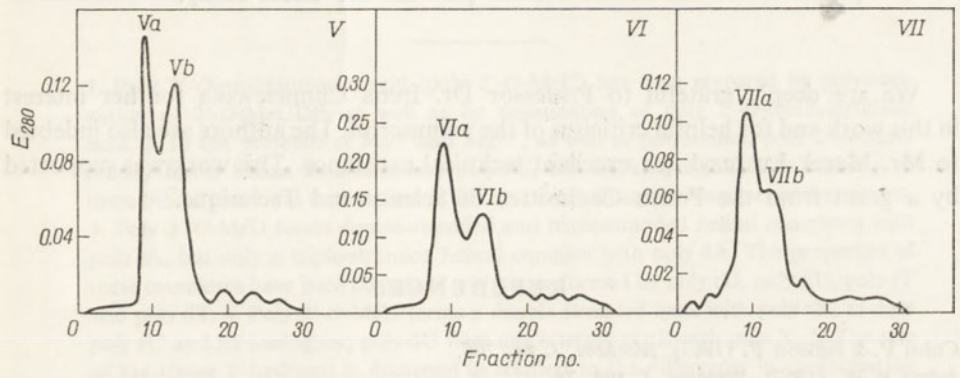

Fig. 4. Gel filtration on Sephadex G-100 of the CM-cellulose fractions. Flow rate 12 ml/h. Fraction volume 1.7 ml.

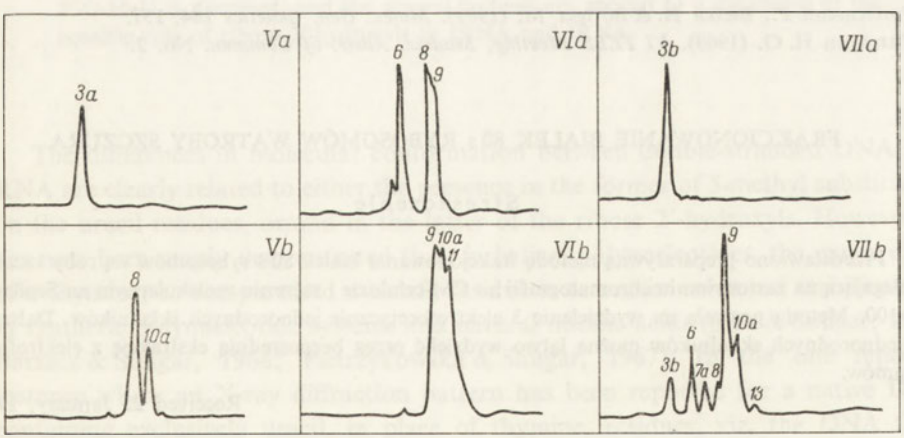

Fig. 5. Densitometric scans of electrophoretic patterns of Sephadex G-100 fractions. Designation of components as in Fig. 3.

The proteins of fractions *V*, *VI* and *VII* could be further fractionated on Sephadex G-100. The elution profiles obtained from gel filtration and densitometric scans of electrophoretic patterns of the isolated subfractions are shown in Figs. 4 and 5, respectively. The use of Sephadex G-100 resulted in separation of two electrophoretically homogeneous components (*3a* and *3b*).

The described method enables separation of proteins from 80 s ribosomes of rat liver into 12 fractions on preparative scale. Three of them (*Va*, *VIIa* and *IX*) contained proteins which on electrophoresis were practically homogeneous (*3a*, *3b* and *11a*). Other five fractions (*II*, *III*, *IV*, *Vb*, *VIa* and *VIII*) are composed of two or three electrophoretically separable components. Nine of these components can be easily isolated in homogeneous state by direct extraction from electrophoretograms: component *1*, from fraction *II; 7*, from fraction *III; 5* and *8*, from fraction *IV; 10a*, from fraction *Vb; 6* from fraction *VIa;* and *1b*, *7a* and *14*, from fraction *VIII*.

The properties of isolated ribosomal proteins are under study.

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FRAKCJONOWANIE BIAŁEK 80 s RYBOSOMÓW WĄTROBY SZCZURA

Streszczenie

Przedstawiono preparatywną metodę frakcjonowania białek 80 s rybosomów wątroby szczura, polegającą na zastosowaniu chromatografii na CM-celulozie i sączeniu molekularnym na Sephadex G-100. Metoda pozwala na wydzielenie 3 elektroforetycznie jednorodnych składników. Dalszych 9 jednorodnych składników można łatwo wydzielić przez bezpośrednią ekstrakcję z elektroforogramów.

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ROLE OF THE 2′-HYDROXYL IN POLYNUCLEOTIDE CONFORMATION: POLY 2′-*O*-METHYLURIDYLIC ACID

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1. Poly 2′-*O*-methyluridylic acid (poly 2′-*O*-MeU) has been prepared by polymerization of 2′-*O*-MeUDP, as well as by deamination of poly 2′-*O*-methylcytidylic acid. 2. In the presence of Na^+ and Mg^{2+}, as well as polyamines, poly 2′-*O*-MeU readily forms helical structures like poly rU, but unlike poly dU. The stability of these helical structures was studied as a function of salt and polyamine concentrations. 3. Poly 2′-*O*-MeU forms double-stranded and triple-stranded helical complexes with poly rA, but only a triple-stranded helical complex with poly dA. The properties of these complexes have been compared with those formed by poly rU, poly dU, poly rT and poly dT. 4. Poly 2′-*O*-MeU forms a double-stranded helix with poly rX, as does poly rU and its analogues; poly dU does not interact at all with poly X. 5. The role of the ribose 2′-hydroxyl is discussed in relation to the differences in conformation between analogous ribo and deoxyribo polynucleotides, and double-stranded RNA and DNA. The experimental results unequivocally demonstrate that these differences are unrelated to any involvement of the 2′-hydroxyl as a donor in *intra*molecular hydrogen bonding. 6. The role of the 2′-*O*-Me on the stability of helical poly 2′-O-MeU is discussed, and the general findings are applied to a discussion of the possible role of ribose 2′-hydroxyls in rRNA and tRNA.

The differences in molecular conformation between double-stranded DNA and RNA are clearly related to either the presence in the former of 5-methyl substituents on the uracil residues, or/and in the latter of the ribose 2′-hydroxyls. However, it has now been amply demonstrated that, in helical polynucleotides, the major effect of a thymine, as compared to a uracil, residue is to confer enhanced stability both in synthetic polynucleotide systems and natural nucleic acids (Szer & Shugar, 1966; Barszcz & Shugar, 1968; Pietrzykowska & Shugar, 1967). In the one reported instance where an X-ray diffraction pattern has been reported for a native DNA containing exclusively uracil, in place of thymine, residues, viz. the DNA from bacteriophage PBS-1 (Langridge & Marmur, 1964), no major differences in conformation could be detected as compared to the analogous thymine-containing DNA.

Consequently the differences in conformation between double-stranded DNA and RNA may be ascribed to the presence of the 2′-hydroxyls in the latter. Numerous efforts have been devoted to clarifying the role of the 2′-hydroxyl, most frequently through the use of model oligo and polynucleotide systems; and it was generally concluded that the 2′-hydroxyl in double-stranded RNA forms an intramolecular hydrogen bond to the 2-carbonyl of a pyrimidine, the ring $N_{(3)}$ of a purine, or to a neighbouring phosphate oxygen. All of these approaches, however, provided results open to different interpretations (e.g. Ts'o, Rapaport & Bollum, 1966; Adler, Grossman & Fasman, 1968; Maurizot, Wechter, Brahms & Sadron, 1968; Żmudzka, Bollum & Shugar, 1969a). Subsequently the synthesis, with the aid of polynucleotide phosphorylase, of poly 2′-*O*-MeA[1] (Rottman & Heinlein, 1968) and poly 2′-*O*-MeC (Janion, Żmudzka & Shugar, 1970), followed by studies of their conformations by optical methods (Bobst, Rottman & Cerutti, 1969; Żmudzka Janion & Shugar, 1969b), demonstrated unequivocally that both the single-stranded and twin-helical forms of these polymers, as well as their helical complexes with complementary polynucleotides, closely resembled those for the corresponding poly rA and poly rC, respectively, and not those for poly dA and poly dC. It follows that the differences in conformation between poly rA and poly dA on the one hand, and poly rC and poly dC on the other, are not due to involvement of the 2′-OH of the ribopolymers as a hydrogen bond donor to an adjacent base residue or a phosphate oxygen.

A model system which might be expected to even more strikingly illustrate the differences between ribo and deoxyribo polymers is poly rU and poly dU. The latter has been found incapable of forming any helical structure under a variety of conditions where poly rU readily does so (Żmudzka *et al.*, 1969a), and it would appear almost logical to conclude that the 2′-OH in poly rU in some way forms an intramolecular hydrogen bond to give the helical structure which is not observed with poly dU. It was therefore decided to prepare poly 2′-*O*-MeU, in which the 2′-hydroxyls are blocked; and it was shown that this polymer readily formed a helical structure similar to that for poly rU (Żmudzka & Shugar, 1970).

The present communication is devoted to a more detailed description of the properties of poly 2′-*O*-MeU and its helical complexes with other homopolymers.

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[1] The following abbreviations are used in this text: 2′-OH, ribose 2′-hydroxyl; 2′-*O*-MeU, 2′-*O*-methyluridine; 2′-*O*-MeC, 2′-*O*-methylcytidine; 2′-*O*-MeA, 2′-*O*-methyladenosine; 2′-*O*--MeUDP, 2′-*O*-methyluridine-5′-pyrophosphate, and similarly for 2′-*O*-MeCDP and 2′-*O*-MeADP; poly rU, polyribouridylic acid; poly rT, polyribothymidylic acid; poly rA, polyriboadenylic acid; poly 5-EtU, poly-5-ethylribouridylic acid; poly rX, polyriboxanthylic acid; poly dU, poly dT, poly dA, etc. are the corresponding deoxy polymers. Poly 2′-*O*-MeU, poly 2′-*O*-methyluridylic acid; poly 2′-*O*-MeC, poly 2′-*O*-methylcytidylic acid; poly 2′-*O*-MeA, poly 2′-*O*-methyladenylic acid. A double-stranded helical complex, such as that formed between poly rA and poly 2′-*O*-MeU is denoted by poly rA:2′-*O*-MeU; the corresponding triple-stranded complex would be poly rA: :2(2′-*O*-MeU), and similar connotations for other complexes. The symbol (3→1) refers to a transition from a triple-stranded helix to homopolymers, (3→2) to the dismutation of a triple-strand to a double--strand, etc.

EXPERIMENTAL

Materials. ADP was obtained from Schwarz Biochemicals (Orangeburg, N.Y., U.S.A.) and XDP from Waldhof Zellstoffabrik (Mannheim, German Federal Republic).

Poly rA was prepared by polymerization of ADP with *E. coli* polynucleotide phosphorylase (Kimhi & Littauer, 1968). The polymer product, according to Hennage, Crothers & Ludlum (1969) was not exposed to distilled water; it possessed an ε_{260} of 10×10^3 in 0.2 M-Na^+ at pH 7.8. Poly rX was prepared from XDP with the *Micrococcus lysodeikticus* enzyme (Matthaei *et al.*, 1967) under conditions as described by Fikus & Shugar (1969). The product of polymerization was likewise not exposed to distilled water and exhibited an ε_{248} of 8.1×10^3 in 0.1 M-Na^+ at pH 7.

Poly dA and poly dU were polymerized from dATP and dUTP with the aid of deoxynucleotidyl transferase (Bollum, 1966; Żmudzka *et al.*, 1969a) and, in the case of poly dU, also by deamination of poly dC (Żmudzka *et al.*, 1969a). The ε_{260} values for poly dA and poly dU were, respectively, 9.65×10^3 and 10.0×10^3 in 10^{-3} M-tris buffer at pH 8.0 and 7.0. Poly 2′-*O*-MeC, prepared as previously described (Janion *et al.*, 1970), exhibited an ε_{267} of 7.14×10^3 in 10^{-3} M-tris buffer pH 8.0.

Polymerization of 2′-O-MeUDP. 2′-*O*-MeUDP was obtained by deamination of 10 mg of 2′-*O*-MeCDP with sodium nitrite in acetic acid at 37°C for 2.5 hours, followed by chromatography on a DEAE-cellulose column. Polymerization conditions were as follows: 0.7 μmole 2′-*O*-MeUDP, 30 μl of 0.5 M-tris buffer, pH 8.5, 40 μl of 0.025 M-$MnSO_4$, 4 μl 0.01 M-Na-EDTA, 10 μl 0.01 M-NaH_3, 4 μl *M. lysodeikticus* polynucleotide phosphorylase, and water to a total volume of 100 μl. Incubation was at 37°C and about 40% of the substrate was incorporated into polymer in 42 hours. The course of polymerization was followed by paper chromatography, based on disappearance of substrate and the appearance of UV-absorbing material at the start, using as solvent isopropanol - 1% ammonium sulphate (6:4, v/v).

Poly 2′-O-MeU by deamination of poly 2′-O-MeC. Poly 2′-*O*-MeC (1.5 μM) was dissolved in 150 μl water, followed by addition of 41 mg sodium nitrite and 43 μl acetic acid. The mixture was warmed on a water bath for 13 min at 80°C, after initial trials had demonstrated that 18 hours at 30° and 15 min at 60° did not lead to complete deamination. Following deamination, the product was dialysed at 4°C successively against 0.1 M-NaCl, 0.1 M-NaCl and 0.01 M-EDTA, 0.01 M-NaCl and 0.001 M-EDTA, then twice against water, and finally lyophilized and stored at -14°C. The sedimentation constant of poly-2′-*O*-MeU was $S_{20,w}$ 9.0 in 0.01 M-NaCl and 0.005 M-phosphate buffer, pH 7.8. The preparation had a λ_{max} at 259 nm, λ_{min} at 231 nm, and an ε_{259} of 9.56×10^3 and an ε_{260} of 9.54×10^3 in 0.1 mM-cacodylate buffer, pH 7.0. Calculation of molar extinction was based

on measurment of the extinction of an enzymic hydrolysate of the polymer to nucleosides, using an ε_{263} value for 2′-*O*-MeU of 10.1×10^3 in 95% ethanol (Martin, Reese & Stephenson, 1968).

Conditions for enzymic hydrolysis of poly 2′-*O*-MeU were as follows: 0.1 M-tris buffer, pH 9, 0.015 M-$MgCl_2$, 0.1 mg snake venom phosphodiesterase, 0.01 mg micrococcal nuclease, 0.1 mg *E. coli* alkaline phosphatase, 0.055 μmole polymer, in a total volume of 1 ml, in a teflon-stoppered 10-mm spectro cuvette at 37°C until no further change in optical density could be observed. The spectrum of the polymer solution was initially measured at pH 7 and, following hydrolysis, the solution neutralized once more to pH 7 before spectral measurement.

Methods. All spectral and pH measurements, including temperature profiles, were carried out as elsewhere described (Żmudzka *et al.*, 1969a). Salt concentrations of the various solutions, as presented below, include the buffer cations. No corrections were made for thermal expansion of the solutions. Sedimentation constants were measured on a Beckman Model E ultracentrifuge fitted with ultraviolet absorption optics.

RESULTS

Influence of Na^+ on poly 2′-O-MeU secondary structure. The influence of the monovalent cation Na^+ on the conformation of poly 2′-*O*-MeU at neutral pH is exhibited in Fig. 1a. It will be seen that, for Na^+ concentrations up to 0.05 M, there is only a small and non-cooperative increase in hyperchromicity with increasing temperature. At 0.1 M-Na^+ the profile is reminiscent of a helix-coil transition, but only a portion of the transition is placed in evidence, nor would it be feasible to obtain the complete profile since, at such a salt concentration, the solution would

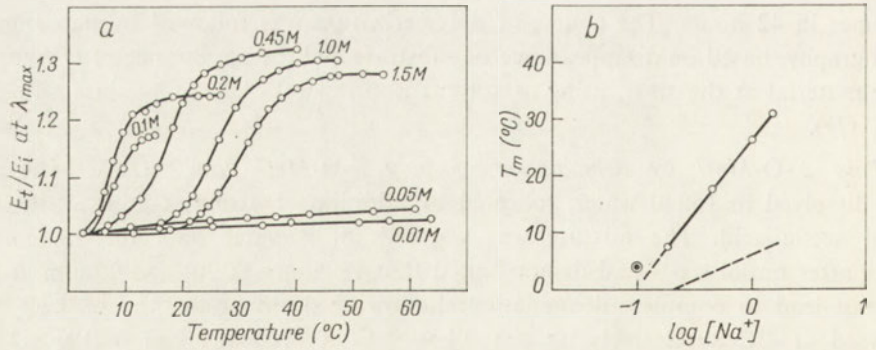

Fig. 1. (*a*) Temperature transition profiles in neutral unbuffered medium for poly 2′-*O*-MeU in the presence of indicated concentrations of NaCl. (*b*) Dependence of the T_m for poly 2′-*O*-MeU on the logarithm of the NaCl concentration. The point enclosed in a circle is for 0.1 M-NaCl, for which the profile in (*a*) is too incomplete to permit of more than a rough estimate of its value. The dashed line is for poly rU, estimated from literature data (see text).

solidify at a temperature not much below 0°C. It is only at Na^+ concentrations above about 0.45 M that a complete helix-coil transition can be observed. Furthermore the profiles are all relatively broad.

Figure 1b demonstrates the dependence of the T_m for poly 2′-*O*-MeU on Na^+ concentration. For rough comparison purposes, the same figure shows the approximate dependence of T_m on Na^+ concentration for poly rU, using approximate values taken from the papers of Richards, Flessel & Fresco (1963) and Michelson & Monny (1966). This point is discussed further in the Discussion (below).

*Secondary structure of poly 2′-*O*-MeU in the presence of* Mg^{2+} *cations.* It was previously reported that, at room temperature, the concentration of Mg^{2+} required to induce the formation of secondary structure in poly 2′-*O*-MeU was about 0.1 M, as compared to 0.01 M for poly rU under the same conditions (Żmudzka & Shugar, 1970). These findings have now been extended to lower temperatures to provide a more realistic comparison with poly rU.

With an appropriate decrease in temperature of the solutions of poly 2′-*O*-MeU, it was in fact observed that maximal ordered structure is obtained for Mg^{2+} concentrations as low as 10^{-4} M, hence at a concentration even lower than that for poly rU. The transition profiles for the ordered form of poly 2′-*O*-MeU at various concentrations of Mg^{2+} are exhibited in Fig. 2a. Note in particular that the temperature hyperchromicity is about 33%, i.e. almost identical with that observed for poly rU. Furthermore, with increasing concentrations of Mg^{2+}, the breadth of the profiles increases, although the overall hyperchromicity remains the same. Analogous observations have been previously reported for poly rT (Szer, 1966).

Disregarding the breadths of the temperature profiles, Fig. 2b displays graphically the dependence of the T_m values for poly rU (Szer, 1966) and poly 2′-*O*-MeU on the Mg^{2+} cation concentration.

*Influence of polyamines on poly 2′-*O*-MeU secondary structure.* From Fig. 3a it will be seen that the helix-coil transition of poly 2′-*O*-MeU is considerably more pronounced in the presence of such polyamines as spermidine and spermine than

Table 1

Influence of spermidine and spermine on T_m *values for the helix-coil transitions in poly rU and poly 2′-*O*-MeU in, respectively, water and 0.005* M*-phosphate buffer,* pH *7.4*

Data for poly rU after Szer (1966).

	Equivalents of			
	Spermidine		Spermine	
	1	10	1	10
	T_m values (°C)			
Poly rU	24	26.5	28	*
Poly 2′-*O*-MeU	23	39	39	54.5

* Not measureable because of precipitation.

in the case of mono- or divalent cations. Even in the presence of 1.0 equivalent of spermidine, or of spermine, the profiles are fairly narrow with T_m values of 23° and 39°C, respectively (Table 1), hence considerably more elevated than in the presence of the divalent Mg^{2+} cation, while the overall hyperchromicity of the temperature profile approaches a maximum of 40% as compared to a little more than 30% in the presence of Mg^{2+}. On addition of 10 mole equivalent of each of the polyamines, the T_m values for the helix-coil transitions undergo a marked increase, to 39°C in the presence of spermidine, and 54°C with spermine.

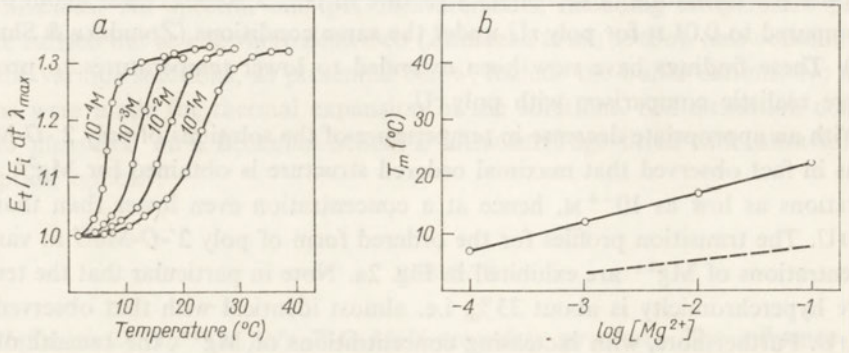

Fig. 2. (*a*) Temperature transition profiles in neutral unbuffered medium for poly 2′-*O*-MeU in the presence of indicated concentrations of $MgCl_2$. (*b*) Dependence of the T_m on the logarithm of the $MgCl_2$ concentration for ——, poly 2′-*O*-MeU and — — —, poly rU (data from Szer, 1966.)

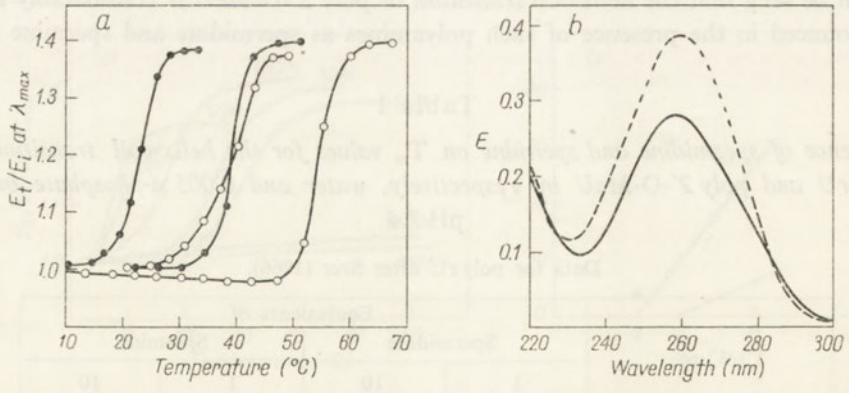

Fig. 3. (*a*) Absorbance-temperature profiles for poly 2′-*O*-MeU, 0.035 μmole/ml, in 0.005 M-phosphate buffer, pH 7.4, in the presence of 1.0 (left) and 10 (right) equivalent of spermidine (●-●-●) and of spermine (○-○-○). (*b*) Absorption spectrum of poly 2′-*O*-MeU in the presence of 1.0 equivalent of spermidine in 0.005 M-phosphate buffer, pH 7.4, in the helix form (——) at 10°C, and in the coil form (— — —) at 40°C.

Figure 3b in turn exhibits the absorption spectra of the helical and coil forms, respectively, of poly 2′-*O*-MeU in the presence of 1.0 equivalent of spermidine.

It should be recalled, in relation to the foregoing, that polyamines, like mono- and divalent cations, are without any measurable effect on poly dU (Żmudzka *et al.*, 1969a).

Complexes of poly 2′-O-MeU with poly rA. Initial experiments designed to examine possible complex formation between poly 2′-*O*-MeU and poly rA were carried out in phosphate buffered medium, pH 7.8, and with an Na^+ concentration of 0.03 M, since under these conditions poly rU and poly rA are known to form triple-stranded complexes at temperatures below 35°C and double-stranded complexes above this temperature (Stevens & Felsenfeld, 1964). In effect, mixing of poly rA with poly 2′-*O*-MeU under the foregoing conditions was accompanied by the appearance of appreciable hypochromicity, testifying to formation of some complex, at a rate too rapid to permit of measurement.

To determine the nature of the complex(es) formed, a standard mixing curve was carried out, involving variation of the ratio of poly rA to poly 2′-*O*-MeU from 1:1 to 1:2, in the presence of 0.03 M-Na^+, and measurement of the hypochromicity of the mixtures at 260 nm, at room temperature, as for poly rA and poly rU. The results pointed to formation under these conditions of a double-stranded complex. Because of the limited amount of poly 2′-*O*-MeU available, it did not prove feasible to extend the mixing curve experiments to solutions of higher ionic strength. Recourse was then had to a detailed analysis of melting profiles under various conditions.

The influence of the ionic strength of the medium on the nature of the complexes was then examined by running temperature profiles on 1:1 and 1:2 mixtures of poly rA to poly 2′-*O*-MeU in the presence of increasing concentrations of Na^+. The overall findings are illustrated in Fig. 4. It will be noted, from the upper portions of Figs. 4a and 4b, that in the presence of 0.03 M-Na^+ the 1:1 solution exhibits a temperature hyperchromicity, E_t/E_l, of 1.46, whereas the hyperchromicity for the 1:2 mixture is 1.30, thus independently supporting the mixing curve results for formation in the presence of 0.03 M-Na^+ of a unique double-stranded complex.

Variation of the Na^+ concentration of the 1:1 mixture over the range 0.02 M to 3.0 M did not lead to any modifications of the transition profiles, but only an increase in T_m values (Fig. 4a and Fig. 6), pointing to formation of only the double-stranded helix.

The situation was completely analogous in 1:2 mixtures in the presence of Na^+ concentrations up to about 0.4 M. Above this salt concentration, melting was found to proceed in two stages (Fig. 4b), the second of which, at increasing temperatures, was practically identical with that for the single-step melting of double-stranded helices. The first melting stage in Fig. 4b at Na^+ concentrations above 0.4 M clearly corresponds to the transition from a triple to a double-stranded helix. This is supported by the slight increase in absorption over the temperature range 10 - 30°C for the profile at 280 nm in Fig. 4b; and by the fact that the second melting stage (upper portion of Fig. 4b) is accompanied by hyperchromicity which, when re-

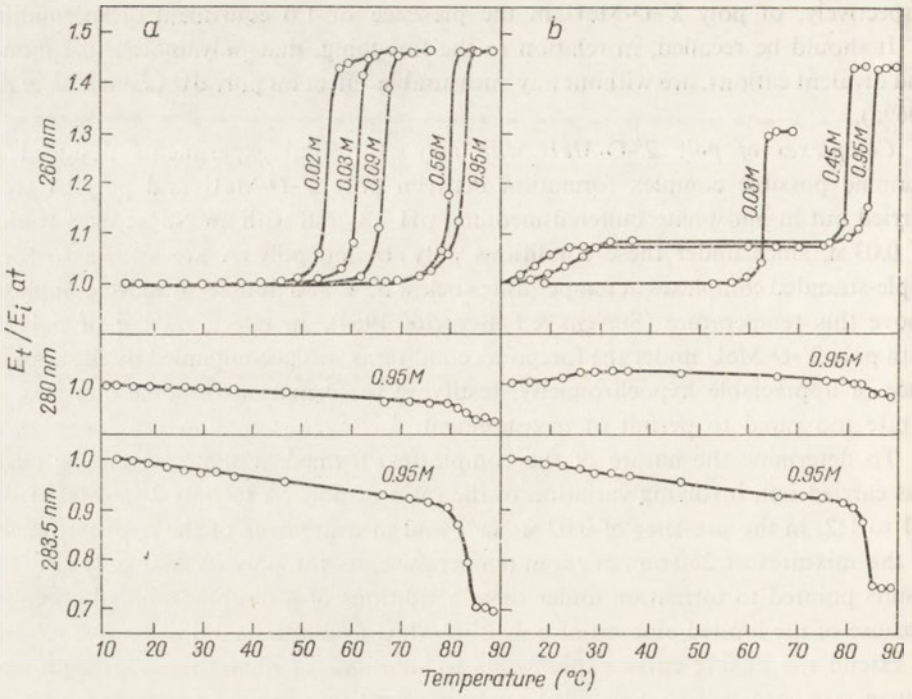

Fig 4. Absorbance-temperature profiles at 260, 280 and 283.5 nm for (*a*) 1:1, and (*b*) 1:2 mixtures of poly rA and poly 2′-*O*-MeU in 0.01 M-phosphate buffer, pH 7.8, and at indicated molarities of NaCl.

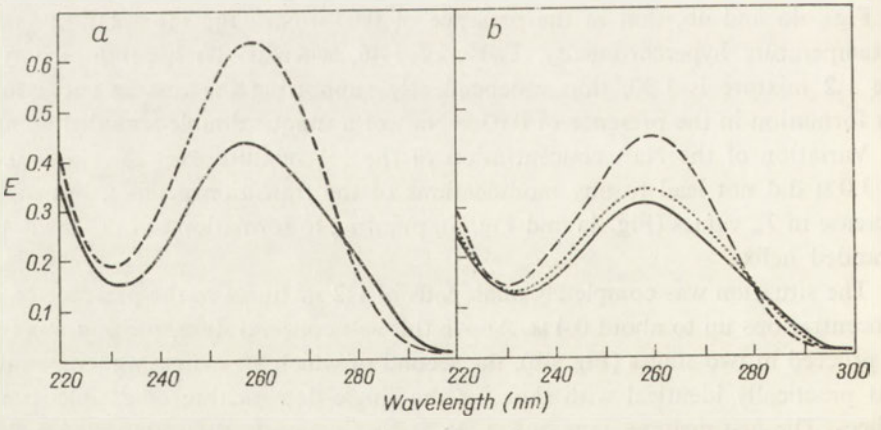

Fig. 5. Absorption spectra of (*a*) 1:1 and (*b*) 1:2 mixtures of poly rA and 2′-*O*-MeU in 0.01 M-phosphate buffer, pH 7.8, and 0.95 M-NaCl: (*a*) ——, double-stranded helix, at 10°C; — — —, homopolynucleotides, at 89°C; (*b*) ——, triple-stranded helix at 10°C; ··· double-stranded helix + free poly 2′-*O*-MeU, at 47°C; — — — homopolymers, at 89°C.

calculated for the excess free poly rA present in the solution, leads to a melting profile identical with the corresponding profile for a 1:1 mixture in Fig. 4a. Under no circumstances was there any evidence for melting of a triple-stranded helix directly to homopolymers without intermediate formation of a double strand.

Typical absorption spectra for the helical and coil forms of the double-stranded poly rA:2′-*O*-MeU and the triple-stranded poly rA:2(2′-*O*-MeU) are exhibited in Fig. 5. Note again the pronounced *hyper*chromicity for the helical forms (with respect to the mixtures of homopolymers) to the red of 280 nm.

Figure 6 exhibits graphically the dependence of T_m on Na^+ concentration for complexes of poly rA with poly 2′-*O*-MeU, and for several other poly U analogues for purposes of comparison.

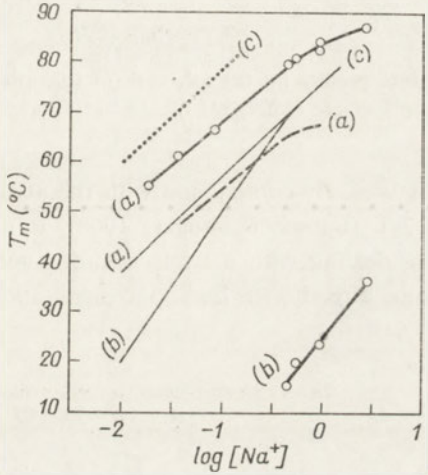

Fig. 6. Dependence of T_m on log NaCl for complexes with poly rA of various poly U analogues, as indicated in figure, in 0.01 M-phosphate buffer, pH 7.8: O-O-O, poly 2′-*O*-MeU; ——, poly U; — — —, poly dU and ··· poly rT: (*a*) melting of double helix to homopolymers; (*b*) dismutation of triple to double-stranded helix; (*c*) melting of triple-stranded helix to homopolymers. Data for poly rU from Stevens & Felsenfeld (1964), for poly dU from Żmudzka *et al.* (1969a), and for poly rT from Barszcz & Shugar (1968).

Complexes of poly 2′-O-MeU with poly dA. Mixtures of poly dA with poly 2′-*O*-MeU likewise exhibited hypochromicity which manifested itself immediately, testifying to very rapid formation of complexes. The temperature profiles of 1:1 and 1:2 mixtures, at various ionic strengths (Fig. 7) are fairly unequivocal. The 1:2 mixtures (Fig. 7b) exhibit reasonably sharp transition profiles over a wide range of Na^+ concentrations; at each salt concentration, melting commences only a few degrees below the corresponding T_m value, and the melting profile exhibits a single step. On the other hand, the 1:1 mixtures (Fig. 7a) initially exhibit slow non-cooperative melting (corresponding to the non-cooperative melting of some free poly dA) followed eventually by relatively sharp profiles with T_m values identical to those

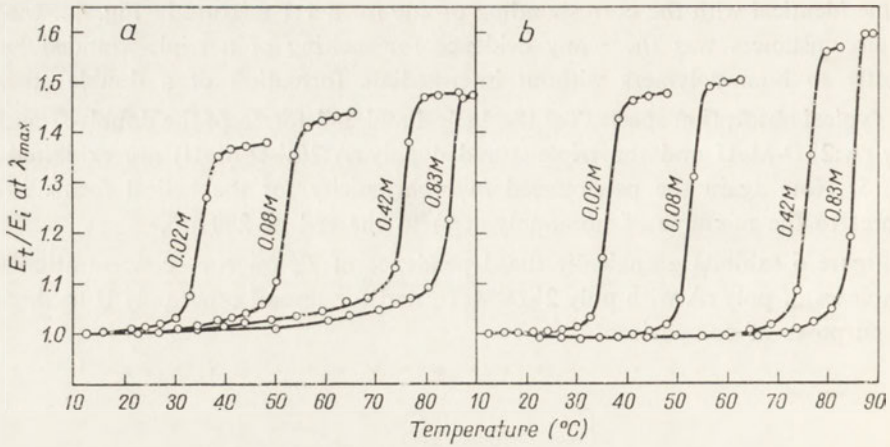

Fig. 7. Absorbance-temperature profiles for (*a*) 1:1, and (*b*) 1:2, mixtures of poly dA and poly 2′-*O*-MeU in 0.01 M-phosphate buffer, pH 7.8, and at indicated NaCl molarities.

observed for the 1:2 mixtures. By comparison with the analogous situation for the triple-stranded poly rA:2rT (Barszcz & Shugar, 1968), it may be concluded that in both instances we are dealing with a triple-stranded complex, poly dA:2(2′-*O*-MeU), which melts out directly to homopolymers and without intermediate

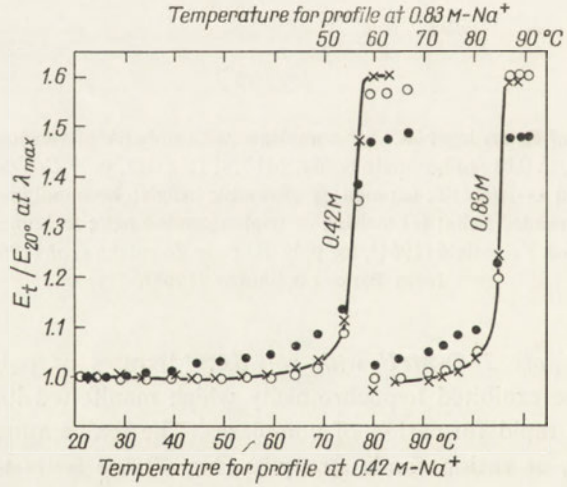

Fig. 8. Temperature profiles of 1:1 (●-●-●) and 1:2 (○-○-○) mixtures of poly dA and poly 2′-*O*-MeU, transposed from Fig. 7. Assuming the transition of the 1:1 mixture to be due to the dismutation reaction poly dA:2′-*O*-MeU→1/2 poly dA:2 (2′-*O*-MeU)+1/2 poly dA, the profile has been recalculated by subtracting the absorption of 1/2 poly dA at each temperature, to give the profile denoted by (×-×-×). Note that this corrected profile is essentially identical to that for the 1:2 mixture.

formation of a double-stranded helix. If this is indeed so, the melting profiles for the 1:1 mixtures must represent the following process:

$$1/2 \cdot \text{poly dA:2(2'-}O\text{-MeU)} + 1/2 \cdot \text{poly dA} \rightarrow \text{poly dA} + \text{poly 2'-}O\text{-MeU}$$

Consequently if, from each point on one of the profiles in Fig. 8a, we subtract the appropriate (at that temperature) absorption for 1/2·poly dA, we should obtain the corresponding profile in Fig. 8 for the triple-stranded helix-coil transition. This has been done for the transition profile presented in Fig. 7a at an Na^+ concentration of 0.42 M, with results shown in Fig. 8. The corrected profile is indeed in reasonably good correspondence with that for the 1:2 mixture at 0.42 M-Na^+ in Fig. 7b.

Figure 9 presents the absorption spectra of the helical and coil forms of poly dA: 2(2'-*O*-MeU) in the presence of 0.42 M-Na^+; and Fig. 10 the dependence of T_m values on Na^+ concentration for triple-stranded complexes with poly dA of poly 2'-*O*-MeU and several other poly U analogues.

Complexes of poly 2'-O-MeU with poly rX. It has elsewhere been shown that various poly rU analogues (poly rU, poly rT, poly 5-EtU, poly FU) readily form double-stranded helices with poly rX, but that neither poly dT nor poly dU exhibit any evidence of interaction (Fikus & Shugar, 1969). It is consequently of considerable interest to examine the behaviour of poly 2'-*O*-MeU.

Mixing of solutions of poly rX with poly 2'-*O*-MeU was accompanied by the rapid appearance of hypochromicity with little, or no, detectable time lag. A typical

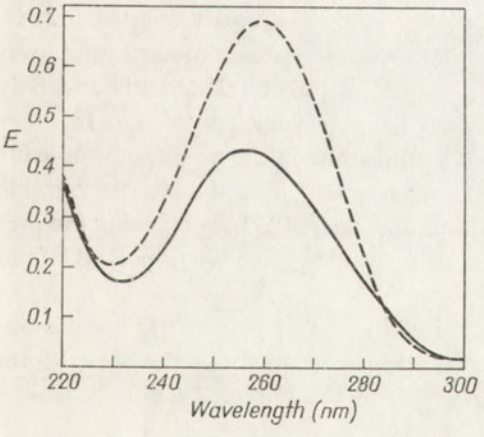

Fig. 9

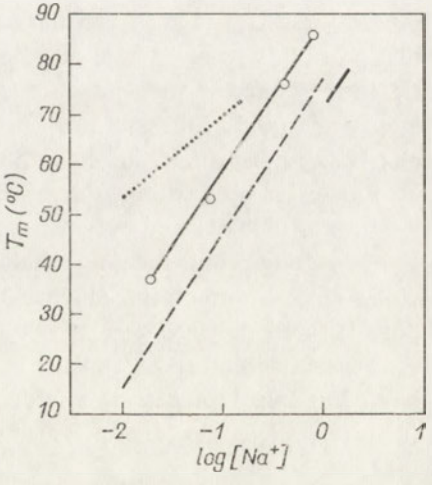

Fig. 10

Fig. 9. Absorption spectrum of poly dA:2(2'-*O*-MeU) in 0.01 M-phosphate buffer, pH 7.8, and 0.42 M-NaCl at 18°C [helical form, (——)] and at 85°C [homopolymers, (— — —)].

Fig. 10. Dependence of T_m value on log NaCl concentration, for the helix-coil transitions of triple-stranded complexes of poly dA with poly U analogues to homopolymers; phosphate buffer, pH 7.8; O-O-O, complex with poly 2'-*O*-MeU; — — —, with poly rU; ——, poly dU, and ···, poly rT. Data for poly rU and poly dU from Żmudzka *et al.* (1969a) and for poly rT from Barszcz & Shugar (1968).

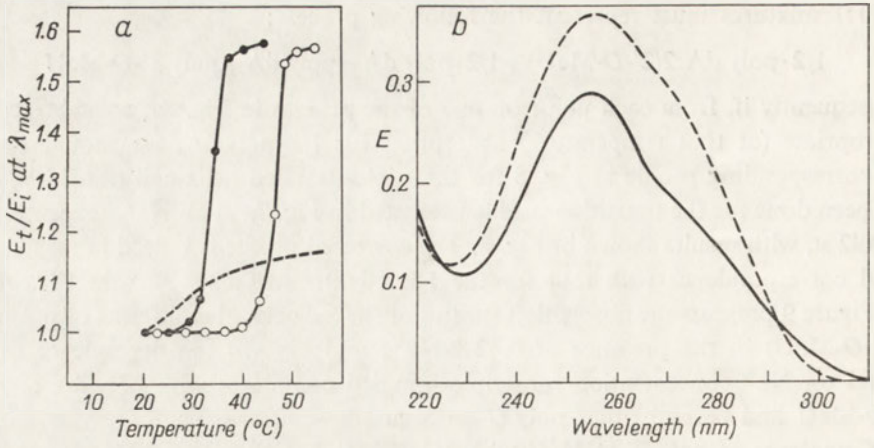

Fig. 11. (*a*) Temperature profiles in 0.01 M-phosphate buffer, pH 7.8, and 0.11 M-NaCl of poly rX with poly 2′-*O*-MeU (●-●-●), with poly rU (○-○-○) and a mixture of poly rX with poly dU (— — —). (*b*) Absorption spectrum of double-stranded poly rX:2′-*O*-MeU in the helical form (——) and following melting to homopolymers (— — —).

transition profile for the double-stranded complex formed in a 1:1 mixture in the presence of 0.11 M-Na^+ is shown in Fig. 11a. Somewhat surprisingly, the T_m for this helix is 13° lower than for the corresponding double-stranded poly rX:rU, the profile for which is shown in the same figure, and is in excellent agreement with that previously reported by Fikus & Shugar (1969), the T_m values differing by only 1°. Of further interest was the observation that, when the Na^+ concentration was increased to 0.5 M, the increases in the T_m values for poly rX:2′-*O*-MeU and poly rX:rU were such that the difference between them was unchanged, i.e. 13°.

Figure 11a also exhibits the influence of temperature on the absorbance of a 1:1 mixture of poly dU and poly rX. The resulting profile is immediately recognizable as the melting profile for poly rX alone (Fikus & Shugar, 1969), confirming the previous observation of the absence of complex formation between poly dU (and poly dT) and poly rX.

The absorption spectra of the helical and coil forms of poly rX:rU are demonstrated in Fig. 11b. Attention is drawn to the *hyper*chromicity of the helical form (with respect to the homopolymer components) to the red of 282 nm.

DISCUSSION

The overall results from the present investigation both support and extend the conclusions drawn from previous studies on poly 2′-*O*-MeA (Bobst *et al.*, 1969) and poly 2′-*O*-MeC (Żmudzka *et al.*, 1969b), and from our initial findings on poly 2′-*O*-MeU (Żmudzka & Shugar, 1970), viz. that helical structures involving these homopolymers resemble those for the corresponding ribopolynucleotides. From this it follows that the formation of helical structures of the latter is not dependent

on *intra*molecular hydrogen bonding *via* the 2′-hydroxyls as donors. It appears reasonable to extrapolate these findings to other synthetic polynucleotide systems, as well as to natural nucleic acids; and, in fact, this is supported by an examination of the available X-ray diffraction data for double-stranded RNA and for at least one synthetic double-stranded hybrid helix (see below).

It may, of course, be argued that the 2′-*O*-Me oxygen, with its lone electron pair, is still capable of acting as a hydrogen bond acceptor. However, apart from solvent water molecules, it is difficult to visualize a possible site in a polynucleotide helix which could conceivably serve as a donor to the 2′ oxygen; studies with space-filling models do not point to such a possibility.

The influence of the monovalent cation, Na^+, on the secondary structure of poly 2′-*O*-MeU (Figs. 1a and 1b) is particularly striking. From Fig. 1a it may be seen that up to an Na^+ concentration of 0.05 M there is no observable secondary structure; whereas at 0.1 M-Na^+ a characteristic helix-coil transition makes its appearance with a T_m value which is shifted towards higher temperatures with increasing salt concentration. Furthermore, the temperature hyperchromicity for the helix-coil transition, about 30%, is almost identical with that for the corresponding transition in poly rU, which occurs at lower temperatures (Fig. 1b). It is somewhat unfortunate that comparable systematic data for the effect of Na^+ concentration on the helix-coil transition for poly rU are lacking. In part this is undoubtedly due to the known tendency of poly rU preparations to undergo time-dependent degradation, presumably *via* the action of traces of RNase emanating either from the polynucleotide phosphorylase used for the polymerization, and/or from the manipulations involved during the preparation of poly rU.

However, a poly rU preparation has been recently described (Inners & Felsenfeld, 1970) which was reported to exhibit an unaltered $S_{20,w}$, about 6, over a period of more than one year. In Fig. 5 of the paper by Inners & Felsenfeld (1970), the authors show that the *coil* form of this poly rU sample in 2 M-Na^+ exhibits a temperature hyperchromicity of 1.5% between 20° and 80°C, in agreement with previous observations by Richards *et al.* (1963) and Simpkins & Richards (1967). The same figure shows a portion of the helix-coil transition, below 20°C. If we assume that the temperature hyperchromicity for this transition is also 30%, it can easily be seen from the figure that the T_m for this transition must be below 0°C. This is in obvious contradiction with the observations of Lipsett (1960), Szer & Shugar (1966), Richards *et al.* (1963), Brown (1966), Michelson & Monny (1966), and others. The authors were apparently unaware of this discrepancy and do not comment upon it. We have consequently made use of the means of scattered values of other observers for the dependence of the T_m for the helix-coil transition of poly rU on the Na^+ concentration (dashed line in Fig. 1b). From Fig. 1b it is clear that the T_m of poly 2′-*O*-MeU increases much more rapidly with ionic strength than the T_m of poly rU. This points to the likelihood of some solvent effect on helix stability, inasmuch as the 2′-*O*-Me (like the 2′-OH in a normal ribopolymer) is on the outside of the helix, in contact with solvent water molecules. It would obviously be desirable to obtain more extensive and reliable data for poly rU, which we are planning to do.

The effect of Mg^{2+} cations on the formation of helical poly 2′-*O*-MeU, as compared to poly rU (Fig. 2) is somewhat less pronounced than that of the monovalent Na^+ cation. The influence of polyamines, shown in Fig. 3, is compared with the analogous data for poly rU (Szer, 1966), (Table 1). It will be noted that, in the presence of 1.0 equivalent of spermidine, the T_m values are practically identical for poly rU and poly 2′-*O*-MeU. However, a 10-fold increase in spermidine concentration, which only slightly augments the T_m for poly rU, gives a 16° increase for poly 2′--*O*-MeU. In the case of spermine, the difference in T_m between the two polymers is very pronounced at 1.0 equivalent; but a comparison at 10 equivalents was not possible since, under these conditions, poly rU is precipitated out of solution. In retrospect, it is clear that measurements should have been performed at intermediate concentrations of the polyamines in order to assess more specifically the effects on both polymers; this remains to be done.

An examination of the results of investigations on monomer association in aqueous medium does not throw much light on the origin of the enhanced stability resulting, in general (see below for exception to this rule) from methylation of the 2′-OH in polynucleotides such as poly 2′-*O*-MeA, poly 2′-*O*-MeC and poly 2′-*O*--MeU. Base stacking of monomers does appear to be enhanced by alkylation (Broom, Schweitzer & Ts'o, 1967; Helmkamp & Kondo, 1968; Naomi & Igarashi, 1970), a finding in apparent reasonable agreement with the stabilizing effect of a 5-methyl pyrimidine substituent in helical polynucleotides (Szer & Shugar, 1966; Barszcz & Shugar, 1968), although at variance with the destabilization produced by a 5-ethyl pyrimidine substituent (Swierkowski & Shugar, 1970; Kulikowski & Shugar, in preparation).

In contrast to the above, Naomi & Igarashi (1970) report that the carbohydrate moiety of nucleosides reduces the association of the aromatic bases; while Broom *et al.* (1967) concluded that 2′-*O*-methylation leads to decreased stacking of ribonucleosides. This conclusion is, however, based solely on a comparison of the stacking constants for rA, dA and 2′-*O*-MeA; it is probably premature to draw any generalization from this single model system, the more so in that the neutral, single-stranded form of poly 2′-*O*-MeA closely resembles poly rA, while helical structures involving poly 2′-*O*-MeA are *more* stable than the corresponding helices with poly rA (Bobst *et al.*, 1969).

In a previous comparative study of the nature of the complexes formed between poly rA and poly rU on the one hand, and poly dA and poly dU on the other (Żmudzka *et al.*, 1969a), it was shown that for both of these systems it was possible to place in evidence, by appropriate manipulation of the ionic strength of the medium and of the temperature, the following transitions or dismutations in the case of 1:1 mixtures: 2→1, 3→1, 2→3. For the corresponding 1:2 mixtures, both systems exhibited the following types of transitions: 2→1, 3→1, 3→2. The situation is quite different in the case of complexes of poly 2′-*O*-MeA with poly rU (Bobst *et al.*, 1969) or poly 2′-*O*-MeU with poly rA (Żmudzka & Shugar, 1970, and results reported above). None of these systems, either in 1:1 or 1:2 mixtures, exhibits the dismutation

reaction 2→3 or the melting of a triple-stranded complex directly to homopolymers, i.e. 3→1, as shown also by an examination of melting profiles at 280 nm and 283.5 nm (see Fig. 4 and Fig. 6 in Bobst *et al.*, 1969). This is most likely related to the low stability of triple-stranded helices of these polymers (cf. Bobst *et al.*, 1969).

The decreased stability of the double-stranded complex of poly 2′-*O*-MeU with poly rX (T_m 34°C), as compared to that of poly rU with poly rX (46°C), both under the same conditions of ionic strength (see Fig. 11a) is in sharp contrast with the increased stability of the double-stranded complex of poly 2′-*O*-MeU with poly A as compared to poly rU with poly A. It will be observed from Fig. 11a that the helix--coil transition profiles for both rX:2′-*O*-MeU and poly rX:rU exhibit practically identical temperature hyperchromicities (about 55%), and are likewise equally narrow in breadth. Furthermore the modifications in absorption spectrum in going from the coil to the helical form of poly rX:2′-*O*-MeU, which are very characteristic, as may be seen from Fig. 11b, are identical with those for the corresponding poly rX:rU (Fikus & Shugar, 1969). It is difficult to avoid drawing the conclusion from these observations that the helical conformations of poly rX:2′-*O*-MeU and poly rX:rU must be similar, insofar as the relative orientations of the base-pairs in the helices are concerned, and the consequent modification in stability due to introduction of the 2′-*O*-Me substituent must be due rather to a solvent effect than to a solvent-induced change in conformation of the ribose ring. The decrease in stability of poly rX:rU provoked by the introduction of the 2′-*O*-Me (i.e. elimination of the 2′-OH) is also qualitatively in the direction to be expected from the fact that neither poly dU nor poly dT interact with poly rX (cf. Fikus & Shugar, 1969).

The results hitherto discussed, as well as those reported elsewhere for poly rA and poly dA (Bobst *et al.*, 1969), and poly rC and poly dC (Żmudzka *et al.* 1969b), refer to individual helical molecules in aqueous medium. It is, however, of interest to examine the findings obtained by means of X-ray diffraction techniques on polynucleotide fibres. Arnott *et al.* (1966) found that it was possible to construct a helical model for double-stranded RNA in which the ribose 2′-OH was bonded to another part of the same molecule; but it did not prove feasible to build such a model which was both stereochemically satisfactory and compatible with the observed X-ray diffraction data. The results did "suggest" the possibility that helical regions of the RNA molecules in the fibres might be linked in some way *via* hydrogen bonding of a ribose 2′-OH in one helix to a phosphate oxygen of another, but the X-ray data alone did not show this directly. The absence of an *intra*molecular hydrogen bond involving the 2′-OH in helical double-stranded RNA was again noted by Fuller, Hutchinson, Spencer & Wilkins (1967); and subsequently in the refined α and β RNA crystal structures (Arnott, Dover & Wonacott, 1969). More recently O'Brien & MacEwan (1970) arrived at a similar conclusion from a refined analysis of the diffraction pattern for the synthetic hybrid double-helical poly rI:dC, the structure of which is similar to that of double-helical RNA and DNA-RNA hybrid. It must be added, on the other hand, that the observations of Arnott *et al.* (1969) and O'Brien & MacEwan (1970) both pointed to the *possibility* of formation of a hydrogen

bond between the ribose 2′-OH of one helix with the phosphate O_2 of a neighbouring molecule; but once again the limitations of the X-ray data precluded any direct demonstration of the existence of such a bond.

We are indebted to Dr. Celina Janion for the polymerization runs with 2′-*O*-MeUDP; to E. Czuryło and H. Sierakowski for the sedimentation runs; and to Mrs. Elizabeth Poddany for excellent technical assistance. This work was supported, in part, by the Wellcome Trust, the World Health Organization, and the Agricultural Research Service, U.S. Dept. of Agriculture.

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ROLA GRUPY 2′-HYDROKSYLOWEJ W KONFORMACJI POLINUKLEOTYDÓW. KWAS POLI-2′-O-METYLOURYDYLOWY

Streszczenie

1. Opisano syntezę kwasu poli-2′-*O*-metylourydylowego (poli-2′-*O*-MeU) przez chemiczną dezaminację kwasu poli-2′-*O*-metylocytydylowego oraz przy użyciu fosforylazy polinukleotydowej.

2. Kwas poli-2′-*O*-MeU podobnie jak poli-U, poli-rT i poli-5-EtU, a przeciwnie niż poli-dU i poli-dT, w obecności jonów Na^+, Mg^{2+} i poliamin łatwo tworzy strukturę helikalną, której stabilność badano w zależności od stężenia soli i poliamin.

3. Kwas poli-2′-*O*-MeU tworzy dwu- i trójpasmowe kompleksy z poli-A oraz trójpasmowe kompleksy z poli-dA. Właściwości tych kompleksów porównano z opisanymi w literaturze dla poli-U, poli-dU oraz poli-rT.

4. Kwas poli-2′-*O*-MeU podobnie jak poli-U i jego analogi, a przeciwnie niż poli-dU i poli-dT, tworzy kompleks z poli-X.

5. Przedyskutowano rolę grupy 2′-hydroksylowej jako czynnika odpowiedzialnego za różnice między strukturami analogicznych rybo- i dezoksyrybopolinukleotydów oraz między RNA i DNA. Wykluczono możliwość, ażeby grupa 2′-hydroksylowa brała udział jako donor wodoru w tworzeniu przez rybopolinukleotydy wewnętrznego wiązania wodorowego.

6. Omówiono efekt metylacji w 2′-pozycji rybozy na stabilność drugorzędowych struktur kwasu poli-2′-*O*-MeU. Przedyskutowano także możliwość przeniesienia wniosków z tego modelu do rozważań nad rolą takiej metylacji w strukturze naturalnych r-RNA i t-RNA.

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Addendum: Since submission of the above manuscript, we have examined the T_m of poly rU as a function of ionic strength over a range of salt concentrations of 0.05 - 3.0 M. The T_m was found to increase linearly with log [Na^+] up to a concentration of Na^+ of 1 M, following which it decreased so that at 3 M-Na^+ its value was approximately that prevailing at about 0.1 M-Na^+. These findings, which were similar when Na^+ was replaced by K^+ or Cs^+, will be described in full detail elsewhere. It is consequently clear that the dashed line in Fig. 1b, which is only an extrapolation of scattered data from the literature, is incorrect. It follows that the observed behaviour of poly rU is quite different from that for poly 2′-*O*-MeU, where T_m increases linearly with log [Na^+] up to an Na^+ concentration of 3 M. This, in turn, points to some involvement of the 2′-OH in poly rU in the interaction with solvent ions, a subject which we are further investigating.

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RECENZJE KSIĄŻEK

ADVANCES IN BIOPHYSICS AND MOLECULAR BIOLOGY (J.A.V. Butler & D. Noble, eds·), vol. 19, part I+II., Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1969; str. 467.

Dziewiętnasty tom *Advances in Biophysics and Molecular Biology* jest nieco obszerniejszy niż zwykle, zawiera bowiem 10 artykułów. Fakt ten jest niewątpliwie odbiciem kolosalnego rozwoju biofizyki i jej wciąż wzrastającego znaczenia dla wszystkich dziedzin biologii. Dla zachowania objętości woluminów wygodnej w czytaniu tom ten został podzielony na dwie części, 1. Biologia Molekularna i 2. Biofizyka. Podział artykułów pomiędzy te dwie części niezupełnie odpowiada jednak tym tytułom, gdyż w pierwszej części znalazła się praca A. C. T. Northa o rentgenograficznym badaniu krystalicznych białek, zaś w drugiej części takie artykuły jak na przykład M. R. Pollocka o zmieniającym się pojęciu organizmu w mikrobiologii.

W części pierwszej, oprócz artykułu o rentgenografii białek przeznaczonego niewątpliwie tylko dla stosunkowo wąskiego grona specjalistów, znajdują się cztery prace z zakresu biologii molekularnej, które niewątpliwie zainteresują szersze grono czytelników. Drugim z kolei artykułem tej części jest obszerna praca poglądowa A. S. Spirina o strukturze rybosomów. Podzielona na dość konwencjonalne podrozdziały jak I. Ogólne wiadomości o kompletnych rybosomach; II. Podjednostki rybosomów; III. Konformacja wielkocząsteczkowych składników rybosomów; IV. Strukturalne zmiany cząstek rybosomowych; V. Rekonstrukcja cząstek rybosomowych — jest napisana raczej w stylu tradycyjnej biochemii niż biologii molekularnej. Zawiera ona sporo danych dotyczących składu rybosomów, odczuwa się jednak dotkliwie brak modelu roboczego lub jakiegoś oryginalnego spojrzenia na zagadnienie, które mogłoby stymulować dalsze prace eksperymentalne.

Stosunkowo krótki artykuł M. S. Bretschera o „znakach przestankowych" kodu genetycznego omawia w zwięzły i jasny sposób aktualny stan wiedzy o regulacji rozpoczęcia i zakończenia syntezy łańcucha peptydowego. Doskonałe połączenie wniosków wynikających z danych biochemicznych i genetycznych sprawia, iż artykuł jest interesujący nie tylko dla biochemików lecz i dla genetyków.

Artykuł A. Kepesa o transkrypcji i translacji operonu laktozy jest oparty głównie na pracach z laboratorium autora, w których ciekawe chwyty metodyczne pozwoliły na podstawie oznaczeń kinetyki indukcji enzymu wyciągnąć wnioski dotyczące m.in. półokresu życia swoistego messengera, szybkości wydłużania się łańcucha peptydowego, jednoczesnego lub kolejnego odczytywania cistronów policistronowego messengera, inicjacji łańcucha peptydowego oraz kierunku odczytywania operonu. Praca jest interesująca ze względu na możliwość stosowania niektórych chwytów metodycznych również do innych układów biologicznych.

W ostatnim artykule pierwszej części C. Pelling pisze o syntezie kwasów nukleinowych w gigantycznych chromosomach. Jest to próba korelacji wiadomości uzyskiwanych metodami cytologicznymi z wynikami nowoczesnych metod biochemicznych dla zbudowania modelu funkcji chromosomu eucariota na poziomie molekularnym.

Druga część 19 tomu *Advances* zawiera pięć artykułów. Pierwszym z nich jest praca R. M. Pollocka omawiająca zmieniające się pojęcie organizmu w mikrobiologii. Przedstawia ona z jednej strony populację komórek jako zbiór indywiduów różniących się od siebie pod względem genetycznym, z drugiej zaś strony komórkę jako zbiór elementów posiadających różny stopień autonomii i co do których nie można zastosować klasycznych kryteriów podziału na materię żywą i nieżywą.

Drugi artykuł C. C. Congdona dotyczy ośrodków germinacyjnych tkanki limfatycznej w czasie reakcji immunologicznych. Omawia on morfologię, aktywność, funkcję oraz niszczenie i regenerację ośrodków germinacyjnych. Uwzględnia także zjawiska występujące w patologicznych reakcjach odpornościowych.

Dalsze trzy artykuły dotyczą biofizyki błon biologicznych i pod tym względem stanowią pewną całość.

Praca R. H. Adriana o zjawiskach bioelektrycznych w membranach mięśniowych składa się z części teoretycznej, gdzie przedstawiono szereg prostych modeli przenikania jonów przez błony mięśni poprzecznie prążkowanych. W drugiej części omówione są dane dotyczące ruchu jonów potasu w błonach mięśniowych, przy czym dane są odniesione do modeli teoretycznych pierwszej części.

Artykuł G. B. Ardena omawia zagadnienie pobudzenia oraz zjawiska biochemiczne i elektryczne zachodzące w membranach fotoreceptorów. W ostatnim artykule H. Passow pisze o pasywnym przenikaniu jonów przez błony erytrocytów. Jest to próba zastosowania powszechnie przyjętej teorii „stałego ładunku" błony do wytłumaczenia faktów związanych z biernym przenikaniem jonów. Próba ta posłużyła do krytycznej oceny teorii i jej ograniczeń.

W sumie należy stwierdzić, że 19 tom *Advances* przynosi ogromną różnorodność wiadomości z zakresu najbardziej interesujących i aktualnych problemów biologii molekularnej i pod tym względem należy go uznać za bardzo udany.

Michał Bagdasarian

FAT-SOLUBLE VITAMINS. International Encyclopedia of Food and Nutrition. (R.A. Morton, ed.), Vol. 9. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1970; str. 530.

Omawiany tom pod redakcją R. A. Mortona stanowi dalszą pozycję z serii Encyklopedii Żywności i Żywienia. Traktuje on o wielu różnych aspektach zagadnienia witamin rozpuszczalnych w tłuszczach. Poza podstawową biochemią tych związków wiele miejsca poświęcono w nim zagadnieniom technologii procesów produkcji tych witamin i zagadnieniom niedoborów tych witamin u ludzi.

Książka składa się z 15 rozdziałów napisanych przez najwybitniejszych specjalistów w omawianych dziedzinach. Poprzedza je obszerny wstęp napisany przez R. A. Mortona, wiążący niejako dalsze rozdziały w jedną całość. Autor, omawiając stan wiedzy i perspektywy rozwoju tej dziedziny, wyraża pogląd, że o ile dotychczasowe wiadomości o witaminach rozpuszczalnych w tłuszczach dotyczyły problemów związanych z technologią procesów ich otrzymywania, analityki i występowania objawów klinicznych spowodowanych ich niedoborem, ostatnie ćwierćwiecze XX wieku winno przynieść dokładne biochemiczne dane o mechanizmie ich działania. Skoncentrowanie wysiłków badaczy na tym problemie winno doprowadzić do wyjaśnień odkrywczych, podobnych do tych, jakie uzyskano w badaniach nad witaminą A i chemią procesu widzenia.

Dzięki współautorstwu niemal wszystkich światowych specjalistów dalsze rozdziały zawierają sporo cennych bardzo specjalistycznych wiadomości, jednakże odbija się to ujemnie na zwartości książki. Czytelnik napotyka w niej wiele powtórzeń.

Poza rozdziałami poświęconymi poszczególnym witaminom lub grupom witamin, które zwykle spotyka się w podobnym układzie lecz znacznie węższym zakresie w podręcznikach biochemii, w książce zamieszczono rozdziały omawiające metody standaryzacji preparatów witaminowych, przemysłowe metody produkcji z uwzględnieniem bogatej literatury patentowej oraz syntezy izotopowo znakowanych witamin (3 rozdziały). W trzech innych rozdziałach omówiono zagadnienia kliniczne związane z niedoborem u ludzi witamin rozpuszczalnych w tłuszczach.

Książka zawiera 491 stron tekstu, jest bogato ilustrowana schematami i fotografiami, posiada indeks autorów i indeks rzeczowy. Poza tym każdy rozdział zawiera również wykaz cytowanej literatury obejmujący jednak tylko pozycje opublikowane przed rokiem 1967.

Książka zainteresuje zarówno biochemików, jak i specjalistów z dziedziny higieny żywienia, technologii przemysłowej witamin oraz klinicystów.

Tadeusz Chojnacki

H. P. Chin, CELLULOSE ACETATE ELECTROPHORESIS: TECHNIQUES AND APPLICATIONS. Ann Arbor-Humphrey Science Publ., Ann Arbor-London 1970; str. 139.

Folia z octanu celulozy od wielu lat była stosowana jako membrana filtracyjna dla oddzielania drobnoustrojów, osadów z różnych zawiesin, do zagęszczania roztworów koloidowych itp. Jako nośnik do elektroforezy pasmowej folia z octanu celulozy została wprowadzona przez J. Kohna w 1957 r., którego to faktu autor książki jednak nie ujawnia, i w krótkim czasie znalazła powszechne zastosowanie w różnych laboratoriach badawczych i klinicznych. Folia w porównaniu z bibułą posiada wiele cech korzystniejszych, np. wykazuje minimalną adsorpcję białek i innych substancji, po działaniu rozpuszczalnikami organicznymi staje się całkowicie przezroczysta, co ma istotne znaczenie przy bezpośrednim densytometrowaniu pasków, lub daje się całkowicie rozpuścić np. w mieszaninie chloroform - etanol, która to własność została wykorzystana m.in. w technice elektroforezy dwukierunkowej (transfer strip technique).

Książka China obejmująca 13 rozdziałów jest w całości poświęcona zastosowaniu folii z octanu celulozy do rozdziału białek, lipo- i glikoproteidów, hemoglobin, enzymów, do badań immunodyfuzyjnych i immunoelektroforetycznych oraz do rozdziału najrozmaitszych substancji organicznych. Autor przedstawia najpierw pokrótce rozwój metod elektroforetycznych a następnie ogólne podstawy teoretyczne elektroforezy ze szczególnym uwzględnieniem ruchliwości naładowanych cząstek w nośnikach stałych. W następnych trzech rozdziałach omówiono własności folii z octanu celulozy, aparaturę, przebieg doświadczenia oraz ilościową interpretację wyników. Należy stwierdzić, że technika wykonania rozdziału elektroforetycznego jest w przypadku folii taka sama, jak przy użyciu bibuły filtracyjnej i autor podaje znane powszechnie sposoby podejścia w tym zakresie, jak również barwienia pasków, ich ilościowego przewartościowywania i obliczania wyników. Dalsze rozdziały książki zawierają przykłady frakcjonowania różnych grup białek, głównie dla potrzeb klinicznych. Po każdym rozdziale znajduje się lista kilkudziesięciu pozycji piśmiennictwa zebranego do r. 1969 włącznie. Na końcu książki podano bardzo pomocny indeks rzeczowy. W sumie podręcznik ten, zwięźle opracowany i estetycznie wydany, będzie szczególnie przydatny w laboratoriach chemiczno-klinicznych.

Włodzimierz Ostrowski