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A SIMPLIFIED METHOD FOR DETERMINATION OF NICOTINAMIDE-ADENINE DINUCLEOTIDES IN LIVER TISSUE

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1. A fluorimetric method for determination of nicotinamide-adenine dinucleotides in liver has been elaborated. The method makes possible the determination of all four individual forms of nucleotides (NAD, $NADH_2$, NADP, $NADPH_2$) using for their separation one enzyme only: the yeast alcohol dehydrogenase. 2. Content of the nicotinamide nucleotides in the liver of rats and rabbits is presented and compared with the data obtained by other authors.

Separate determination of all four forms of nicotinamide-adenine dinucleotides (NAD, $NADH_2$, NADP, $NADPH_2$) in tissue extracts has been carried out chiefly using spectrophotometric methods: Glock & McLean (1955), Villee (1962), Klingenberg (1963), Slater, Sawyer & Sträuli (1964), and fluorimetric methods: Lowry, Roberts & Kapphahm (1957), Bassham, Birt, Hems & Loening (1959), Lowry, Passonneau, Schulz & Rock (1961a), Pande, Bhan & Venkitasubramanian (1964), Jouany (1965). In both kinds of methods at least two enzymes are necessary: one specific for NAD, usually alcohol dehydrogenase, and another enzyme specific for NADP, usually G-6-P dehydrogenase. In some methods additional enzymes are necessary, such as specific oxido-reductases (Glock & McLean, 1955), diaphorase (Villee, 1962), glutamate dehydrogenase (Lowry *et al.*, 1961a), lactate dehydrogenase (Klingenberg, 1963).

The procedure proposed here is based mainly on the classical fluorimetric methods of Lowry *et al.* (1957) and Bassham *et al.* (1959). A modification was introduced in order to limit the use of enzymes to one only, the easily obtainable yeast alcohol dehydrogenase.

EXPERIMENTAL

The principle of the method. In the proposed procedure for separate fluorimetric determination of all four forms of nicotinamide nucleotides in tissues three steps are essential: (1), selective extraction of the reduced and oxidized forms of nucleotides from the tissue; (2), separation of NAD from NADP and $NADH_2$ from $NADPH_2$; (3), development and measurement of the fluorescence.

The extraction of tissue with cold acid permits to obtain unchanged NAD and NADP, whereas $NADH_2$ and $NADPH_2$ are destroyed. Treatment with hot alkali allows $NADH_2$ and $NADPH_2$ to be obtained unchanged, whereas NAD and NADP are destroyed. In the present work the extraction of the oxidized and reduced forms is carried out according to Gordon (1963) using respectively 5% trichloroacetic acid and 0.1 M-sodium carbonate.

The separation of NAD from NADP in the acidic extract is achieved after enzymic reduction of NAD by an ethanol-alcohol dehydrogenase system, followed by alkaline and acidic extraction for $NADH_2$ and NADP, respectively. The same principle is applied for the separation of $NADH_2$ from $NADPH_2$, except that the reduced forms are first subjected to non-enzymic oxidation with phenazine methosulphate, as proposed by Gordon (1963).

The determination of the nucleotides is based upon the measurement of the fluorescence of the products obtained from the oxidized nucleotides by treatment with strong alkali (Bassham *et al.*, 1959).

The scheme of the procedure is presented in Fig. 1.

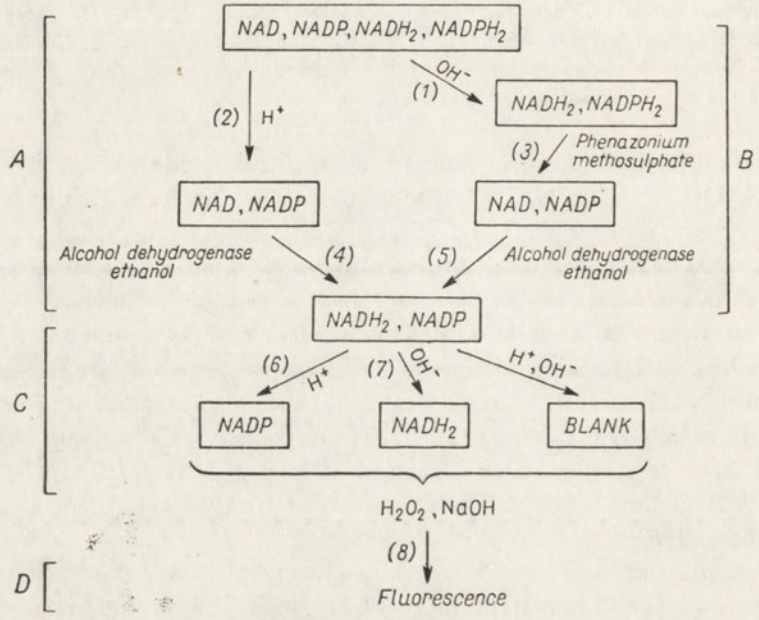

Fig. 1. Scheme of the procedure.

The parameters of the method

Parallel alkaline and acid extractions of nucleotides proposed by Gordon (1963), permit an efficient separation of the reduced and oxidized forms to be achieved, as shown in Table 1.

For the extraction of the two reduced forms from the tissue (step *1*) 0.1 M-Na_2CO_3 is used and the process is carried out in a boiling-water bath. To separate $NADH_2$

Table 1

The recovery of $NADH_2$ and NAD under conditions of alkaline and acid extraction

To liver samples (1 g. of tissue for alkaline and 1.5 g. for acidic extraction) 1000 mμmoles of NAD or 500 mμmoles of $NADH_2$ was added and the extraction was performed with hot 0.1 M-Na_2CO_3 or cold 5% trichloroacetic acid as described in the text. In each extract the sum of NAD+NADP was determined, enzymic separation of NAD from NADP being omitted. The endogenous nucleotides were estimated in separate extracts.

Extraction	Number of determinations	Recovery ± S.D. (%)	
		$NADH_2$	NAD
Alkaline	5	101 ± 8	0
Acidic	5	0	101 ± 7

Table 2

The dependence of the recovery of NAD added to the acidic extract (step 2) on the temperature of decomposition of NADP (step 7)

To an acid extract of 1.5 g. of liver, 400 mμmoles of NAD and 400 mμmoles of NADP were added, then NAD was enzymically reduced (step *4*) and NADP decomposed by treatment with NaOH (step *7*) at 60° and 40°. The endogenous nucleotides were determined in a separate sample.

Temperature	Number of determinations	Recovery ± S.D. (%)	
		NAD	NADP
60°	8	84 ± 9	0
40°	8	98 ± 2	0

Table 3

Oxidation of $NADH_2$ by different concentrations of phenazine methosulphate at different reaction time

To 15 ml. samples of alkaline extracts of 1 g. of liver, 225 mμmoles of $NADH_2$ and different amounts of phenazine methosulphate (0.2 ml.) were added and incubated at 20° (step *3*). After deproteinization, the sum of NAD+NADP was determined. The amount of endogenous nucleotides was determined separately. Mean values of 3 measurements are given.

Phenazine methosulphate concentration (μM)	Percentage of $NADH_2$ oxidized after		
	15 min.	30 min.	45 min.
6.6	89	106	102
20.0	102	98	98
40.0	98	108	100

from NADP (step *7*), 0.2 vol. of 1 N-NaOH is added to the solution and a lower temperature has been found to give higher yield (Table 2).

The yield of the non-enzymic oxidation of the reduced nucleotides (step *3*) has been checked for various concentrations of phenazine methosulphate and various time periods of oxidation. Under all conditions applied, complete oxidation of the nucleotides was obtained (Table 3).

The enzymic reduction of NAD was practically complete when carried out at pH 8.0 - 8.8 and at concentrations of alcohol dehydrogenase ranging from 10 to 100 μg. per ml. of the extract (Table 4). This concentration of the enzyme is sufficient for both steps of the procedure (*4* and *5*), i.e. also when an excess of phenazine methosulphate is present.

To develop the fluorescence of the final solution (step *8*) the procedure of Bassham *et al.* (1959) was followed, but the volume of the solution was increased. The fluorescence curve for different concentrations of NAD is shown in Fig. 2. When $NADH_2$ is present in the sample to be estimated, the treatment with hydrogen peroxide included in the original procedure leads to its complete oxidation (Table 5).

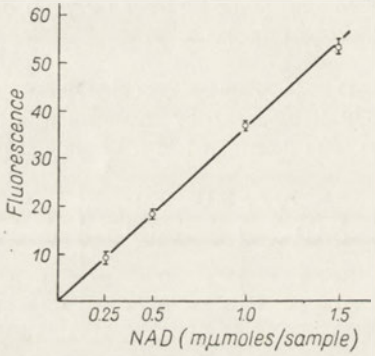

Fig. 2

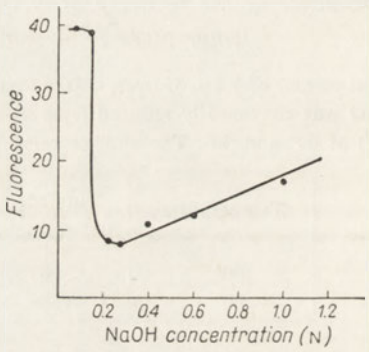

Fig. 3

Fig. 2. The fluorescence of samples containing different amounts of NAD (step *8*). Samples of 0.3 ml. containing indicated amounts of NAD were mixed with 0.6 ml. of $NaOH-H_2O_2$ solution. After incubation at 38° for 60 min., 1.4 ml. of water was added and fluorescence was measured.

Fig. 3. The fluorescence of the blank with different concentrations of NaOH used for decomposition of the oxidized nucleotides. To 0.5 ml. samples obtained by procedures *A* and *B* (steps *1* - *5*), and after 5 min. incubation with 0.1 ml. of 1 N-HCl at room temperature, 0.25 ml. of appropriate NaOH solution was added to give the indicated final concentrations.

Blanks are prepared by applying both acidic and alkaline destruction of the nucleotides in the same sample. Figure 3 shows that the concentration of NaOH used in this procedure does influence considerably the final result. At concentrations above 0.2 N although the destruction of NAD is complete, the fluorescence increases due to other factors.

To check the efficiency of the proposed procedure, different amounts of standard solutions of NAD and NADP were added to the tissue samples prior to extraction,

Table 4

Enzymic reduction of NAD at different pH values and various concentrations of alcohol dehydrogenase

The sample contained: 0.5 M-ethanol, 1 mM-EDTA, 67 mM-sodium pyrophosphate, 0.02 mM-NAD, 0.5 ml. of the acidic extract from liver (final volume 3 ml.) or 1.5 ml. of the alkaline extract (final volume 5 ml.), and indicated amounts of alcohol dehydrogenase (ADH). pH of the samples were adjusted by means of 1 N-HCl or 1 N-NaOH. After 5 min. incubation at 20° the increase in extinction at 340 mμ was measured. Mean values of 2 - 5 measurements are given.

ADH (16.5 μg./ml.)		pH 8.6	
pH	yield (%)	ADH (μg./ml.)	yield (%)
8.0	90	8.25	96
8.3	95	16.5	96
8.6	95	33.0	95
8.8	94	60	95
—	—	100	95

Table 5

The development of $NADH_2$ fluorescence due to oxidation with $NaOH-H_2O_2$ *solution as compared with NAD fluorescence*

Samples of 0.3 ml. containing indicated amounts of NAD or $NADH_2$ were mixed with 0.6 ml. of $NaOH-H_2O_2$ solution. After incubation at 38° for 60 min., 1.4 ml. of water was added and the fluorescence was measured. Mean values of 3 determinations are given.

NAD or $NADH_2$ (mμmoles)	Fluorescence of	
	NAD	$NADH_2$
0.4	15	14
0.7	25	26
1.0	38	38

Table 6

The recovery of NAD and NADP

To 1.5 g. of liver the indicated amounts of NAD and NADP were added and the acidic extraction performed as described in the text. NAD, NADP and sum of NAD+NADP were determined separately. The endogenous nucleotides were determined in a separate sample. Mean values of 2 determinations are given.

Added (mμmoles)		Recovered					
NAD	NADP	NAD		NADP		NAD+NADP	
		mμmoles	%	mμmoles	%	mμmoles	%
400	400	396	99	396	99	856	107
900	150	927	103	141	94	987	94
600	600	558	93	750	95	1090	91
1200	600	1160	97	583	98	1640	91
Mean ± S.D.			98 ± 5		97 ± 10		96 ± 9

and a satisfactory recovery was obtained (Table 6). The recovery of $NADH_2$ added to tissue samples was studied in the same way. For amounts ranging from 250 to 1000 mμmoles the recovery was complete (mean value of 10 determinations was 101%).

Description of the method

Reagents: (*a*), ethanol solution: 0.1 M-sodium pyrophosphate, 0.75 M-ethanol, 1.5 mM-EDTA. (*b*), $NaOH-H_2O_2$ solution, to be prepared immediately before use: to 2.7 ml. of cold (0 - 4°) 10 N-NaOH, 0.3 ml. of 1% H_2O_2 is added. (*c*), Phenazine methosulphate (Koch-Light, Colnbrook, Bucks, England), 10 mM-solution in water. This solution is stable about one week when stored in the dark at 0 - 4°. (*d*), Alcohol dehydrogenase from yeast (Boehringer, Mannheim, West Germany). The preparation was stored at 4° and diluted with water immediately before use. (*e*), NAD (Boehringer), 0.40 mM standard solution in water, stable about one month when stored at 0 - 4°. The concentration was determined spectrophotometrically at 260 mμ and after reduction at 340 mμ using the following molar extinction coefficients: ε_{260} 18.0×10^6, ε_{340} 6.22×10^6 (Bergmeyer *et al.*, 1963). (*f*), $NADH_2$ (Boehringer), 0.45 mM standard solution in 0.1 M-Na_2CO_3, freshly prepared. The concentration was determined spectrophotometrically at 340 mμ. (*g*), Fluorescence standard: quinine sulphate, 0.05 μg./ml. in 0.1 N-H_2SO_4.

Material. Rats are killed by decapitation and immediately (within 1 min.) samples of liver are taken for the extractions.

A. Acidic extraction (*step 2*) *and enzymic reduction* (*step 4*). A sample of liver, about 1.5 g., is homogenized in 7 ml. of cold (0 - 2°) 5% trichloroacetic acid using Potter's glass homogenizer, the tube being cooled with ice. The homogenate is centrifuged at 8000 *g* for 15 min. at 0 - 2°. The supernatant, decanted quantitatively, is adjusted to 10 ml. with 5% trichloroacetic acid; 0.5 ml. of the above extract is neutralized with 0.16 ml. of 1 M-tris, then 2 ml. of the ethanol solution (*a*) and 0.32 ml. of water are added. After mixing, 120 μg. of alcohol dehydrogenase (in 0.02 ml.) is added. The sample is left for 5 min. at 20°. From this mixture four parallel 0.5 ml. samples are taken for the determination of NAD, NADP, the sum NAD+NADP and the blank, and treated as described in procedure *C*.

B. Alkaline extraction (*step 1*). A sample, 1 g., is heated in 10 ml. of 0.1 M-Na_2CO_3 on a boiling-water bath for 1 min. and then homogenized for 45 sec. The homogenate is cooled on ice and 4 ml. of 1 M-phosphate buffer (pH 7.4) is added. After centrifugation at 16 000 *g* for 15 min. at 0 - 2° the supernatant is decanted quantitatively and adjusted to 15 ml. with water.

Oxidation (step *3*): to the above solution 0.2 ml. of phenazine methosulphate is added, kept at 20° for 30 min., then 1.7 ml. of 100% (w/v) trichloroacetic acid is added, centrifuged at 8000 *g* for 15 min. at 0 - 2°, and the supernatant is adjusted to 20 ml. with water. At this stage the nucleotides present originally in the reduced form are converted into their corresponding oxidized forms.

Reduction (step *5*): to 1.5 ml. of the above solution, 3.5 ml. of an alkalized ethanol solution [0.2 ml. of 2 N-NaOH and 3.3 ml. of the ethanol solution (*a*)]

and 300 μg. of alcohol dehydrogenase (in 0.05 ml.) are added and the sample is left for 5 min. at 20°. Then four 0.5 ml. samples are taken, as in procedure *A*.

C. Separation of $NADH_2$ and NADP (step 6 and 7). Both procedures, *A* and *B*, applied for the extraction of oxidized and reduced nucleotides from the tissue, give a mixture of $NADH_2$ and NADP. For separate determination of these nucleotides, the destruction of NADP is effected by alkali treatment and the destruction of $NADH_2$ by acid treatment. The four 0.5 samples obtained by procedure *A* and the four obtained by procedure *B* are treated in the following way. For determination of $NADH_2$, 0.1 ml. of 1 N-NaOH is added and the sample is heated at 40° for 15 min. For determination of NADP, 0.1 ml. of 1 N-HCl is added and the sample is left at room temperature for 10 min. For determination of the sum of $NADH_2$ and NADP, 0.5 ml. of water is added. To the sample which is to serve as a blank, 0.1 ml. of 1 N-HCl is added; after 10 min. at room temperature, 0.25 ml. of 1 N-NaOH is added and the sample is heated at 40° for 15 min.

D. Development and measurement of fluorescence. The volume of each sample prepared as described above, is adjusted to 1 ml. with water, then from each two parallel 0.3 ml. samples are taken and 0.6 ml. of the $NaOH\text{-}H_2O_2$ solution (*b*) is added. After mixing, the samples are heated at 38° for 60 min., 1.4 ml. of water is added and the fluorescence is measured using a Farrand model A fluorimeter (Farrand Optical Co. Inc., New York, U.S.A.), and 3 ml. cuvettes, with primary and secondary filters no. 5860 and nos. 4308 and 3389, respectively. The primary filter prescribed isolates the Hg line at 365 mμ (Lowry *et al.*, 1957). The secondary filter complex has maximal transmission at 480 mμ (Udenfriend, 1962). The fluorimeter is standardized with quinine sulphate solution (0.05 μg/ml. in 0.1 N-H_2SO_4).

Standard aqueous solutions of 0.40 mM-NAD and 0.45 mM-$NADH_2$ were used for calibration throughout the whole procedure described above.

E. Calculations. For calculations of nicotinamide nucleotides concentration in the liver tissue the following formula is used:

$$\text{mμmoles/g. wet weight of tissue} = \frac{F_t - F_{tb}}{(F_s - F_{sb})w}\, d$$

where the fluorescence of the respective samples is designated by subscripts: F_t, proper sample derived from the tissue; F_{tb}, appropriate blank sample; F_s, standard; F_{sb}, standard blank; w is the wet weight of the tissue used for extraction; d, dilution factor, 400 and 449 for oxidized and reduced nucleotides, respectively.

Content of nicotinamide nucleotides in liver

The method described above has been used for determination of nicotinamide nucleotides in livers of rats and rabbits. In the experiment were used: 12 female rats of Wistar strain, 8 months old, body weight 220±20 g., weight of liver 7.9 ± 1.1 g.; 7 male rabbits, 1 year old, body weight 3.5 ± 0.2 kg., weight of liver 79±16 g. The results are presented in Table 7, in which the data obtained by other authors are also included.

Table 7

The content of nicotinamide nucleotides in the liver according to different authors and methods used

Methods: I, spectrophotometric; II, fluorimetric.

Animal	NAD	$NADH_2$	NADP	$NADPH_2$	Method	Authors
	mμmoles/g. of wet tissue					
Rat	654	178	34	308	I	Glock & McLean (1955)
	498	119	41	269	I	Slater *et al.* (1964)
	390	170	15	350	I	Villee (1962)
	485 ± 33	133 ± 24	25 ± 6	251 ± 24	II	Bassham *et al.* (1959)
	628	252	115	502	II	Lowry *et al.* (1961a)
	664 ± 10	394 ± 21	124 ± 21	319 ± 21	II	Jouany (1965)
	300 ± 43	82 ± 4	79 ± 4	254 ± 13	II	Pande *et al.* (1964)
	447 ± 40	199 ± 38	96 ± 15	409 ± 56	II	Present paper
Rabbit	612	235	—	—	I	Jedeikin & Weinhouse (1955)
	717 ± 61	227 ± 81	136 ± 29	397 ± 135	II	Present paper

DISCUSSION

We succeeded in elaborating a procedure for determination of nicotinamide-adenine dinucleotides in which one enzyme only is applied, namely the inexpensive and easily available alcohol dehydrogenase from yeast. This was achieved by the introduction of non-enzymic oxidation of the reduced forms of nucleotides (Fig. 1, step *3*), due to which in both paths of analysis NAD and NADP are obtained as the only components to be determined. The oxidation of the reduced nucleotides eliminates the errors which may arise due to oxidation of these compounds in alkaline extracts (Bassham *et al.*, 1959).

Determination of NAD in the presence of NADP is dependent on the efficiency of the enzymic reduction of NAD. The 95% recovery obtained may be regarded as complete taking into account the presence of some 5% of an enzymically inactive form in the preparation of NAD.

The fluorimetric method is not specific for the nucleotides studied. The alkaline fluorescence results from the presence of nicotinamide-ribose binding. Thus, the nicotinamide mononucleotide and nicotinamide-riboside may also contribute to the measurement (Ciotti & Kaplan, 1957). The above compounds are, however, destroyed in alkaline medium, thus they do not influence the determination of $NADH_2$ and $NADPH_2$ as well as of NAD which is determined after enzymic reduction and heating with alkali (Lowry, Passonneau & Rock, 1961b). Only the determination of NADP is influenced by the mononucleotide. It has to be mentioned that N_1-methylnicotinamide practically does not interfere with the determination as it does not show fluorescence in strong alkali, in contrast to its behaviour in media containing butanone or acetone (Levitas *et al.*, 1947; Burch *et al.*, 1955; Pande *et al.*, 1964).

It is known that certain substances present in the tissue such as α-ketoglutarate, pyruvate, oxalate, glucose, fructose, and others decrease the fluorescence developed from nucleotides in alkaline media (Lowry *et al.*, 1957; Bassham *et al.*, 1959). Their influence, however, is depressed by dilution of the extracts applied in this procedure, which seems to be confirmed by practically complete recovery of the standard nucleotides from the extracts.

The method gives reproducible results when the conditions of analysis are strictly observed. It is particularly important to begin the extraction as soon as possible because of rapid enzymic decomposition of liver nucleotides after the death of the animal (Jouany, 1965). The time of heating the tissue in sodium carbonate and immediate neutralization of the extract are also of importance: too short heating leads to incomplete decomposition of the oxidized forms, whereas one too long renders possible the decomposition of the reduced forms due to spontaneous oxidation in alkaline extracts (Bassham *et al.*, 1959; Lowry *et al.*, 1961a, b; Pande *et al.*, 1964).

To avoid errors which may arise due to these processes it is proposed that the standards of NAD and $NADH_2$ be analysed throughout the whole procedure in every series of determinations and that they be accepted as a reference for calculations. The presence of standards at the successive steps of the procedure makes possible to detect any arising error at this step at which it originated.

The sensitivity of the method is approximately 0.1 mμmole in the final sample. Taking into account the dilutions introduced in the procedure, an initial concentration of 50 mμmoles per 1 g. of fresh tissue may be determined. The sensitivity may be to some extent increased by taking larger samples of the extract for the final fluorimetric measurement.

In Table 7 are presented the concentrations of nicotinamide nucleotides in livers of rats and rabbits, obtained by various authors both by spectrophotometric and fluorimetric methods. The results differ considerably, probably due to differences both in the analytical procedures and different animal strains used. It is known that in rats sex and age have considerable influence upon the concentration of nicotinamide nucleotides in the liver (Slater *et al.*, 1964; Jouany, 1965). However, from the data collected in Table 7 it appears that the concentrations of NADP determined fluorimetrically are higher than those found spectrophotometrically. With respect to the other nucleotides, the values found fluorimetrically vary within wider limits as compared with the spectrophotometric data.

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UPROSZCZONA METODA OZNACZANIA NUKLEOTYDÓW NIKOTYNOAMIDOWYCH W WĄTROBIE ZWIERZĄT

Streszczenie

1. Opracowano metodę fluorymetrycznego oznaczania nukleotydów nikotynoamidowych w wątrobie zwierząt. Metoda umożliwia oznaczenie wszystkich czterech form nukleotydów (NAD, NADP, $NADH_2$, $NADPH_2$) przy zastosowaniu do ich rozdziału tylko jednego enzymu: dehydrogenazy alkoholowej z drożdży.

2. Oznaczono zawartość nukleotydów nikotynoamidowych w wątrobie szczurów i królików i porównano z wynikami innych autorów.

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KRYSTYNA BOBRZECKA, JANINA RYBARSKA and W. OSTROWSKI

THE ROLE OF AROMATIC AMINO ACIDS IN THE ACTIVITY OF ACID PHOSPHOMONOESTERASE FROM HUMAN PROSTATE

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Acid phosphomonoesterase from prostate was treated with reagents modifying aromatic amino acids; *N*-bromosuccinimide caused complete and irreversible inactivation of the enzyme, whereas *p*-hydroxy-5-nitrobenzylbromide, *p*-diazobenzenosulphonic acid, photo-oxidation and carboxymethylation had little or no effect. Tartrate afforded some protection of enzyme activity. The obtained results suggest that tryptophan residues play an important role in the catalytic function of the enzyme.

Acid phosphomonoesterase (EC 3.1.3.2) from prostate has been observed to lose its enzymic activity after being treated with iodine monochloride (Bobrzecka, Ostrowski & Rybarska, 1968). On the basis of spectrophotometric titration of native and iodinated enzyme it was possible to calculate that iodination of about 30% of tyrosine residues to monoiodotyrosine leads to about 80% inactivation of the enzyme. For further examination of the role of aromatic amino acids in the catalytic function of prostatic phosphatase, the enzyme was treated with various compounds reacting with histidine, tryptophan and tyrosine, and its activity determined. It was found that treatment of the prostatic phosphatase with *N*-bromosuccinimide[1] led to complete and irreversible loss of enzymic activity. HNBBr, *p*-diazobenzenosulphonic acid, iodoacetate, iodoacetamide as well as photo-oxidation in the presence of 1 mM-Rose Bengal had little or no effect on the activity of the enzyme.

MATERIALS AND METHODS

The acid phosphomonoesterase obtained from hypertrophic human prostate glands by the earlier described procedures (Ostrowski & Tsugita, 1961; Ostrowski, 1968), was homogeneous as it was shown by ultracentrifugation, disc electrophoresis and immunoelectrophoresis.

[1] Abbreviations used: NBS, *N*-bromosuccinimide; HNBBr, 2-hydroxy-5-nitrobenzylbromide; *p*-DBSA, *p*-diazobenzenosulphonic acid.

Enzymic activity was determined in 0.05 M-acetate buffer of pH 5 using *p*-nitrophenylphosphate (Sigma 104, Biochem. Co., St. Louis, Mo., U.S.A.) as substrate. To 200 µl. of 0.02 M-substrate solution, 2 to 50 µl. of enzyme solution was added and incubated at 37° for 1 - 3 min. The reaction was stopped by adding 15 volumes of 0.1 N-NaOH and extinction of liberated *p*-nitrophenol was measured at 400 mµ in a Uvispec (Hilger & Watts, London, England) spectrophotometer. Activity was expressed in µmoles of *p*-nitrophenol split by 1 ml. of enzyme solution. Inhibition was calculated as percentages in relation to the activity of the untreated enzyme.

Modification of the enzyme was carried out by the following reagents: *p*-diazobenzenosulphonic acid and *N*-bromosuccinimide, prepared as described by Vogel (1964); iodoacetate and iodoacetamide, products of Light & Koch (Colnbrook, England); 2-hydroxy-5-nitrobenzylbromide, obtained by the method of Koshland, Karkhanis & Latham (1964). Photo-oxidation was carried out in the presence of Rose Bengal preparation (obtained from the collection of the Jagiellonian University, Kraków) as described by Westhead (1965).

The excess of inhibitors was removed from enzyme solution by dialysis against 0.1 M-NaCl solution or by filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden). The column (20×1 cm.) was equilibrated with 0.1 M-acetate buffer of pH 5.5 and contained 30 mg% of crystalline bovine serum albumin (Sigma, Biochem. Co., St. Louis, Mo., U.S.A.). Fractions of 0.3 to 0.5 ml. were collected at a rate of 5 ml. per hour in the cold room.

RESULTS AND DISCUSSION

Coupling reaction with p-*diazobenzenosulphonic acid.* To 200 µl. of 0.025 M-tris-HCl buffer solution of pH 8.2, containing 1 to 15 mM-*p*-DBSA, 2 µl. of enzyme solution was added at 2°. The final concentration of enzyme was 0.01 mg. protein per ml. The sample was incubated at 2° for 60 min., then 50 µl. was withdrawn and incubated with 200 µl. of substrate solution for 3 min. as described in Methods. At 1 mM concentration of *p*-DBSA the enzymic activity was decreased by about 30%. At 7.5 mM concentration of the inhibitor the enzyme was inhibited by 50% and at 15 mM by 86%. The inhibition remained unchanged after 24 hr. dialysis against 0.1 M-NaCl solution. Under the conditions of incubation with *p*-DBSA, there is a possibility of formation of azotyrosine and azohistidine (Pontremoli, Grazi & Accorsi, 1967). The simultaneously observed loss of activity is in agreement with the participation of tyrosyl residues in the catalytic function of phosphatase, as it was suggested by the experiments with iodination of the enzyme (Bobrzecka *et al.*, 1968).

Photochemical oxidation. Photochemical oxidation of the phosphatase was carried out in the presence of Rose Bengal in 0.05 M-tris-HCl buffer solution of pH 8.3. A sample of 1 ml. containing 10 µg. of protein and 1 - 3 µg. of dye was irradiated at 10° with the light of a 500 Watt lamp focussed on the sample. After 30 min. irradiation, 50 µl. of the solution was taken for determination of enzymic activity

and 7 to 20% inactivation was observed. This experiment indicates that either histidine residues are little affected by photo-oxidation in the presence of 10 to 30-fold molar excess of the dye, or the oxidation of histidine does not influence the enzymic activity.

Carboxymethylation. The reaction was carried out at 22° using iodoacetate and iodoacetamide. In 0.05 M-citrate buffer solution of pH 5.5 and at enzyme concentration of 30 μg. per ml., 20 mM-iodoacetate caused only 11.5% inhibition after 5.5 hr., and 27.5% inhibition after 20 hr. of incubation. It should be noted that the reagent was not removed from the reaction mixture before the determination of enzyme activity. No inhibition was found at all when the enzyme was incubated with 10 mM-iodoacetamide in 0.05 M-tris-HCl buffer solution of pH 8.65, even after 24 hr. of incubation. These results are in agreement with those of Tsuboi & Hudson (1955), and indicate that carboxymethylation has little effect on the enzymic activity of prostatic phosphatase, and histidine does not seem to be an essential amino acid for catalytic function of this enzyme.

Treatment with NBS and HNBBr. The experiments described below support the view that tryptophan is an important amino acid in the catalytic function of prostatic phosphatase. NBS abolished the enzyme activity completely and irreversibly in 0.1 M-acetate buffer solution at pH 4 and 20° (cf. Fig. 1); complete inhibition

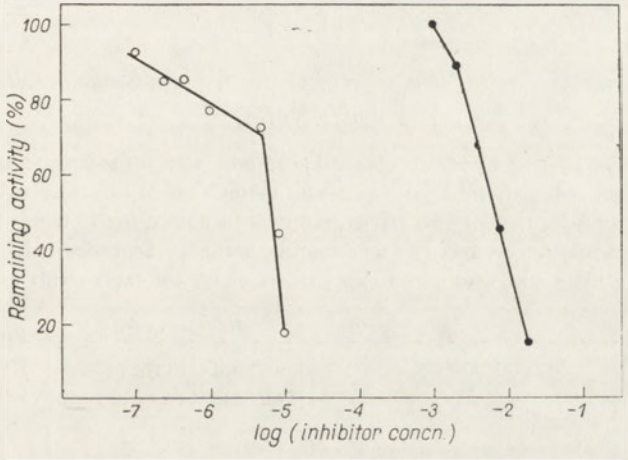

Fig. 1. The effect of (○), NBS and (●), HNBBr concentration on prostatic phosphatase activity. Enzyme, 20 μg./ml., was incubated for 3 min. (NBS) or 30 min. (HNBBr) at 20° with indicated concentration of the reagents in 0.1 M-acetate buffer solution of pH 4. In the case of reaction with HNBBr the sample contained 10% (v/v) of methanol. The enzyme activity was determined at pH 5 after removal of inhibitors by gel filtration.

being achieved at 0.02 mM concentration of the reagent. The relationship between percentage of inhibition and logarithm of the NBS concentration shows a two-step reaction. The first step up to 5 μM concentration of NBS represents about 30%

of inhibition, which is probably due to the oxidation of tryptophan residues; however, oxidation of tyrosine residues can not be excluded (Viswanatha & Lawson, 1961). The second step occurs at higher concentration of NBS and is supposed to be connected with a non-specific reaction, as for example cleavage of peptidyl bonds (Spande, Green & Witkop, 1966).

HNBBr, which also is reported to be a specific reagent for tryptophan (Koshland *et al.*, 1964), caused 85% of inhibition at the final concentration of about 10 mM, after 30 min. incubation at 20° (Fig. 1). Only high concentrations of this inhibitor were effective because of its marked instability in aqueous solution. About 50% inhibition was achieved within the first 3 min. of reaction and after further 30 min. of incubation there was only 10% drop in activity.

Experiments with *p*-DBSA and HNBBr were performed also in the presence of tartrate as the competitive inhibitor of prostatic phosphatase (Abul-Fadl & King, 1949). The enzyme (10 - 100 μg./ml.) was first incubated with 0.1 M-tartrate for 15 min., then treated with the appropriate reagent and after incubation under the described conditions (see Table 1) filtered on the Sephadex G-25 column, or dialysed against 0.1 M-NaCl and total enzyme activity was determined. In the presence of tartrate, the inhibition by *p*-DBSA was lower by 21% and the inhibition by HNBBr lower by 73% than in the absence of this competitive inhibitor (Table 1).

Table 1

The influence of tartrate on the inhibition of prostatic phosphomonoesterase by p-*DBSA and HNBBr*

Expt. I: the enzyme, 10 μg./ml., was incubated for 15 min. with 0.1 M-tartrate, then with 7.5 mM-*p*-DBSA for 90 min. at 0°; after dialysis against 0.1 M-NaCl for 24 hr., the activity of the enzyme was determined. Expt. II: the enzyme, 100 μg./ml., was incubated for 15 min. with 0.1 M-tartrate, then with 8 mM-HNBBr for 30 min. at room temperature; after Sephadex G-25 gel filtration, the enzyme activity was determined. The results of typical experiments are given.

	Inhibitor	Inhibition (%)		Protection from inhibition (%)
		without tartrate	in the presence of tartrate	
Expt. I	*p*-Diazobenzenosulphonic acid	50	38	21
Expt. II	2-Hydroxy-5-nitrobenzyl-bromide	33	9	73

The above described experiments seem to indicate that tryptophan residue, along with tyrosine residue (Bobrzecka *et al.*, 1968) influence the function of the prostatic phosphatase. The experiments show the importance of both tyrosine and tryptophan for catalytic function of prostatic phosphomonoesterase, but do not solve the question whether they directly take part in the active site of this enzyme or stabilize only its active conformation.

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ROLA AROMATYCZNYCH AMINOKWASÓW W AKTYWNOŚCI KWAŚNEJ FOSFOMONOESTERAZY STERCZU LUDZKIEGO

Streszczenie

Kwaśną fosfomonoesterazę z gruczołu krokowego poddano działaniu odczynników reagujących z aminokwasami aromatycznymi. *N*-bromosukcynimid powoduje zupełną i nieodwracalną inaktywację enzymu, podczas gdy *p*-hydroksy-5-nitrobenzylobromid, kwas *p*-dwuazobenzenosulfonowy, fotooksydacja i karboksymetylacja wykazują niewielki efekt lub w ogóle nie wpływają na aktywność enzymu. Winian jako inhibitor kompetytywny ochrania aktywność enzymu przed wpływem kwasu *p*-dwuazobenzenosulfonowego i *p*-hydroksy-5-nitrobenzylbromidu. Uzyskane wyniki sugerują, że reszty tryptofanu spełniają istotną rolę w katalitycznej funkcji enzymu.

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ON THE PROPERTIES OF ETHANOLAMINEPHOSPHATE CYTIDYLYLTRANSFERASES IN ANIMAL TISSUES

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1. Ethanolaminephosphate cytidylyltransferase (EC 2.7.7.14) catalysing the formation of CDP-ethanolamine was shown to be present in chicken kidney and gut mucosa, i.e. in the tissues in which formation of serine ethanolamine phosphate is known to occur. The kinetics, molecular weights, optimum pH and effect of some possible activators and inhibitors of the enzyme were studied. 2. Some of the unnatural analogues of CDP-ethanolamine containing other aminoalcohols instead of ethanolamine were tested in the enzymic synthesis of serine ethanolamine phosphate.

Cytidine diphosphate ethanolamine (CMP-PE)[1] is involved in two main synthetic pathways leading to phosphoric acid diesters. The better known and principal one leads to formation of phosphatidylethanolamine (Kennedy & Weiss, 1956). From the studies of Rosenberg's group another process of the utilization of CMP-PE became evident, i.e. the formation of L-serine ethanolamine phosphate (Rosenberg, Ennor & Sugai, 1964). The occurrence of this compound is restricted to fish, birds, reptiles and amphibians (Rosenberg & Ennor, 1961).

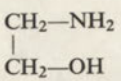
It has been shown that SEP is the biological precursor of lombricine (2-guanidinoethyl-2-amino-2-carboxyethyl hydrogen phosphate) which is formed by the transfer of the amidino group of arginine to SEP (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959). Lombricine was first isolated from earthworms (*Lumbricus terrestris*) by Van Thoai & Robin (1954), who held the view that *N*-phosphoryllombricine is the phosphagen of the species.

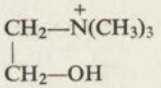
Some characteristics of the formation of CMP-PE in mammalian tissues where its only role consists in taking part in the reaction catalysed by diglyceride ethanolaminephosphotransferase (EC 2.7.8.1) are known. No data are, however, available on the CMP-PE synthetizing system in those organisms where it also is involved in the formation of water-soluble ethanolamine phosphodiesters. The aim of the

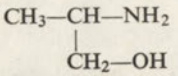
[1] Abbreviations used: PAPr, 2-aminopropan-1-ol phosphate; PAiPr, 1-aminopropan-2-ol phosphate; PC, choline phosphate; PE, ethanolamine phosphate; PGE, 2-guanidinoethanol phosphate; PAMPr, 2-amino-2-methylpropan-1-ol phosphate; CMP-PAPr, CMP-PAiPr, CMP-PE and CMP-PGE are the CMP derivatives of the respective aminoalcohol phosphates. SEP, serine ethanolamine phosphate.

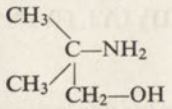
present paper was to characterize this enzyme in chicken and rat tissues by kinetic data, gel filtration and the effect of some possible activators and inhibitors.

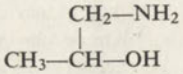
Some unnatural analogues of CMP-PE containing instead of ethanolamine another aminoalcohol (see Scheme 1), were also tested in the enzymic synthesis

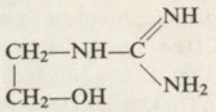

Ethanolamine (E)

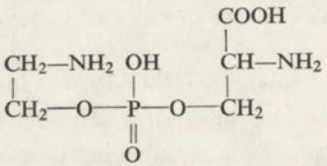

Choline (C)


2-Aminopropan-1-ol (APr)


2-Amino-2-methylpropan-1-ol (AMPr)


1-Aminopropan-2-ol (AiPr)


2-Guanidinoethanol (GE)


Serine ethanolamine phosphate (SEP)

Scheme 1.

of analogues of serine ethanolamine phosphate. This part of work was kindly performed for us by Mr. A. K. Allen at the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra.

MATERIALS AND METHODS

Chemicals. [^{32}P]Orthophosphate was the product of Institute of Nuclear Research, Warszawa, Poland. Ethanolamine phosphate [PE] labelled with ^{32}P and choline phosphate (PC) were prepared as described by Ansell & Chojnacki (1966). [β-^{32}P]-Cytidine diphosphate ethanolamine (CMP-^{32}PE) was prepared according to Chojnacki & Metcalfe (1966). 1-Aminopropan-2-ol phosphate (PAiPr) and 2-amino-2-methyl-propan-1-ol phosphate (PAMPr) were of the same origin as those used by Chojnacki & Ansell (1967). Cytidine diphosphate-2-guanidinoethanol (CMP-^{32}PGE), cytidine diphosphate 2-aminopropan-1-ol (CMP-^{32}PAPr) and cytidine diphosphate 1-amino-propan-2-ol (CMP-^{32}PAiPr) were prepared according to Chojnacki & Ansell (1967) and Chojnacki (1964). ATP, CTP, UTP and NAD were from Sigma Chemical Company (St. Louis, Mo., U.S.A.). Sephadex G-100 and G-200 were Pharmacia

Ltd. (Uppsala, Sweden) products. Charcoal (Zakł. Elektr. Węgl., Racibórz, Poland) was treated according to Threlfall (1957).

Protein standards for molecular weight estimation (ribonuclease A from bovine pancreas, human albumin and human γ-globulin) were of the same origin as those used by Chojnacki, Radomińska-Pyrek & Korzybski (1967).

Analytical. Phosphorus, ^{32}P, and protein were analysed as described previously by Chojnacki *et al.* (1967).

Animals and homogenate. White Rock chickens of both sexes, 12 weeks old and white Wistar rats, 6 weeks old, weighing 200 - 220 g. were used. The chicken gut mucosa was prepared as described by Rosenberg & Ennor (1965). The 105 000 *g* supernatants were obtained from rat liver, rat brain, chicken kidney and chicken gut mucosa as previously described (Chojnacki *et al.*, 1967).

Gel filtration. Five-ml. sample of the 105 000 *g* supernatant was applied to Sephadex G-100 column (1.2 × 100 cm.) or Sephadex G-200 column (1.5 × 100 cm.). Elution was performed with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5, and 1 ml. fractions were collected.

Assay for CMP-PE synthesis. The standard incubation medium contained in a total volume of 0.5 ml.: 30 mM-sodium phosphate buffer, pH 7.5, 15 mM-magnesium chloride, 0.2 mM-CTP, 0.1 mM-^{32}PE (specific activity 4×10^5 to 3.3×10^6 counts/min./μmole) and 105 000 *g* supernatant in amounts: 50 μl. from rat liver and 100 μl. from rat brain, chicken kidney and chicken gut mucosa. The activity of ethanolaminephosphate cytidylyltransferase was measured as previously described (Chojnacki *et al.*, 1967).

SEP biosynthesis. The activity of SEP synthetase was estimated by Mr. Allen of the John Curtin School of Medical Research, Australian National University using the technique described by Allen & Rosenberg (1968).

RESULTS

Gel filtration. The elution patterns from Sephadex G-100 of the ethanolaminephosphate cytidylyltransferase from rat liver, chicken kidney and chicken gut mucosa are shown in Fig. 1. Similar results were obtained on Sephadex G-200. In all our experiments a double peak of enzymic activity was observed irrespectively of the origin of enzyme. The enzymes from rat liver, chicken kidney and chicken gut mucosa had the same molecular weight, 40 000, as calculated according to Andrews (1964) for the fraction present in the main peak.

The effect of pH on ethanolaminephosphate cytidylyltransferases activity was studied by varying the sodium phosphate buffer in the standard assay over the pH range 5.6 - 8.0. The enzymes from rat liver, chicken kidney and chicken gut mucosa showed two peaks of optimum activity, one at pH 6.2 and another at pH 7.7, whereas the enzyme from rat brain showed a single maximum at pH 7.7 - 7.8 (Fig. 2).

Kinetic studies. All studied tissue extracts exhibited distinct activity of ethanolaminephosphate cytidylyltransferase. The substrate affinity of the studied enzymes were similar for PE and CTP. When the concentration of CTP was constant and con-

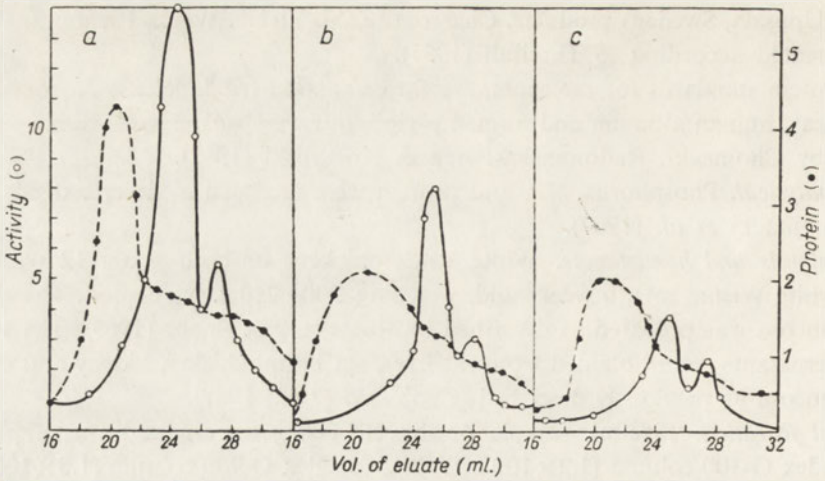


Fig. 1. Sephadex gel filtration of the 105 000 *g* supernatant from (*a*), rat liver; (*b*), chicken kidney; and (*c*), chicken gut mucosa. The column (1.2×100 cm.) was eluted with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5. Fractions of 1 ml. were collected and assayed for ethanolaminephosphate cytidylyltransferase activity by the standard procedure described in the text. The activities are expressed in mμmoles of synthetized CMP-PE per 1 ml., and the amount of protein in mg./1 ml.

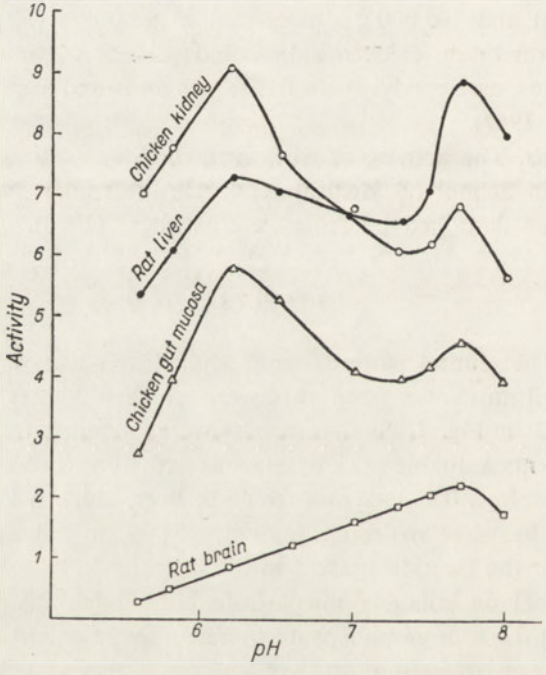


Fig. 2. Effect of pH on the activity of ethanolaminephosphate cytidylyltransferase. The experimental conditions were the same as those of assay method, except that the pH of the system was varied from 5.6 to 8.0 using appropriate sodium phosphate buffers. The pH of the incubation mixture was measured with a Radiometer-Copenhagen type 22 pH-meter. (●), Rat liver enzyme; (○), chicken kidney enzyme; (△), chicken gut mucosa enzyme; (□), rat brain enzyme. Activities are expressed in mμmoles of synthetized CMP-PE.

centration of PE varied the K_m values for PE were 77 mM for rat liver enzyme, 62 mM for chicken kidney enzyme and 43.5 mM for chicken gut mucosa (Fig. 3a). When the concentration of PE was maintained constant and the concentration of CTP varied, the K_m values for CTP were: 44.7 mM, 40 mM and 44.5 mM for the rat liver, chicken kidney and chicken gut mucosa enzymes, respectively (Fig. 3b).

The effect of analogues of PE and CTP on the formation of CMP-PE. No effect of phosphorylaminoalcohols was observed when either PAiPr, PAMPr or PC were present in the reaction mixture at concentrations of 0.05 - 0.5 mM. The enzymic reaction of PE with CTP was unaffected by the presence of ATP, UTP and NAD at concentrations of 0.05 - 0.5 mM. In fact slight stimulation (about 20%) was observed with our preparations of ATP and UTP. NAD did not affect the reaction at all.

The specificity of the SEP synthetizing system. The specificity of CMP-PE synthetizing system has been studied previously (Chojnacki, 1964; Chojnacki & Ansell,

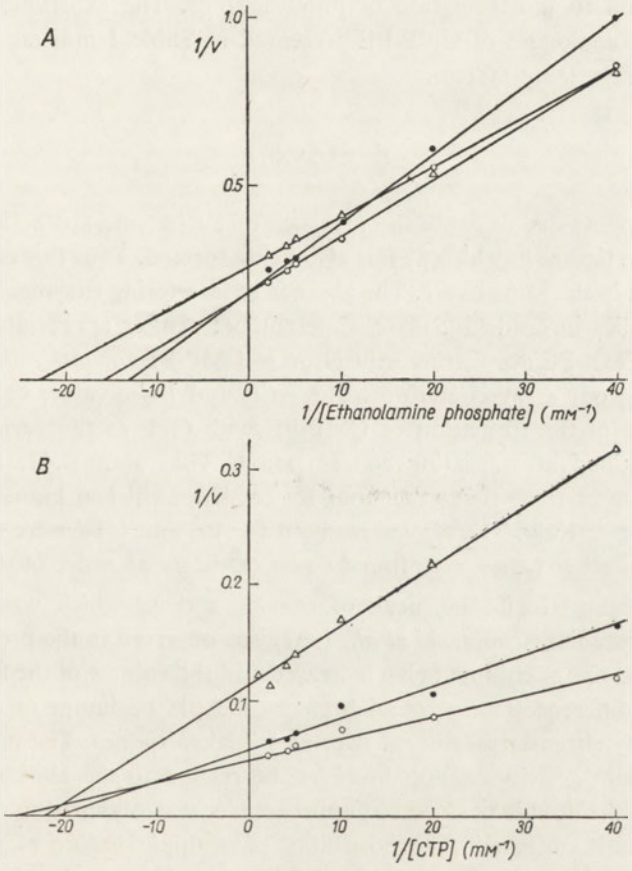


Fig. 3. Lineweaver and Burk plots for the synthesis of CMP-PE catalysed by the enzyme from (●), rat liver; (○), chicken kidney; and (△), chicken gut mucosa. Standard incubation mixture except that in *A*, 0.025 - 0.5 mM-^{32}PE and in *B*, 0.025 - 0.5 mM-CTP were present. Incubation for 5 min. at 37°. *v*, mμmoles of synthetized CMP-PE per sample.

Table 1

Incorporation of ^{32}P *from CMP-*[^{32}P]*bases into SEP and lipids in the chicken gut microsomes*

Substrate used	Radioactivity (counts /min. /μmole)	SEP			Lipids		
		counts /min.	mμmoles /hr.	mμmoles /mg. protein /hr.	counts /min.	mμmoles /hr.	mμmoles /mg. protein /hr.
CMP-^{32}PE	2.1×10^6	8 650	4.3	2.62	11 092	5.3	3.23
CMP-^{32}PGE	2.95×10^6	36	0.01	0.01	17 515	5.9	3.60
CMP-^{32}PAPr	1.62×10^6	160	0.10	0.06	1 044	0.65	0.40
CMP-^{32}PAiPr	1.77×10^6	690	0.40	0.24	4 280	2.65	1.31

1967) in relation to the formation of phospholipids. The experiments with some aminoalcoholic analogues of CMP-PE presented in Table 1 indicate the specificity of the SEP synthetizing system.

DISCUSSION

Our experiments demonstrate the presence of an enzyme synthetizing CMP-PE in those chicken tissues in which SEP is also being formed. Thus this active substrate can be supplied in the same tissue. The absence of interfering enzymes made possible the kinetic studies in crude 105 000 *g* supernatant: neither appreciable decomposition of PE or CMP-PE nor further utilization of CMP-PE for biosynthetic processes was observed in our assay conditions. The estimated V_{max} values enabled calculation of the rate of the formation of CMP-PE with CTP as the variable substrate and PE maintained at saturating concentration. They were 0.42, 1.15 and 1.72 μmoles per gram of fresh tissue per hour for rat liver, chicken kidney and chicken gut mucosa, respectively. The K_m values both for PE and CTP were similar. Molecular weights of all enzymes were found to be of the same order (40 000).

The phenomenon of double peak of enzyme activity which was undetectable in our previous studies (Chojnacki *et al.*, 1967) was observed in the presented experiments due to the size of column being increased and the volume of the fractions being decreased. No differences were found between the pH optimum of ethanolaminephosphate cytidylyltransferases in rat liver and chicken tissues. The presence of two peaks of optimum activity cannot, however, be related to the ability of the tissue to form SEP as the double peak of optimum activity was obtained in rat liver where no such process is observed. The possibility of multiple nature of ethanolaminephosphate cytidylyltransferases as inferred from the pH curve has been supported in gel filtration experiments. The use of other methods of protein fractionation might solve this problem. The previous work on the specificity of rat brain and liver ethanolaminephosphate cytidylyltransferase (Chojnacki & Ansell, 1967) has shown

that the enzyme is fairly specific in relation to aminoalcohol moiety of the cytidine diphosphate base. The present experiments with two of the previously used analogues indicate that this is also true for chicken liver and gut. Moreover, from the present experiments it can be concluded that propanolamine phosphates have no inhibitory effect on the phosphoethanolamine activating enzyme. The results with CMP-PGE, CMP-PAPr and CMP-PAiPr in the experiments on the formation of SEP and phospholipids in chicken gut mucosa support the previous results in respect to phospholipids formation (Chojnacki, 1964; Chojnacki & Ansell, 1967). While the incorporation of unnatural phosphorylaminoalcohols from their cytidylyl derivatives into phospholipids was considerable, in the reaction with serine only CMP-PE could act effectively. This is in accord with the results of Allen & Rosenberg (1968) who employed for the synthesis of SEP other cytidine diphosphate bases.

The autor expresses her sincere gratitude to Doc. Dr. T. Chojnacki for guidance in the course of this work.

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O WŁASNOŚCIACH CYTYDYLILOTRANSFERAZ ETANOLOAMINOFOSFORANOWYCH W TKANKACH ZWIERZĘCYCH

Streszczenie

1. Stwierdzono występowanie cytydylilotransferazy etanoloaminofosforanowej (EC 2.7.7.14) katalizującej powstawanie CDP-etanoloaminy w nerce i śluzówce jelita kurczęcia, tj. w tkankach gdzie zachodzi synteza serynoetanoloaminofosforanu. Przeprowadzono badania kinetyczne, wpływu pH i inhibitorów oraz oznaczono ciężar cząsteczkowy badanego enzymu.

2. Zbadano specyficzność reakcji enzymatycznej syntezy serynoetanoloaminofosforanu w tkankach kurczęcia, stosując aminoalkoholowe analogi CDP-etanoloaminy.

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HYDROXYLATION OF TRYPTOPHAN BY RAT-LIVER EXTRACTS

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1. Hydroxylation of tryptophan in crude liver extracts is inhibited by phenylalanine and phenylpyruvate. The inhibition by phenylalanine is of the competitive type. The inhibition by phenylpyruvate is bi-phasic; in the first phase (up to 1.6 mM-phenylpyruvate concentration) the inhibition appears to be uncompetitive, in the second phase (from 4 mM-inhibitor concentration) competitive. 2. Intraperitoneal administration of phenylalanine and tryptophan causes a 50% decrease of tryptophan hydroxylation by liver extracts 2 and 4 hr., respectively, after the injection.

Freedland, Wadzinski & Waisman (1961) reported on formation of 5-hydroxyindoles by rat-liver extract in the presence of nicotinamide-adenine dinucleotide, oxygen and tryptophan at relatively high concentration. At the same time Renson, Goodwin, Weissbach & Udenfriend (1961) demonstrated using phenylalanine hydroxylase (Fraction I) from rat liver and tetrahydrofolate dehydrogenase (Fraction II) from sheep liver, the pteridine cofactor and $NADPH_2$, that the enzyme system which catalyses the hydroxylation of phenylalanine catalyses also the hydroxylation of tryptophan. It may be therefore supposed that the impairment of phenylalanine hydroxylase in inborn phenylketonuria is connected with a decrease in 5-hydroxytryptophan formation.

It has been shown in previous studies that transient phenylketonuria can be induced in the rat by intraperitoneal administration of a single dose of phenylalanine (Bełżecka, 1965; Bełżecka, Jakubiec & Pużyńska 1966, 1967). After phenylalanine administration the following features characteristic of phenylketonuria were observed: a 50-fold increase in the content of phenylalanine in blood, excretion of phenylpyruvate in urine, and a 70% decrease of phenylalanine hydroxylase activity in liver.

In connection with the above findings, in the present work the effect of administration *in vivo* of phenylalanine and tryptophan on the activity of tryptophan hydroxylation by liver extract was studied. Moreover, the effect of phenylalanine and phenylpyruvate on tryptophan hydroxylation *in vitro* was examined.

MATERIALS AND METHODS

Animals. White rats 4 - 5 months old, weighing 180 - 200 g. were used.

Liver extract. The animals were killed by decapitation, the liver was immediately removed and homogenized with 2 volumes of 0.9% KCl as described previously (Bełżecka *et al.*, 1967). The 13 000 *g* (60 min.) supernatant was used for experiments, and is further referred to as liver extract. The extract obtained from 1 g. of liver contained 90 - 120 mg. of protein.

Assay for tryptophan hydroxylation activity. The assay was performed after Freedland *et al.* (1961). The standard incubation mixture contained in a final volume of 2.5 ml.: liver extract, 30 - 40 mg. of protein (1.0 ml.), 1 μmole of NAD, 10 μmoles of nicotinamide, 1 M-Na,K-phosphate buffer, pH 6.7 (0.2 ml.) and 45 μmoles of L-tryptophan. After incubation at 37° with shaking, the reaction was stopped by adding 1 ml. of 20% trichloroacetic acid, and the formed 5-hydroxyindoles, designated further as 5-HTP, were determined by the α-nitroso-β-naphthol colorimetric method after Renson, Weissbach & Udenfriend (1962). It was found that 5 ml. of ethylene dichloride was sufficient to remove the excess of α-nitroso-β-naphthol, and that it could be replaced by the same amount of methylene chloride. The presence of 8 mM-phenylalanine, 0.14 mM-tyrosine, 16 mM-phenylpyruvate and 0.4 mM-*p*-hydroxyphenylpyruvate in the final colorimetric sample had no effect on the reaction of standard 5-hydroxytryptophan with α-nitroso-β-naphthol.

Assay for phenylalanine hydroxylase activity. The liver extract was incubated under the same conditions as given for the determination of tryptophan hydroxylation, except that 8 μmoles of L-phenylalanine per sample was used as substrate. The tyrosine formed was determined colorimetrically according to Ceriotti & Spandrio (1957) as a complex with α-nitroso-β-naphthol.

It was found that the presence of 0.2 mM tryptophan in the colorimetric sample interfered with formation of the tyrosine - α-nitroso-β-naphthol complex; therefore it proved impossible to study the effect of tryptophan on phenylalanine hydroxylation *in vitro* and *in vivo*. The presence of 0.02 mM-5-hydroxytryptophan in the colorimetric sample did not interfere with complex formation.

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

Special reagents. L-Tryptophan (Aldrich Chem. Co. Inc., Milwaukee, Wis., U.S.A.); L-phenylalanine, sodium phenylpyruvate and DL-5-hydroxytryptophan (Nutr. Biochem. Corp., Cleveland, Ohio, U.S.A.); DL-5-methyltryptophan (Fluka A. G., Buchs, Switzerland); NAD (C.F. Boehringer, Mannheim, West Germany); nicotinamide and trichloroacetic acid (Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland); L-tyrosine (Zakłady Farmaceutyczne Polfa, Warszawa, Poland); *p*-hydroxyphenylpyruvate (Sigma Chem. Comp., St. Louis, Mo., U.S.A.); and α-nitroso-β-naphthol (E. Merck, Darmstadt, West Germany).

RESULTS

Hydroxylation of tryptophan and phenylalanine by rat-liver extract

The rate of tryptophan hydroxylation was linear for at least 30 min. of incubation, and the maximum activity was observed after 60 min. (Fig. 1). Saturation of the enzyme with substrate occurred both after 30 and 60 min. in the presence of 18 mM-tryptophan (45 μmoles/sample, Fig. 2). Hydroxylation of tryptophan, like

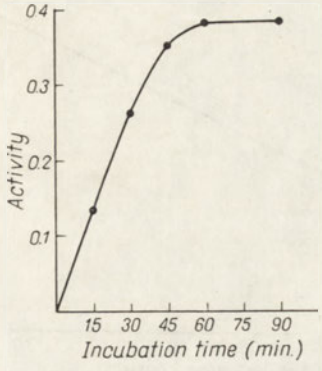


Fig. 1

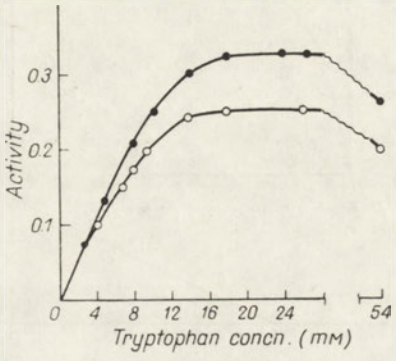
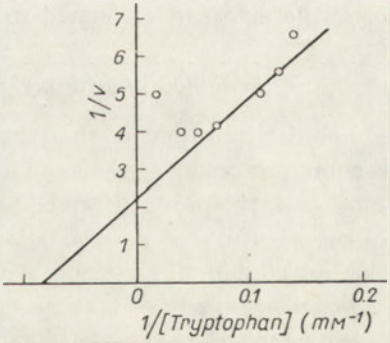


Fig. 2

Fig. 1. The effect of time of incubation on the activity of tryptophan hydroxylation. The standard incubation mixture contained in a volume of 2.5 ml.: 1 ml. of the liver extract (30 - 40 mg. protein), 1 μmole of NAD, 10 μmoles of nicotinamide, 1 M-Na,K-phosphate buffer, pH 6.7 (0.2 ml.) and 45 μmoles of tryptophan. The activity is expressed as μmoles of 5-HTP formed/100 mg. protein.

Fig. 2. The effect of substrate concentration on the activity of tryptophan hydroxylation. The standard incubation mixtures with tryptophan concentrations varying from 2.4 to 54 mM, were applied. The activity is expressed as μmoles of 5-HTP formed/100 mg. protein at (○), 30 min. and (●), 60 min. incubation.

Fig. 3. Lineweaver-Burk plot of the effect of substrate concentration on the activity of tryptophan hydroxylation. The standard incubation mixtures with tryptophan concentrations varying from 7 to 54 mM, were used. The activity, *v*, is expressed as μmoles of 5-HTP formed/100 mg. protein/30 min.



that of phenylalanine (Udenfriend & Cooper, 1952; Bełżecka *et al.*, 1967), was inhibited by high substrate concentration. In the presence of 54 mM-tryptophan a 20% decrease in enzymic activity was observed. At 18 mM-tryptophan concentration the activity was linear with 8 - 40 mg. of protein during 30 and 60 min. incubation. The maximum activity corresponded to formation of 0.22±0.05 μmoles

of 5-HTP/100 mg. protein/30 min., and 0.32±0.08 μmoles/60 min. (mean values of 18 and 43 determinations, respectively).

Despite the difficulties in interpreting kinetic data obtained with a crude enzyme preparation, an approximate K_m value for tryptophan of 12 mM was calculated from the Lineweaver-Burk plot shown in Fig. 3. There is evidence of substrate inhibition with large tryptophan concentrations.

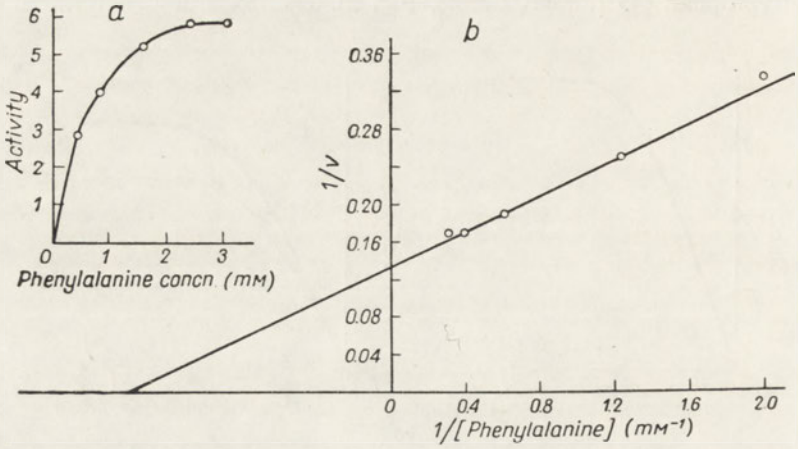


Fig. 4. (*a*), Effect of substrate concentration on the activity of phenylalanine hydroxylation and (*b*), Lineweaver-Burk plot of the same data. The standard incubation mixture as described in Methods containing 0.4 - 3.2 mM-phenylalanine was used. The activity, *v*, is expressed as μmoles of tyrosine formed/100 mg. protein/30 min.

Under the same assay conditions the rate of phenylalanine hydroxylation was linear for 30 min. At the saturating concentration of phenylalanine, 2.5 mM, the activity was 6 μmoles of tyrosine formed/100 mg. of protein/30 min. (Fig. 4a). The approximate K_m value determined for phenylalanine was much lower than for tryptophan and amounted to 0.7 mM (Fig. 4b).

Inhibition of hydroxylation of tryptophan in rat-liver extract

For determination of the type of inhibition, an incubation time at which the reaction proceeded at the initial velocity rate could not be applied because the amounts of the product formed were so low that it was difficult to achieve the necessary accuracy of determinations. Therefore in most instances the incubation time was 60 min., i.e. the time at which the maximum activity was observed.

Freedland *et al.* (1961) demonstrated that hydroxylation of tryptophan is competitively inhibited by phenylalanine. The same was found in our experiments. Phenylalanine at 0.8 mM concentration inhibited the hydroxylation of tryptophan by 50% and at 4 mM concentration the inhibition was complete (Fig. 5, curve *1*). The results of this experiment plotted according to Lineweaver & Burk, 1/*v* *versus* 1/[S], and according to Dixon, 1/*v* *versus* [I] (Webb, 1963) indicate that the inhibition by phenylalanine is of the competitive type (Fig. 6a, b).

The hydroxylation of tryptophan was also inhibited by 5-methyltryptophan, an analogue of 5-HTP. At concentrations of 4, 8 and 16 mM, DL-5-methyltryptophan inhibited the formation of 5-HTP by 20, 27 and 31%, respectively (Fig. 5, curve *2*).

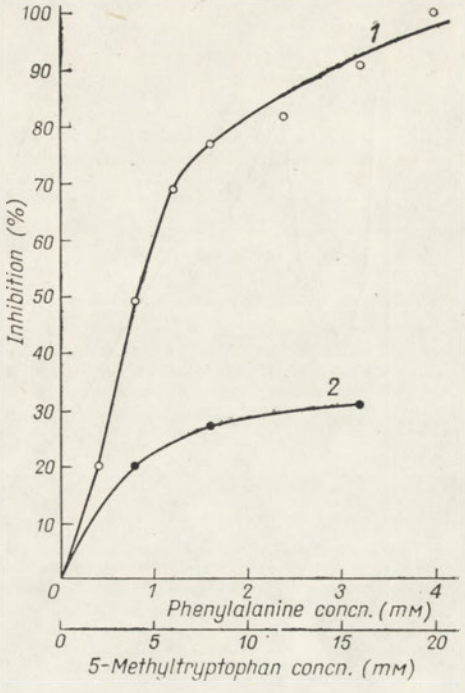


Fig. 5. Inhibition of tryptophan hydroxylation by (○), phenylalanine and (●), 5-methyltryptophan. To the standard incubation mixtures various amounts of inhibitors were added. Incubation time 60 min.

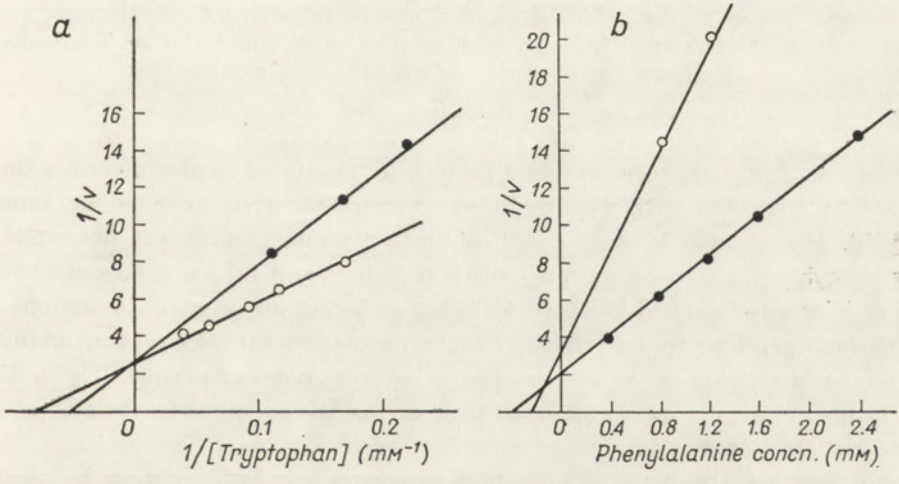


Fig. 6. Graphical determination of the inhibiting effect of phenylalanine on tryptophan hydroxylation. (*a*), Lineweaver-Burk plots for the tryptophan hydroxylation (○), uninhibited and (●), inhibited by 0.1 mM-phenylalanine. Standard incubation mixtures contained 4.5 - 28 mM-tryptophan. (*b*), The method of Dixon. The incubation mixtures contained tryptophan at (○), 9 mM, and (●), 18 mM concentration; and 0.4 - 2.4 mM-phenylalanine. The activity, *v*, is expressed as μmoles of 5-HTP formed/100 mg. protein/60 min.

Phenylpyruvate, which has been found to inhibit the hydroxylation of phenylalanine (Bełżecka *et al.*, 1967), inhibited also the hydroxylation of tryptophan. Fig. 7 illustrates the effect of varying concentrations of phenylpyruvate and various

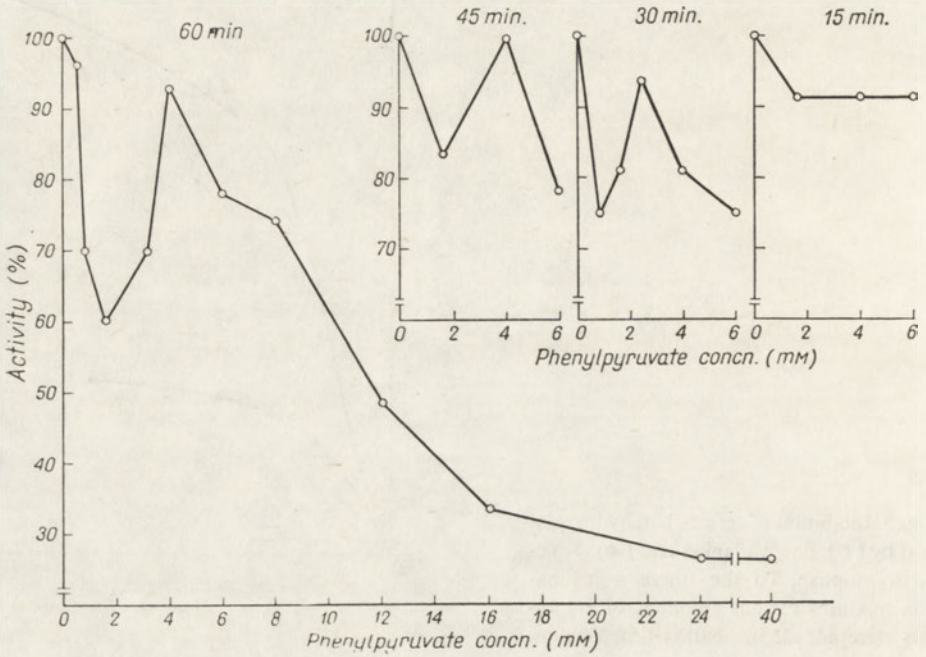


Fig. 7. The effect of phenylpyruvate concentration on the activity of tryptophan hydroxylation at different time of incubation. To standard incubation mixtures, 0.4 - 40 mM-phenylpyruvate was added, and incubated for 15, 30, 45 and 60 min., as indicated in the Figure. The results are expressed in percentages of the uninhibited reaction.

ncubation time on the formation of 5-HTP. After 15 min. of incubation only a small, about 10%, inhibition by phenylpyruvate was found, irrespective of the amount applied. However, at longer incubation times a peculiar effect was observed. At low phenylpyruvate concentration, 0.8 mM at 30 min. and 1.6 mM at 45 and 60 min., the activity was inhibited by 20 to 40%, but at higher inhibitor concentrations the inhibition appeared to be smaller, and in the presence of 4 mM-phenylpyruvate the starting activity of the extract was recovered. At concentrations higher than 4 mM, phenylpyruvate again inhibited the reaction but complete inhibition was not observed even at 40 mM.

To determine the type of inhibition of tryptophan hydroxylation by phenylpyruvate, two sections of the plot of hydroxylase activity (at 60 min. incubation) *versus* inhibitor concentration were examined (see Fig. 7). The first section represents the inhibition at phenylpyruvate concentrations up to 1.6 mM, and the second the inhibition observed with 4 - 40 mM-phenylpyruvate. The Lineweaver-Burk plots (Fig. 8) with two phenylpyruvate concentrations corresponding to the first

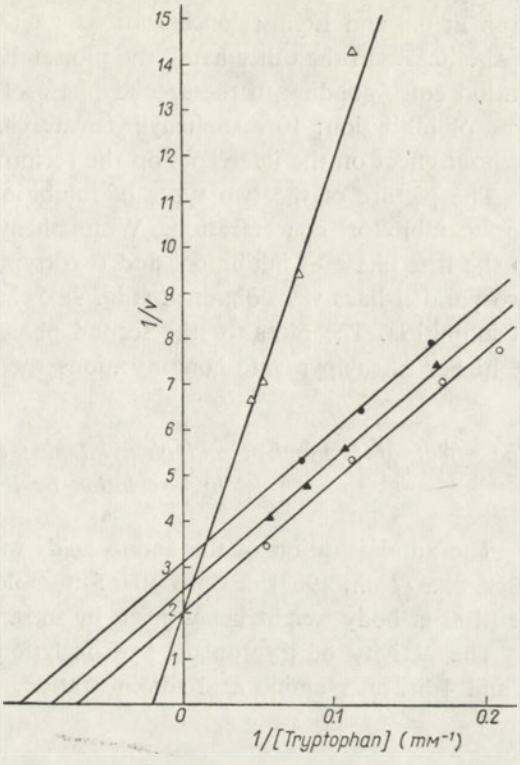


Fig. 8. Lineweaver-Burk plots of the inhibitory effect of phenylpyruvate on tryptophan hydroxylation. The standard incubation mixtures contained 5 - 18 mM-tryptophan. (○), Without inhibitor, with (▲), 0.8 mM, (●), 1.6 mM and (△), 10 mM-phenylpyruvate. The activity, *v*, is expressed as μmoles of 5-HTP formed/100 mg. protein/60 min.

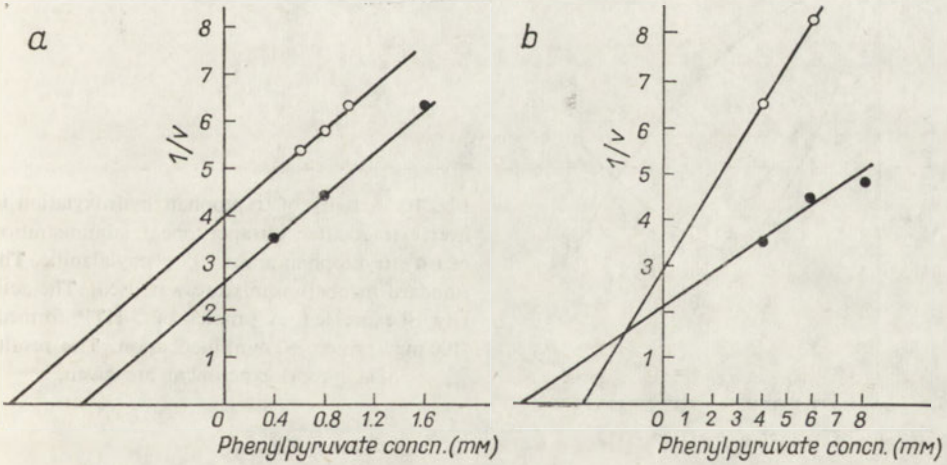


Fig. 9. Dixon plots on the inhibitory effect of phenylpyruvate on tryptophan hydroxylation. The standard incubation mixture contained: (○), 9 mM and (●), 18 mM-tryptophan, and varying concentrations of phenylpyruvate. (*a*), Phenylpyruvate concentrations belonging to the first phase of inhibition (up to 1.6 mM); (*b*), concentrations belonging to the second phase of inhibition (4 - 8 mM). The activity, *v*, is expressed as μmoles of 5-HTP formed/100 mg. protein/60 min.

phase of inhibition give an indication of uncompetitive inhibition (Webb, 1963). Both at 0.8 and 1.6 mM concentrations phenylpyruvate did not change the slope of the lines. On the other hand, the plot at 10 mM-phenylpyruvate, i.e. the concentration corresponding to the second phase of inhibition, indicates the competitive type of inhibition; 10 mM-phenylpyruvate changed the slope of the line but was without effect on the intercepts on the reciprocal velocity axis.

The picture of the two types of inhibition was also obtained by plotting $1/v$ *versus* inhibitor concentration. With phenylpyruvate concentrations belonging to the first phase of inhibition, and two tryptophan concentrations, 9 and 18 mM, two parallel lines were obtained (Fig. 9a), characteristic of the uncompetitive type of inhibition. The plots for the second phase of inhibition (Fig. 9b) suggest that at higher phenylpyruvate concentrations the inhibition is competitive.

The effect of tryptophan and phenylalanine administration in vivo *on tryptophan hydroxylation by liver extract*

The administration of the amino acids was performed as described previously (Bełżecka *et al.*, 1967), a dose of 0.5 m-mole of L-tryptophan or L-phenylalanine per 100 g. body weight being given by intraperitoneal injection.

The activity of tryptophan hydroxylation in liver extracts was measured at 2 and 4 hr. after amino acid administration. Two hours after tryptophan injection

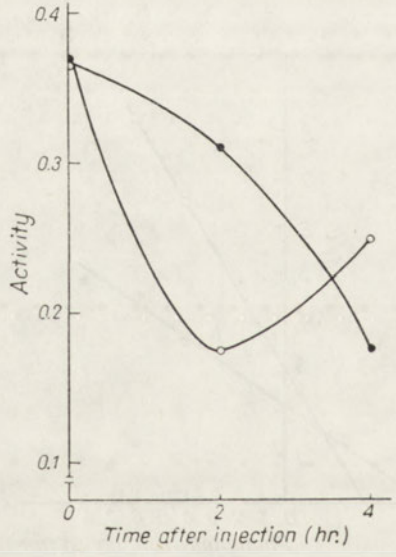


Fig. 10. Activity of tryptophan hydroxylation in liver extract after intraperitoneal administration of (●), tryptophan and (○), phenylalanine. The standard incubation mixture was used. The activity is expressed as μmoles of 5-HTP formed/ /100 mg. protein/60 min. incubation. The results of a typical experiment are given.

the activity was decreased by 16%, and after 4 hr. by 50% as compared with the control rat. With phenylalanine the highest, 50%, inhibition was observed 2 hr. after the injection, and then the activity increased (Fig. 10). This difference in the effect of the two amino acids may be due to differences in the rate of their accumula-

tion in the liver. However, it should be noted that in the previous study concerning the effect of phenylalanine administration on phenylalanine hydroxylase (Bełżecka *et al.*, 1967) the greatest inhibition, 70%, was observed at 4 hr. after the injection and lasted for a further 4 hr.

DISCUSSION

The inhibiting effect of phenylpyruvate on tryptophan hydroxylation by liver extract was reported by Freedland *et al.* (1961). In our previous work it was found that phenylpyruvate inhibits the phenylalanine hydroxylation. The activity of liver extract decreased linearly with increasing (1.6 - 11 mM) inhibitor concentration, and in the presence of 11 mM-phenylpyruvate the inhibition of phenylalanine hydroxylation was complete (Bełżecka *et al.*, 1967). The course of inhibition of tryptophan hydroxylation was quite different, and with increasing phenylpyruvate concentration a bi-phasic inhibition, indicating the occurrence of two types of inhibition, was observed. The course of this inhibition is unparalleled by the action of any of the so far known inhibitors; it is difficult to interpret this phenomenon on the basis of the present studies performed on crude liver extracts. It could be suggested that phenylpyruvate modifies the conformation of the enzyme. The possibility of interaction with the two enzymes, i.e. 4-hydroxylase (Fraction I) and tertahydrofolate dehydrogenase (Fraction II) composing the phenylalanine hydroxylating system should be also considered.

Phenylalanine inhibited the hydroxylation of tryptophan competitively, which is obviously dependent on the greater affinity of the enzyme for phenylalanine than for tryptophan. In the presence of 4 mM-phenylalanine, i.e. at the saturating substrate concentration, no formation of 5-HTP was observed. We were unable to determine the extent of simultaneous conversion of phenylalanine to tyrosine. Tryptophan inhibited the formation of the tyrosine - α-nitroso-β-naphthol complex, which served for the colorimetric estimations of tyrosine. The attempts at removing tryptophan from the deproteinized incubation mixture before the tyrosine determination were unsuccessful.

The competitive inhibition of tryptophan hydroxylation by phenylalanine seems to indicate the common active centre of the enzyme for both substrates. Freedland (1963) demonstrated, however, that *in vitro* ascorbic acid and Fe^{2+} ion activated the hydroxylation of tryptophan whereas EDTA acted as an inhibitor; neither of these substances had any effect on phenylalanine hydroxylation.

Phenylalanine administered to rats decreased the activity of tryptophan hydroxylation in liver. This finding could explain the lowered serotonin content in blood serum of phenylketonuric children demonstrated by Pare, Sandler & Stacey (1957). However, it is difficult to explain the presence of even small amounts of serotonin in blood serum. The tryptophan hydroxylation activity is very low and when, as in phenylketonurics, it is inhibited, no formation of 5-HTP, the precursor of serotonin, can be expected. Renson *et al.* (1962) found that the decreased content of serotonin in rat brain after phenylalanine injection did not return to normal

value on simultaneous administration of 5-HTP. The authors assumed that phenylalanine inhibited the transport of 5-HTP from liver to brain. The suggestion was also put forward that other tissues could contain a tryptophan hydroxylation-catalysing enzyme other than the hydroxylation system present in liver. Green & Sawyer (1966) demonstrated in the mitochondrial fraction of brainstem the presence of an enzyme which converted [^{14}C]tryptophan through [^{14}C]5-HTP to [^{14}C]serotonin. The enzyme activity was not affected by 80 μM-L-tyrosine or L-phenylalanine, 100 μM-2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridin, 100 μM-$NADPH_2$, or 13 mM-NaH_2PO_4. In our previous studies (Bełżecka *et al.*, 1967) it was demonstrated that phenylalanine administered *in vivo* decreased the DOPA decarboxylase activity in rat brain. Thus it seems possible that in patients with phenylketonuria the activity of 5-HTP decarboxylase in brain is lowered, leading to the decreased serotonin content with all its clinical implications.

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HYDROKSYLACJA TRYPTOFANU W WYCIĄGACH WĄTROBY SZCZURA

Streszczenie

1. Hydroksylacja tryptofanu w nieoczyszczonych wyciągach wątroby szczura jest hamowana przez fenyloalaninę i fenylopirogronian. Hamowanie fenyloalaniną ma charakter kompetycyjny. Hamowanie fenylopirogronianem jest dwufazowe; w pierwszej fazie (do 1.6 mM stężenia fenylopirogronianu) hamowanie jest akompetycyjne, w drugiej fazie (od 4 mM stężenia inhibitora) kompetycyjne.

2. Dootrzewnowe obciążenie fenyloalaniną i tryptofanem powoduje odpowiednio po 2 i 4 godz. po podaniu 50% obniżenie hydroksylacji tryptofanu w wyciągach wątroby.

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TERESA LASKOWSKA-KLITA

PURIFICATION OF *p*-HYDROXYPHENYLPYRUVATE HYDROXYLASE AND ITS NATURAL INHIBITOR FROM LIVER OF THE FROG, *RANA ESCULENTA*

Department of Physiological Chemistry, Medical School, Krakowskie Przedmieście 26/28, Warszawa 64

1. *p*-Hydroxyphenylpyruvate hydroxylase (EC 1.14.2.2) which is present in frog liver in proenzyme form, was purified after activation by trypsin treatment. Molecular weight of the hydroxylase determined by gel filtration is 85 000 and K_m is 0.5 mM. 2. The natural inhibitor of the frog enzyme was purified and obtained in electrophoretically homogeneous form. The inhibitor is a low-molecular protein, with isoelectric point below pH 3.6. The inhibitor is not specific for frog-liver enzyme and inhibits the hydroxylation of *p*-hydroxyphenylpyruvate by enzymic preparations from other animals.

In the previous paper (Laskowska-Klita & Mochnacka, 1967) the presence of the inactive form of *p*-hydroxyphenylpyruvate hydroxylase [*p*-hydroxyphenylpyruvate, ascorbate:oxygen oxidoreductase (hydroxylating), EC 1.14.2.2] in the tadpole and in liver of the adult frog has been demonstrated. The occurrence of the inactive form of the enzyme was found to be independent of the seasonal changes in physiological condition of the frog. The active enzyme was obtained by trypsin digestion or as a result of autolysis. No enzyme activity could be observed in frog eggs even after trypsin digestion. The inhibitor released from the proenzyme was isolated and found to be a non-diffusible, thermostable compound, soluble in 2.5% trichloroacetic acid, and resistant to the action of trypsin and carboxypeptidase. In the present work the enzyme and inhibitor were further purified and their kinetics studied.

MATERIALS AND METHODS

Special reagents. α,α'-Dipyridyl and 2,6-dichlorophenol indophenol (Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland), *p*-hydroxyphenylpyruvic acid (Koch-Light Lab. Ltd., Colnbrook, England), reduced glutathione and crystalline trypsin (Nutr. Biochem. Corp., Cleveland, Ohio, U.S.A.); before use, trypsin was activated according to Baines, Baird & Elmore (1964). Sephadex G-25, G-50 and G-100 was from Pharmacia (Uppsala, Sweden), Whatman DEAE-cellulose from Balston Ltd. (Maidstone, Kent, England).

For molecular weight determination the following standards were used: ribonuclease A from bovine pancreas, salt-free, protease-free, mol. wt. 13 700 (Sigma, St. Louis, Mo., U.S.A.); cytochrome *c* from horse heart, mol. wt. 13 000 (Reanal, Budapest, Hungary); bovine serum albumin, fraction V, mol. wt. 67 000 (Pentex, Kankakee, Ill., U.S.A.); pyruvate kinase from rabbit muscle, mol. wt. 166 000 (Serva, Heidelberg, West Germany).

Material. Adult frogs, *Rana esculenta*, were collected in autumn and kept at 4°. The experiments were continued from October till April. The animals were killed and the liver immediately isolated, placed in ice, and extracts for enzyme and inhibitor isolation were prepared as described in Results.

Analytical methods. For protein determination three methods were used: the turbidimetric tannin method of Mejbaum-Katzenellenbogen (1955), spectrophotometric according to Warburg & Christian (1941) and colorimetric according to Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin fraction V was used as standard for turbidimetric and colorimetric determinations.

Electrophoresis was performed on Whatman no. 3 *MM* paper (18×57 cm.) in 0.01 M-acetate buffer, pH 3.6, for 80 min. at 20 v/cm. On the electrophoretogram the UV-absorb ng substances were located under UV light, and protein after spraying with a 0.2% solution of ninhydrin in acetone.

Determinations of enzyme activity were carried out according to Zannoni & La Du (1959). The standard incubation mixture contained: 20 μmoles of reduced glutathione, 2 μmoles of α,α′-dipyridyl, 25 μg. of dichlorophenol indophenol, 1.6 μmoles of *p*-hydroxyphenylpyruvate, enzyme preparation (0.5 to 5 mg. of protein), and 0.1 M-Na,K-phosphate buffer, pH 6.5, to a final volume of 2.6 ml. After 20 min. incubation at 37°, the enzyme activity was measured by the decrease in the amount of substrate, determined by the enol (aromatic) borate-tautomerase method according to Lin, Pitt, Civen & Knox (1958).

Activity of the inhibitor was expressed as percentages of inhibition in relation to the uninhibited enzymic reaction.

RESULTS

Purification of the enzyme

All manipulations were carried out in a cold room at 4°. The liver (30 g.) was homogenized with 270 ml. of 0.25 M-sucrose in a Potter-type glass homogenizer with a polyacryl pestle. Nuclei and mitochondria were removed by centrifugation for 15 min. at 600 *g* and 12 000 *g*. In a control experiment, the supernatant was centrifuged at 105 000 *g* for 1 hr. at 0°; the sedimented microsomes were found to contain no *p*-hydroxyphenylpyruvate hydroxylase. In subsequent experiments the post-mitochondrial supernatant was brought to pH 5.2 with cold 0.1 N-acetic acid and after 10 min. at 4° the precipitated protein and microsomes were centrifuged off at 12 000 *g* for 15 min. The supernatant was neutralized with cold 0.1 N-

NaOH and fractionated with ammonium sulphate. The precipitate at 0.4 - 0.7 saturation was collected, dissolved in 2 ml. of 0.01 M-Na,K-phosphate buffer, pH 7.3, and applied to a Sephadex G-50 column (2.5×55 cm.). In the eluate two protein fractions were obtained: the main fraction *I* in which the enzyme was present, and a smaller fraction *II* with the inhibitor activity (Fig. 1). The application of a column higher than that used in the previous work (Laskowska-Klita & Mochnacka, 1967) resulted in separation of the *p*-hydroxyphenylpyruvate hydroxylase activity of fraction *I* into two peaks, *A* and *B*. The activity in peak *B* was higher than in peak *A*. Digestion with trypsin, at a concentration of 100 μg./ml. of the eluate, increased the activity in peak *A* but had no effect on peak *B*. Thus it appears that peak *A* is a mixture of the enzyme activated by autolysis and the inactive enzyme, whereas peak *B* contains only the active enzyme.

When the 0.4 - 0.7 ammonium sulphate saturation fraction was submitted to trypsin digestion prior to Sephadex gel filtration, a single active peak was obtained (Fig. 2) with the elution volume corresponding to that of peak *B*. The fractions

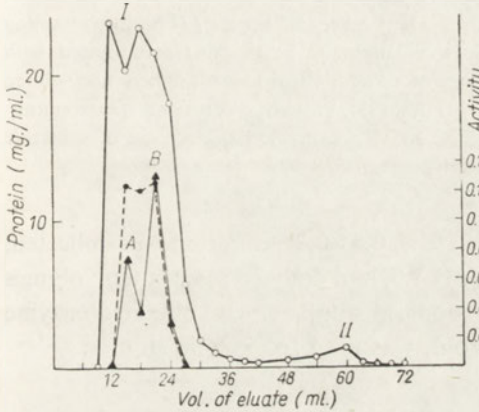

Fig. 1

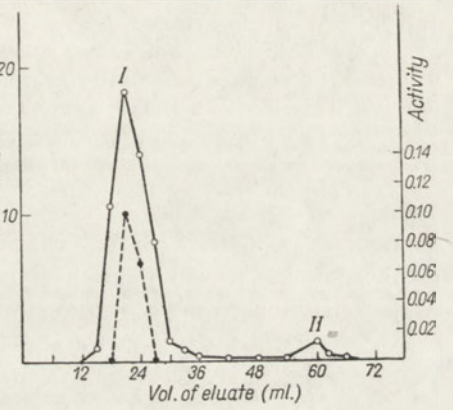

Fig. 2

Fig. 1. Sephadex G-50 gel filtration of the 0.4 - 0.7 ammonium sulphate saturation fraction. The solution of 350 mg. of protein (2 ml.) was applied to the column (2.5×55 cm.) equilibrated with 0.01 M-Na,K-phosphate buffer, pH 7.3, and the same buffer was used for elution. Fractions of 3 ml. were collected at intervals of 12 min. The protein (○) was assayed spectrophotometrically. The activity of *p*-hydroxyphenylpyruvate hydroxylase was assayed (▲), directly and (●), after trypsin digestion, and expressed as decrease in the amount of substrate in μmoles/mg. protein/20 min.

Fig. 2. Sephadex G-50 gel filtration of the 0.4 - 0.7 ammonium sulphate saturation fraction after trypsin digestion. A sample of 1.5 ml. containing 200 mg. of protein was applied to the column (2.5×55 cm.), then the elution and determinations were carried out as described for Fig. 1. (○), Protein; (●), *p*-hydroxyphenylpyruvate hydroxylase activity.

eluted at 19 - 26 ml. were pooled and further purified on DEAE-cellulose column, the protein being eluted with 0.01 M and 0.06 M-Na,K-phosphate buffer, pH 7.3 (Fig. 3). This procedure removed from the preparation 80% of inactive protein which emerged in the first 90 ml. of the eluate. Fractions from 93 - 115 ml. containing the *p*-hydroxyphenylpyruvate hydroxylase activity, were pooled and treated with

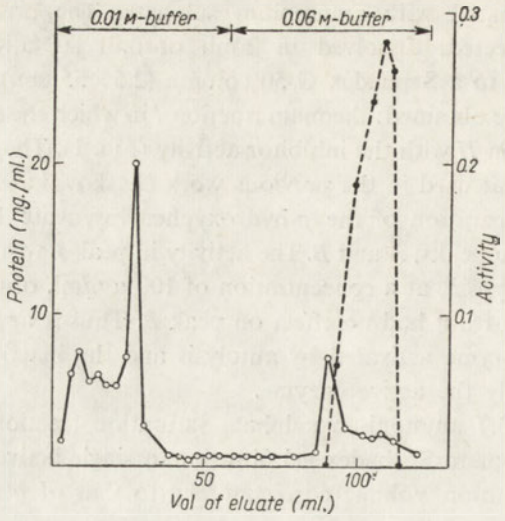

Fig. 3. DEAE-cellulose column chromatography of the Sephadex G-50 peak *I* (Fig. 2) containing *p*-hydroxyphenylpyruvate hydroxylase activity. To the column (1.5×14 cm.) equilibrated with 0.01 M-Na,K-phosphate buffer, pH 7.3, 250 - 280 mg. of protein (7 ml.) was applied and eluted with 0.01 M and then with 0.06 M-phosphate buffer, pH 7.3. (○), Protein, determined spectrophotometrically; (●), *p*-hydroxyphenylpyruvate hydroxylase activity, expressed as decrease of substrate in μmoles/mg. of protein.

ammonium sulphate at pH 7.3. The precipitate at 0.4 - 0.7 saturation was collected, dissolved in 0.01 M-Na,K-phosphate buffer, pH 7.3, and dialysed against ten volumes of the same buffer for 18 hr. to remove ammonium sulphate. The obtained enzyme preparation, which was purified about 80-fold, was used for kinetic studies.

Purification of the inhibitor

Fraction *II* from Sephadex G-50 gel filtration (Figs. 1, 2), which contained the inhibitor, was contaminated with some substances absorbing at 260 mμ. It was found that digestion by ribonuclease (10 μg. of RNase/ml.) at room temperature for 10 min. had no effect on the activity of the inhibitor.

In the previous work (Laskowska-Klita & Mochnacka, 1967) it has been demonstrated that the inhibitor is soluble in 2.5% trichloroacetic acid and resistant to trypsin digestion. To purify the inhibitor, the fractions eluted at 57 - 63 ml. from several Sephadex G-50 filtrations were pooled and treated with trypsin (100 μg./ml.) at room temperature for 1 hr. Trypsin and the undigested proteins were precipitated by 2.5% trichloroacetic acid and discarded by centrifugation. The supernatant was brought to pH 7.3 with 0.1 N-NaOH and dialysed for 18 hr. against 30 volumes of water. The obtained preparation was freeze-dried, then dissolved in 0.01 M-Na,K-phosphate buffer, pH 7.3, and applied to a Sephadex G-25 column (Fig. 4). The inhibitor was present in the fractions eluted at 9 - 27 ml. In these fractions, which still contained some impurities absorbing at 260 mμ, protein was assayed by three

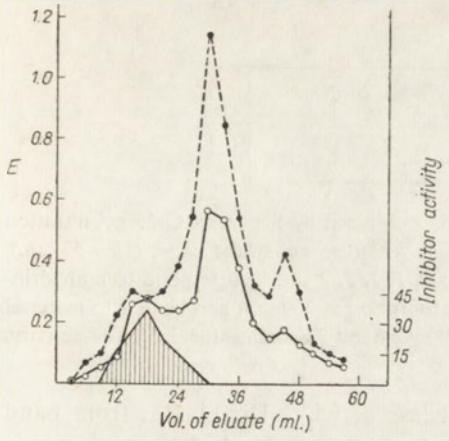

Fig. 4

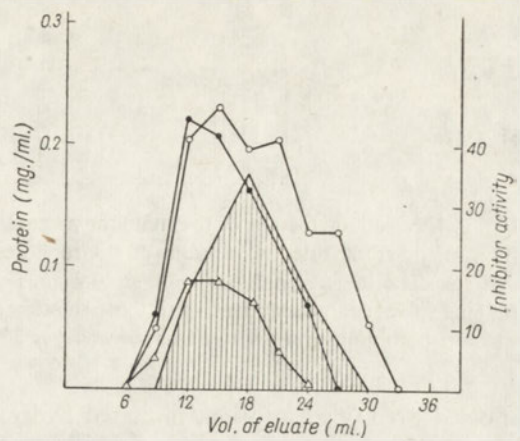

Fig. 5

Fig. 4. Sephadex G-25 gel filtration of the inhibitor preparation purified by trypsin digestion. The column (1.4×45 cm.) was equilibrated and eluted with 0.01 M-Na,K-phosphate buffer, pH 7.3. Fractions of 3 ml. were collected, the extinction was read at (●), 260 mμ and (○), 280 mμ, and the activity of the inhibitor assayed using 0.4 ml. of the eluate fraction. The inhibitor activity (shaded area) is expressed as percentages of the uninhibited enzymic reaction.

Fig. 5. Determination of protein by three methods in the inhibitor-containing fractions (see Fig. 4). (△), Tannin method; (●), spectrophotometric method; (○), method of Lowry *et al.* The shaded area represents inhibitor activity.

methods (Fig. 5). The amounts of protein determined by the turbidimetric tannin method (Mejbaum-Katzenellenbogen, 1955) not only were lower than those obtained by the method of Lowry *et al.* (1951) but moreover in some protein fractions in which the inhibitor activity had been demonstrated, no reaction with tannin was observed. These discrepancies were not due to differences in the accuracy of the two methods, since parallel determinations made on standard bovine albumin gave in either case the same results. It seems possible, however, that the inhibitor molecules are too small to react with tannin. Mejbaum-Katzenellenbogen & Lorenc-Kubis (1966) in their studies on insulin observed that the phenylalanine chain B (mol. wt. 3600) precipitated with tannin, whereas the glycyl chain A, possessing lower molecular weight (2400), did not react with tannin.

In further experiments, the amount of inhibitor protein was determined by the method of Lowry *et al.* (1951).

The inhibitor-containing fractions, eluted from Sephadex G-25 between 9 - 27 ml. were pooled, dialysed against 30 vol. of water for 18 hr. at 4°, freeze-dried, then dissolved in a small volume of water and submitted to paper electrophoresis at pH 3.6 (Fig. 6). Eight bands were obtained; two of them were ninhydrin-negative and showed UV absorption; six were ninhydrin-positive, one of them showing also absorption at 260 mμ. The bands were eluted with water and assayed for the presence of the inhibitor. Only the eluate from the ninhydrin-positive band 7, which

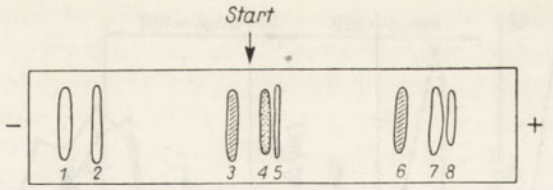

Fig. 6. Electrophoretogram of the inhibitor preparation obtained by Sephadex G-25 gel filtration (Fig. 4) and freeze-dried. Conditions of electrophoresis: Whatman no. 3 *MM* paper (1.8×57 cm.), 0.01 M-acetate buffer, pH 3.6, 80 min. at 20 v/cm. Bands *1*, *2*, *5*, *7* and *8* correspond to ninhydrin-staining substances, bands *3* and *6* to those showing absorption at 260 mμ, and band *4* to material which is both ninhydrin-positive and absorbing at 260 mμ; band *7* contains the inhibitor activity.

showed no UV absorption, inhibited hydroxylase activity. The eluates from band *7* of several electrophoretograms were pooled and freeze-dried. This preparation of the inhibitor, after being dissolved in water, was used for further investigations.

Determinations of molecular weight

By gel filtration on Sephadex G-100 according to Andrews (1964), a value of 85 000 was obtained for the purified *p*-hydroxyphenylpyruvate hydroxylase (Fig. 7). Attempts were also made to determine the molecular weight of the inhibitor. Although the exact value could not be obtained, it is certainly lower than 5000.

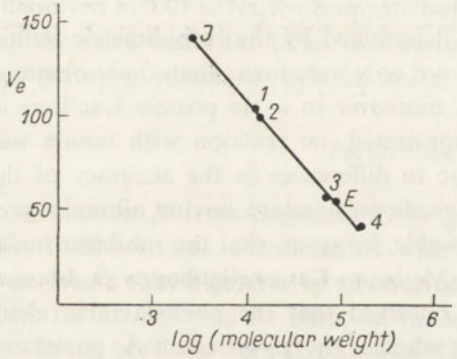

Fig. 7. Determinations of molecular weight by Sephadex G-100 gel filtration. The column (1.8× 52 cm.) was equilibrated with 0.01 M-Na,K-phosphate buffer, pH 7.3. The proteins were dissolved in the equilibration buffer. Standard proteins 3) mg. each): *1*, cytochrome *c*; *2*, ribonuclease; *3*, ox albumin; *4*, pyruvate kinase. *E*, The *p*-hydroxyphenylpyruvate hydroxylase preparation after DEAE-cellulose chromatography (20 mg. of protein); *I*, the inhibitor (3.5 mg. of the purified preparation). The elution volume of the protein was assayed by protein determination, and of the enzyme and inhibitor also by measuring their activity.

Kinetic studies

The kinetic measurements were made with the partially purified preparations of *p*-hydroxyphenylpyruvate hydroxylase and the electrophoretically homogeneous preparations of the natural inhibitor.

The enzymic reaction was linear with time for 20 min. at 37° at protein concentrations up to 5 mg. per sample (Fig. 8A,B), and substrate concentrations up to 0.46 mM (Fig. 9). Therefore in kinetic studies an incubation period of 20 min. was applied, and the amount of enzyme protein was 3.5 mg. per sample.

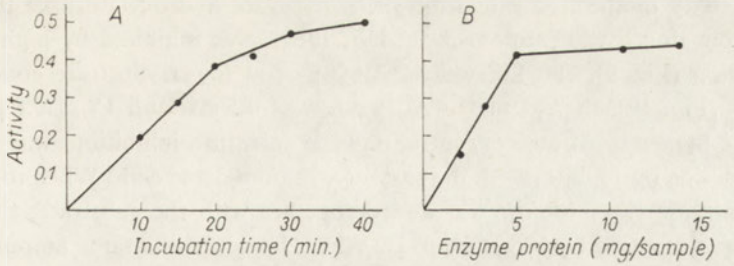

Fig. 8. The effect of time and protein concentration on hydroxylation of *p*-hydroxyphenylpyruvate by the purified enzyme preparation. Standard incubation mixtures contained: *A*, 4.7 mg. of enzyme protein; *B*, varying amounts of enzyme protein, incubation time being 20 min. The activity is expressed as decrease of substrate in μmoles per sample.

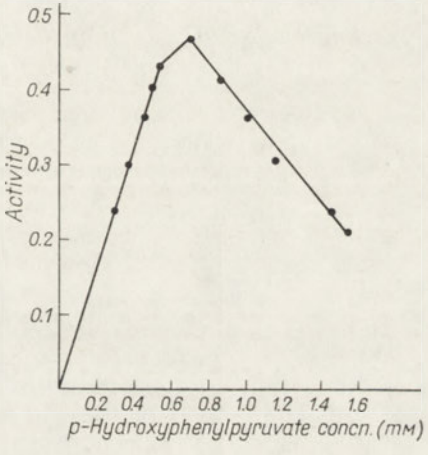

Fig. 9. Effect of substrate concentration on the enzymic reaction. The standard incubation mixture contained 3.5 mg. of enzyme protein and varying concentrations of *p*-hydroxyphenylpyruvate. Incubation time 20 min. at 37°. The activity is expressed as decrease of substrate in μmoles per sample.

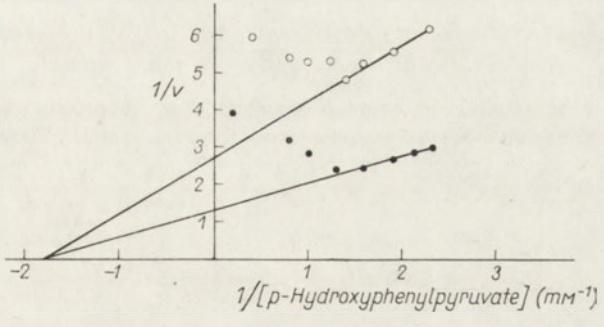

Fig. 10. Double reciprocal plots of velocity against substrate concentration (●), without and (○), with the natural inhibitor. The enzyme was the purified *p*-hydroxyphenylpyruvate hydroxylase preparation (3.5 mg./sample). The inhibitor was the purified, electrophoretically homogeneous preparation (0.35 mg./sample), and was preincubated with the enzyme for 20 min. at 37°. The velocity, *v*, is expressed as decrease of substrate in μmoles/20 min./sample at 37°.

The activity of the frog *p*-hydroxyphenylpyruvate hydroxylase, like that of the enzyme from dog liver (Zannoni & La Du, 1959), was inhibited by high substrate concentration (Fig. 9). The Lineweaver-Burk plot at lower substrate concentration was linear (Fig. 10), the calculated K_m value was 0.5 mM and V_{max} 0.8 μmole per sample. At higher substrate concentrations the substrate inhibition was apparent.

The reaction of inhibitor with the enzyme was time-dependent. When to the whole reaction mixture the inhibitor was added together with the substrate, the enzyme activity was decreased by 18% (Table 1). When, however, the same amount of inhibitor was preincubated with the enzyme for 20 min. and then the other components and substrate were added, the inhibition amounted to 70%.

The Lineweaver-Burk plots of the uninhibited and inhibited reaction showed the non-competitive type of inhibition (Fig. 10). The inhibitor decreased the V_{max} and had no effect on the K_m value (Dixon & Webb, 1964).

Table 1

Effect of preincubation of the enzyme with inhibitor on p-*hydroxyphenylpyruvate hydroxylase activity*

The standard incubation mixture contained 3.5 mg. protein of *p*-hydroxyphenylpyruvate hydroxylase and, where indicated, the inhibitor (0.7 mg. protein). The incubation time was 40 min. at 37°. The activity is expressed as decrease in the amount of substrate in μmoles/mg. of enzyme protein/40 min.

Inhibitor	Activity	Inhibition (%)
Omitted	18.2	0
Added at the beginning of incubation	15.0	18
Preincubated with enzyme for 20 min.	5.8	69

Table 2

Effect of the inhibitor isolated from frog liver on the activity of p-*hydroxyphenylpyruvate hydroxylase in liver extracts of some animals*

Standard reaction mixture containing the indicated amount of extract protein was used. The purified inhibitor preparation was preincubated with the extract for 20 min. at 37°. The activity is expressed as decrease in the amount of substrate in μmoles/mg. protein/20 min. at 37°.

Liver extract of	Protein (mg./sample)	Initial activity	Inhibitor added			
			0.47 mg. protein		0.79 mg. protein	
			Activity	Inhibition (%)	Activity	Inhibition (%)
Frog	3.5	13.1	7.3	45	5.5	58
Rat	1.2	37.0	28.0	25	24.0	35
Pigeon	0.7	25.0	14.0	40	12.0	52
Ox	1.2	10.3	4.3	60	3.6	65

Specificity of the inhibitor from frog liver

Extracts from the livers of rat, ox and pigeon were prepared in the same way as the extract from frog liver, and the supernatants after acidification to pH 5.2 were used for experiments. Preincubation with the inhibitor for 20 min. decreased the activity in all enzymic preparations (Table 2).

DISCUSSION

p-Hydroxyphenylpyruvate hydroxylase in frog liver is present, like in the dog (La Du & Zannoni, 1956), pig (Hager, Gregerman & Knox, 1957), rat and guinea pig (Goswami, 1964), in the soluble fraction of the cell. Occurrence of the inactive form of this enzyme seems to be peculiar to the frog, as it has not been observed either in any of the above-mentioned animals or in the snail, lizard or fish (Michałek-Moricca, 1965).

The inhibitor isolated from the frog-liver proenzyme, purified to an electrophoretically pure form and free from UV-absorbing substances, is a protein of molecular weight probably not exceeding 3000, with isoelectric point below pH 3.6. The inhibitor is not specific for the frog enzyme only, and inhibits also the *p*-hydroxyphenylpyruvate hydroxylases of rat, ox and pigeon.

The frog *p*-hydroxyphenylpyruvate hydroxylase was purified by ammonium sulphate fractionation, Sephadex G-50 gel filtration and DEAE-cellulose column chromatography. Attempts to purify the frog enzyme with chloroform-ethanol as applied by Zannoni & La Du (1959) for the dog enzyme, were unsuccessful. The treatment of the post-mitochondrial supernatant from frog liver with a chloroform-ethanol mixture (2:5 ,v/v) for 15 min. at -5°, although at first it enhanced the enzyme activity, made impossible further purification because the protein obtained after Sephadex G-50 gel filtration proved to be inactive.

Affinity of the frog-liver *p*-hydroxyphenylpyruvate hydroxylase for the substrate is smaller than that of the enzymes from the dog (Zannoni & La Du, 1959) and rat (Lin *et al.*, 1958). For the latter enzymes the K_m value was 0.02 mM, whereas the K_m for the frog enzyme was higher and amounted to 0.5 mM.

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OCZYSZCZANIE HYDROKSYLAZY *p*-HYDROKSYFENYLOPIROGRONIANU I JEJ NATURALNEGO INHIBITORA Z WĄTROBY ŻABY, *RANA ESCULENTA*

Streszczenie

1. Hydroksylazę *p*-hydroksyfenylopirogronianu (EC 1.14.2.2) występującą w wątrobie żaby w formie proenzymu oczyszczono 80-krotnie po aktywacji trypsyną. Wartość K_m enzymu wynosi 0.5 mM, a ciężar cząsteczkowy oznaczony metodą sączenia na żelu 85 000.

2. Naturalny inhibitor z wątroby żaby uzyskano w postaci elektroforetycznie homogennej. Inhibitor jest białkiem niskocząsteczkowym o punkcie izoelektrycznym poniżej pH 3,6. Inhibitor nie jest specyficzny dla enzymu żaby i hamuje hydroksylację *p*-hydroksyfenylopirogronianu przez preparaty enzymatyczne z innych zwierząt.

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POLYRIBOSOMES AND THE SYNTHESIS OF ANTIALBUMIN ANTIBODIES IN SPLEEN CELLS OF RABBITS HYPERIMMUNIZED WITH SERUM ALBUMIN

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1. Spleen cells of rabbits hyperimmunized with bovine serum albumin synthesize proteins which have the electrophoretic mobility of γ-globulins. About half of the newly synthesized polypeptides are precipitated by antigen - antibody mixture. 2. Gradient sedimentation of extracts from spleen labelled with [^{14}C]isoleucine showed that the amino acid is first incorporated into polyribosomes and then into the single ribosome or monosome fraction. 3. The radioactivity of the polyribosome fraction derived from hyperimmunized animals may be precipitated with antigen - antibody mixture (up to 80% of total counts). Only about 14% of total counts is precipitated by antigen - antibody mixture in control experiments with non-immunized rabbits. 4. The sedimentation coefficients of polyribosomes from spleen cells suggest that they contain messenger RNA long enough to code for entire light and heavy chains of immunoglobulins.

Groups of ribosomes held by a single strand of messenger RNA have been recognized as the sites of protein synthesis in mammalian and bacterial systems (Rich, Warner & Goodman, 1963). The number of ribosomal particles in these groups depends upon the length of the message and a rough estimate of this length may be obtained therefore by measuring the sedimentation coefficients of polyribosomes (Rich, Penman, Becker, Darnell & Hall, 1965).

In recent years several classes of polyribosomes which synthesize specific proteins have been identified. These include polyribosomes which synthesize haemoglobin (Warner, Knopf & Rich, 1963), virus coat protein (Scharff, Shatkin & Levintow, 1963), β-galactosidase (Kiho & Rich, 1964, 1965), myosin (Heywood, Dowben & Rich, 1967) and histidinol phosphate phosphatase (Bagdasarian & Cieśla, to be published).

There seems to be little doubt at present that also γ-globulins are synthesized on polyribosomes (Bagdasarian, Bagdasarian & Borecka, 1966; Becker & Rich, 1966; Shapiro, Scharff, Maizel & Uhr, 1966; Talal, 1966; Tawde, Scharff & Uhr, 1966). The existence of polyribosome-like structures in lymphoid tissues has been established as well as the increase of the polyribosome peak after immunization it is not clear however whether the newly synthesized polypeptides on these poly,

ribosomes represent the antibody directed against the antigen used for immunization and whether the increased specific radioactivity of the above polyribosomes reflects the synthesis of a new species of messenger RNA.

In this report we present some evidence indicating that spleen polyribosomes derived from rabbits hyperimmunized with bovine serum albumin carry out the synthesis of antialbumin antibodies.

MATERIAL AND METHODS

Special chemicals. Bovine serum albumin (BSA)[1], Fraction V (Armour Laboratories, Kankakee, Ill., U.S.A.); L-[U-^{14}C]isoleucine, 24.7 mc per m-mole (Radiochemical Centre Amersham, Bucks., England); crystalline pancreatic ribonuclease (Boehringer & Soehne, Mannheim, West Germany). Cellulose acetate strips for electrophoresis (Oxo Ltd., London, England); Millipore HA filters (Millipore Filter Corp., Bedford, Mass., U.S.A.).

Animals. Inbred white rabbits ("Popielno" strain), average weight 2.5 kg., were hyperimmunized by 8 intravenous injections (20 mg. each) of BSA made every third day. Six weeks after the last dose the animals received a booster injection of BSA. The titer of antibodies determined by tannic acid haemagglutination test (Boyden, 1957) reached 1:1 000 000 in five days. This corresponded to about 5 mg. of antibody per 1 ml. of serum as determined by quantitative precipitation (Kabat & Mayer, 1961).

Methods. The spleens were labelled with L-[U-^{14}C]isoleucine by injecting 5 μc of the amino acid in 0.1 ml. of water directly into the spleen of a rabbit in ether anaesthesia before the circulation of blood had stopped. The spleens were then removed, chilled and disrupted in buffer A [5 mM-tris, pH 7.4 - 60 mM-KCl - 10 mM-$Mg(CH_3COO)_2$] with the aid of a steel mesh. The above procedure described by Becker & Rich (1966) gave better labelling and less degradation of polyribosome fraction than other methods commonly used in previous studies.

The radioactivity in protein was determined as follows: protein was precipitated with 5% trichloroacetic acid (TCA), then washed three times with 5% TCA, once with acetone and once with ether. Proteins precipitated with BSA - anti-BSA mixture were washed three times with cold 0.9% NaCl, once with ethanol and once with ether. The air-dried protein was dissolved in concentrated formic acid and 0.3 ml. of this solution was added to a mixture of 11 ml. of 0.3% *p*-diphenyl-oxazole in toluene and 3.5 ml. of absolute ethanol. Up to 600 μg. of protein could be held as transparent solution in this mixture. The mixtures were counted in a liquid scintillation counter with an efficiency of 45%. In some experiments protein precipitates were poured on Millipore filters, washed with 5% TCA, then with ethanol and counted in a low background (2 counts per minute) gas-flow counter with an efficiency of 20%.

[1] Abbreviations: BSA, bovine serum albumin; TCA, trichloroacetic acid; EDTA, ethylenediaminetetra-acetic acid.

Sucrose density gradient centrifugation of polyribosomes was performed according to the method of Martin & Ames (1961). Sucrose was dissolved in buffer A. Linear gradients of 4 ml. of 15% to 30% sucrose were centrifuged at 39 000 rev./min. at 0° and 30 ml. gradients of 15% to 30% sucrose at 25 000 rev./min. and 0° in MSE centrifuge. The gradients were fractionated by syphoning from the bottom of the tubes. Fractions of 0.1 ml. were collected from the 4 ml. gradients and 0.75 ml. from the 30 ml. gradients by drop counting.

Immunoelectrophoresis was conducted by the method of Grabar & Williams (1955). Zone electrophoresis on acetyl cellulose strips was performed by the method of Kohn (1958).

RESULTS

Characterization of protein synthesized by spleen cells

The electrophoresis of serum of normal and hyperimmunized rabbits showed that after hyperimmunization the content of γ-globulin fraction increased markedly (Fig. 1). The immunoelectrophoresis revealed one precipitation arc in the region of γ-globulins (Fig. 2).

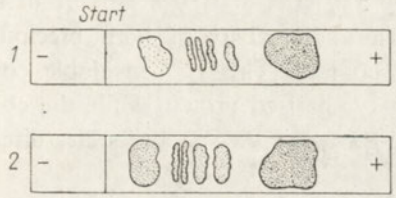

Fig. 1

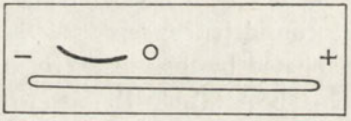

Fig. 2

Fig. 1. Electrophoresis of rabbit serum. Serum, 4 µl., was applied to cellulose acetate strips (18×2.5 cm.) and subjected to electrophoresis at pH 8.9 for 13 hr. at 6 v/cm. and 4°. Buffer: 0.65 M-tris - 0.02 M-EDTA - 0.098 M-boric acid. The electrophoretogram was stained with Amido Black. *1*, Serum from control (non-immunized) animal; *2*, serum from the animal hyperimmunized with BSA.

Fig. 2. Immunoelectrophoresis of serum of a rabbit hyperimmunized with BSA. Electrophoresis was carried out by the method of Grabar & Williams (1955).

To characterize the protein newly synthesized in the spleen cells of hyperimmunized rabbits the spleen was labelled with [^{14}C]isoleucine for 5 min., then removed, chilled and homogenized. The homogenate was centrifuged at 78 000 *g* for 2 hr. and the supernatant used for further analysis.

Zone electrophoresis of this supernatant showed one major fraction with the mobility of γ-globulins. When the radioactivity of the electrophoretic fractions was determined by cutting the strip into fragments and counting the individual fragments in liquid scintillation counter, the γ-globulin fraction showed the highest activity

while other fractions had only a negligible number of counts (Fig. 3). These results indicate that the proteins synthesized by spleen cells consist predominantly of γ-globulins and that very little of other proteins is synthesized by these cells.

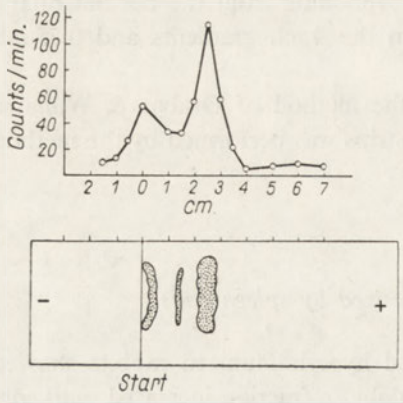

Fig. 3. Electrophoresis of the soluble fraction of spleen cells of a rabbit hyperimmunized with BSA. Spleen was labelled with [^{14}C]isoleucine and the high-speed supernatant prepared as described in Methods. Ten µl. of the supernatant was subjected to electrophoresis as described in Fig. 1. The electrophoretogram was cut into strips and individual strips counted in a liquid scintillation counter.

For further immunological characterization of the proteins produced by the spleen, equal portions of the labelled „high-speed supernatant" derived from spleens of hyperimmunized rabbits, were precipitated by 5% TCA and by a mixture of BSA and rabbit anti-BSA serum in equivalency. The radioactivity of both precipitates was then determined in liquid scintillation counter. The TCA-insoluble counts were considered to represent the total newly synthesized protein while the counts precipitated by the mixture of BSA and anti-BSA, the specific antibodies directed against BSA (Table 1).

Table 1

Incorporation of [^{14}C]*isoleucine into proteins of spleen cells of hyperimmunized rabbits*

The spleens were labelled with [^{14}C]isoleucine, homogenized and centrifuged at 78 000 *g* for 2 hr. Equal portions of supernatant were precipitated with TCA and with antigen - antibody mixture. The radioactivity of precipitates was determined in liquid scintillation counter.

No. of rabbit	Counts/min.		Per cent of counts precipitated with antigen-antibody mixture
	Precipitated by TCA	Precipitated by antigen-antibody mixture	
1	270	180	67
2	420	190	45
3	362	285	78

It is evident from the results of these determinations that a considerable part of total protein synthesized by spleen cells of immunized animals consists of specific antibodies directed against the antiged used for immunization. Other proteins

are also synthesized by spleen cells and their amount, estimated by subtracting the counts specifically precipitated with antigen - antibody mixture from total counts in protein (Table 1), constitutes 22 to 55%.

The detection of polyribosomes in spleen cells

Spleen homogenate labelled with [^{14}C]isoleucine was prepared as described in Methods and centrifuged for 10 min. at 10 000 *g* to remove cell debris, nuclei and mitochondria. The supernatant, 0.2 ml., was layered on 4 ml. sucrose gradients and centrifuged at 39 000 rev./min. for 2 hr. at 0°. The distribution of ultraviolet absorption and radioactivity in this gradient is presented in Fig. 4A. The estimation of sedimentation coefficients by the method of Martin & Ames (1961) as well

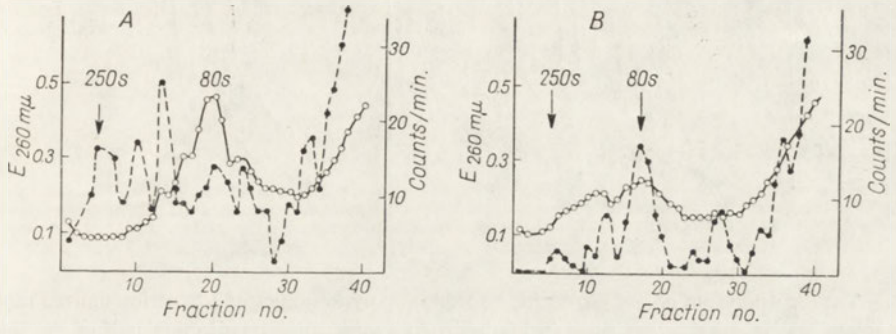

Fig. 4. Gradient sedimentation of post-mitochondrial supernatant of spleen cells of a hyperimmunized rabbit. (*A*), Spleen cells were labelled with [^{14}C]isoleucine for 5 min. as described in Methods. Post-mitochondrial supernatant, 0.2 ml., was applied on 4 ml. sucrose gradients 15% to 30% and centrifuged at 39 000 rev./min. for 2 hr. at 0°. Fractions of 0.1 ml. were collected from the bottom of tubes and their extinction at 260 mμ determined. Protein was then precipitated with TCA, the precipitates collected on Millipore filters and counted in a low background counter. (*B*), Same as *A* but 2 μg./ml. of crystalline pancreatic ribonuclease was added to the post-mitochondrial supernatant immediately before centrifugation. (○), Extinction at 260 mμ; (●), radioactivity.

as comparisons with parallel runs of purified ribosomes showed that the highest ultraviolet absorbing peak with sedimentation coefficient of 80s represents the fraction of single ribosomes or monosomes (i.e. individual ribosomal particles with a strand of messenger RNA attached to each particle). The labelling of fractions heavier than monosomes and their specific activity, which is higher than that of the 80s fraction, suggest that this material with sedimentation coefficients of up to 250s is composed of polyribosomes. The treatment of post-mitochondrial supernatant with 2 μg./ml. of pancreatic ribonuclease before gradient centrifugation leads to the disappearance of radioactivity from the heaviest fractions thus confirming the suggestion that individual ribosomes in the heavy labelled fractions of gradient are held together by RNA (Fig. 4B).

In rabbit spleen cells, unlike in mammalian cells of other origin, the 80s fraction becomes very quickly and highly labelled. It is necessary to label the cells with a very short pulse in order to obtain the incorporation into heavy fractions only. The results of one of these experiments are presented in Fig. 5. The sucrose gradient centrifugation of post-mitochondrial supernatant of spleen cells labelled for 1 min.

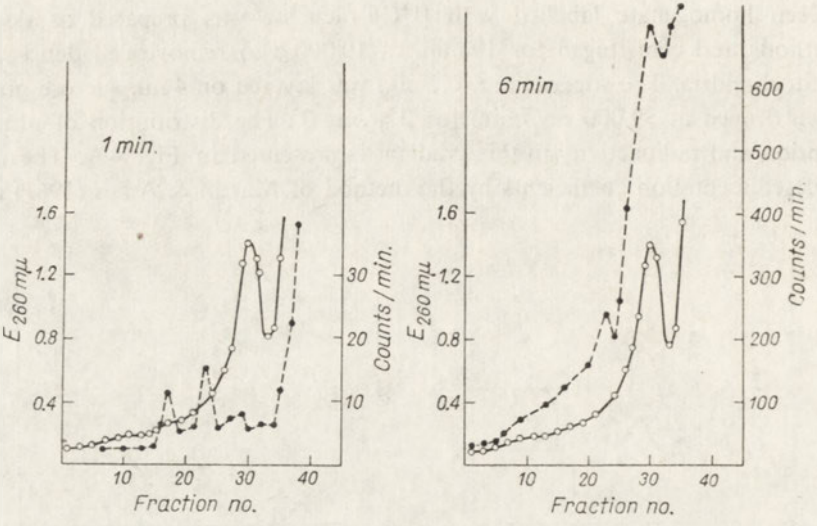

Fig. 5. The incorporation of [^{14}C]isoleucine into spleen polyribosomes of a hyperimmunized rabbit. The spleen was labelled as described in Methods. At 1 min. after the injection half of the spleen was cut off, chilled and homogenized. The other half was removed after 6 min. and treated in the same way. Then 0.2 ml. of post-mitochondrial supernatant from each portion of labelled spleen were layered over 4 ml. of 15% to 30% sucrose gradient and centrifuged for 1 hr. at 39 000 rev./min. at 0°. Gradients were analysed as in Fig. 4. (○), Extinction at 260 mμ; (●), radioactivity.

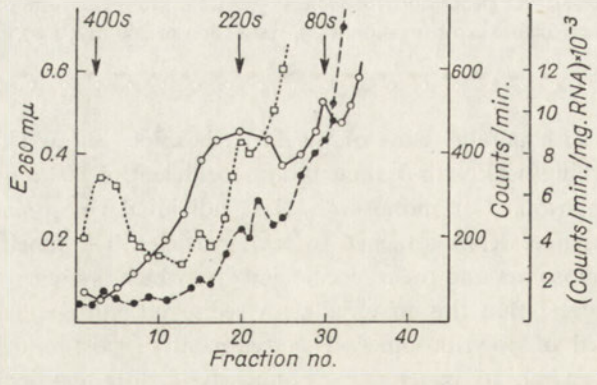

Fig. 6. Specific activity of spleen polyribosomes of a hyperimmunized rabbit. The spleen was removed at 5 min. after injection of [^{14}C]isoleucine, homogenized and centrifuged as in Fig. 5. (○), Extinction at 260 mμ; (●), radioactivity, counts/min.; (□), specific radioactivity, counts/min./mg. RNA.

revealed high radioactivity in polyribosome fraction and very little activity under the monosome peak while after 6 min. of labelling not only the polyribosomes but also the 80s fraction became highly labelled. The reason for the early and extensive labelling of the 80s fraction is at present unclear. Our experimental evidence does not permit to decide whether the 80s fraction represents the monosomes, i.e. single ribosomes with attached messenger RNA which are actively engaged in protein synthesis as in the case of reticulocytes (Lamfrom & Knopf, 1965), or single ribosomes derived from polysomes as a result of nuclease action. However, the existence of rapidly labelled heavy polyribosomes suggests that protein synthesis in spleen cells can take place on large polysomes which in turn implies the existence of messenger RNA long enough for coding of γ-globulin subunits. This is best seen if the results of a gradient sedimentation of spleen polysomes are also represented in terms of specific radioactivity as in Fig. 6.

Detection of newly synthesized antibody on polysomes

To characterize the newly synthesized proteins detected on polysomes we compared the total TCA-precipitable radioactivity with the activity that could be precipitated with antigen - antibody mixture. The post-mitochondrial supernatant of labelled spleen cells derived from a hyperimmunized rabbit was sedimented through a 30 ml. sucrose gradient. The fractions from one gradient were precipitated with TCA and the fractions from a parallel gradient with the BSA - anti-BSA mixture. The results of these determinations are presented in Fig. 7A and 7B. The high ultraviolet absorbing peak in fraction 37 corresponds to single ribosome fraction. Fractions 15 to 35 consist of polyribosomes. The profile of radioactivity precipitated by the antigen - antibody mixture (Fig. 7B) is similar to the profile of total TCA-

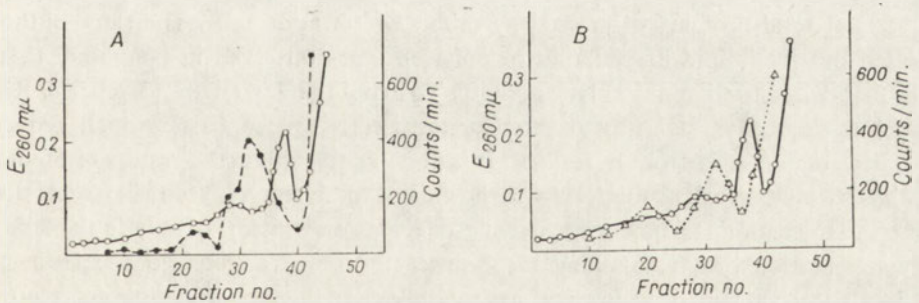

Fig. 7. Gradient sedimentation of polyribosomes from spleen cells synthesizing anti-BSA antibodies. The spleen was labelled with [^{14}C]isoleucine for 5 min. One ml. portions of post-mitochondrial supernatant were centrifuged through 30 ml. of 15% to 30% sucrose gradient at 25 000 rev./min. for 90 min. Fractions of 0.7 ml. were collected from the bottom of the tubes. After the estimation of extinction at 260 mμ fractions from one gradient (*A*) were precipitated with TCA and from a parallel gradient (*B*) with BSA - anti-BSA mixture. The radioactivity was determined in liquid scintillation counter. (○), Extinction at 260 mμ; (●), TCA-insoluble radioactivity; (△), radioactivity precipitated by BSA - anti-BSA mixture.

insoluble radioactivity which represents total newly synthesized polypeptide chains (Fig. 7A). Control experiments run in parallel with spleen of non-immunized animals showed very little non-specific coprecipitation (Fig. 8). The antigen - antibody

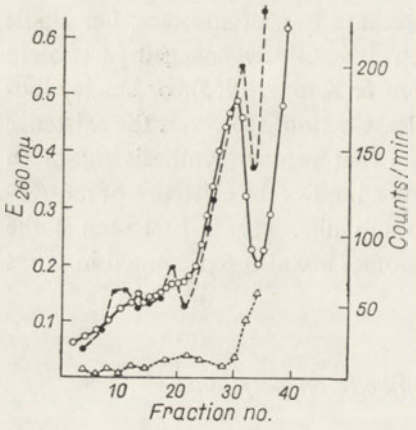

Fig. 8. Gradient sedimentation of polyribosomes of labelled spleen cells of a control (non-immunized) rabbit. Conditions are as in Fig. 7. (○), Extinction at 260 mμ; (●), TCA-insoluble radioactivity; (△), radioactivity precipitated with BSA - anti-BSA mixture.

mixture precipitated non-specifically only about 14% of total TCA-insoluble counts while in the case of immunized animals the number of counts precipitated by the antigen - antibody mixture reached 86% of total TCA-insoluble radioactivity.

DISCUSSION

In a number of papers published recently evidence is presented which indicates that γ-globulins are synthesized on polyribosomes. Manner, Gould & Slayter (1965) showed that lymph node cells of immunized rats contain polyribosomes which synthesize protein. Similarly Tawde *et al.* (1966) showed that γ-globulins constitute 25 to 75% of total protein synthesized by rabbit lymph node cells. The same authors pointed out that rapid degradation of polyribosomes observed in lymphoid tissue is probably due to high nucleolytic activity. To avoid the degradation Becker & Rich (1966) devised a special way of homogenization of spleen tissue which reduces liberation of nucleases from lysosomes. It is possible to explain the results of Stenzel, Phillips, Thompson & Rubin (1964), who failed to detect polysomes larger than dimers in spleen homogenates, by extensive degradation which probably took place in their preparations. Rapid labelling of the single ribosome fraction in our experiments could also be explained by enzymic hydrolysis although it is impossible to rule out the existence of monosomes (Lamfrom & Knopf, 1965) actively engaged in protein synthesis. The difference in sedimentation coefficients of monosomes and single ribosomes would be difficult to detect by sucrose gradient method used in the present work.

There is little doubt at present that both light and heavy chains of γ-globulins are synthesized on polyribosomes, i.e. on a specific template of messenger RNA. The works of Askonas & Williamson (1966) and of Shapiro *et al.* (1966) suggest

that the genes of light and heavy chains are transcribed to different molecules of messenger RNA and it is therefore possible to identify two separate classes of polyribosomes, one for light and one for heavy chains.

The results of the present work have confirmed the existence of polyribosomes in spleen cells, and in some experiments a double polysomal peak was obtained which probably represents the separation of the two above-mentioned classes of polyribosomes. In addition evidence is presented that specific antibody directed against the antigen with which the animal was hyperimmunized is also synthesized on large polyribosomes. This suggests that the message of their primary structure is encoded in a specific template of informational RNA and therefore the step which determines the specificity of newly synthesized antibody, which depends upon the primary structure of light and heavy chains (Lennox & Cohn, 1967), takes place probably before the translation of messenger RNA.

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POLIRYBOSOMY SYNTETYZUJĄCE PRZECIWCIAŁA ANTYALBUMINOWE W KOMÓRKACH ŚLEDZIONY KRÓLIKÓW HIPERIMMUNIZOWANYCH ALBUMINĄ SUROWICY

Streszczenie

1. Komórki śledziony królików hiperimmunizowanych albuminą surowicy wołu syntetyzują białka wykazujące ruchliwość elektroforetyczną γ-globulin. Około połowy nowo powstających polipeptydów daje się wytrącić mieszaniną antygen - przeciwciało.

2. Wirowanie w gradiencie sacharozy ekstraktów śledziony znakowanej [^{14}C]izoleucyną wykazało, że aminokwas jest włączany najpierw do frakcji polirybosomów a następnie do pojedynczych rybosomów lub monosomów.

3. Radioaktywność frakcji polirybosomów uzyskanych ze zwierząt hiperimmunizowanych może być wytrącona mieszaniną antygen - przeciwciało (do 80% całkowitej ilości impulsów). W przypadku polisomów ze zwierząt kontrolnych (nie immunizowanych) tylko 14% całkowitej ilości impulsów daje się wytrącić mieszaniną antygen - przeciwciało.

4. Wielkość współczynników sedymentacji polirybosomów z komórek śledziony pozwala sądzić, że zawierają one informacyjne RNA wystarczająco długie aby kodować nie tylko lekkie lecz i ciężkie łańcuchy immunoglobulin.

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PROPERTIES OF POLY-XANTHYLIC ACID AND ITS REACTIONS WITH POTENTIALLY COMPLEMENTARY HOMOPOLYNUCLEOTIDES

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1. The preparation of poly-riboxanthylic acid (poly-X) with the aid of polynucleotide phosphorylase from two different sources is described. 2. The structure of this poly-X, and of three commercial preparations (all of which exhibited essentially similar properties) was examined over the pH range 4.0 (in which the monomer residues are in the neutral form) to 8.0 (monomer residues in monoanionic form). The anomalous effect of the divalent cation Mg^{2+} on the structure of poly-X is described. 3. The polymer is fully resistant to pancreatic ribonuclease, but is completely hydrolysed to mononucleotides by ribonuclease T_1. 4. It undergoes thermal degradation to shorter chains, with no release of monomers, at elevated temperatures ($>60°$), by a mechanism similar to that for thermal degradation of poly-rA, and the biological implications of which are discussed. 5. Poly-X readily forms twin-stranded helices with poly-rU and several analogues of the latter. The relative stabilities of these were examined under different conditions and their significance evaluated. 6. Poly-X does not complex with either poly-dU or poly-dT; the structural and genetic implications of this are briefly reviewed. 7. Poly-X does not form any complex with poly-rC, poly-dC, or poly-dI. It does form the following triple-stranded helices: poly-(X+2rI), poly-(2X+1rA), and poly-(2X+1dA); the processes leading to formation of these complexes, and their properties, are described. 8. The remarkable ability of xanthosine (the deamination product of guanosine) to base-pair with a variety of other bases (as already noted previously by Michelson and Monny) is discussed in relation to its reported apparent inertness in biological and genetic processes.

The application of synthetic polynucleotides to studies on the structure and function of nucleic acids is fairly widespread, and considerable information may be derived from investigations on synthetic polymers containing base analogues not normally encountered in natural nucleic acids. The homopolymer poly-X[1],

[1] The following abbreviations are used in this text: poly-X, poly-riboxanthylic acid; poly-rA and poly-dA, poly-ribo- and deoxyribo-adenylic acids; poly-rU and poly-dU, poly-ribo- and deoxyribo-uridylic acids; poly-rT and poly-dT, poly-ribo- and deoxyribo-thymidylic acids; poly-EtU, poly-ribo-5-ethyluridylic acid; poly-FU, poly-ribofluorouridylic acid; poly-C, poly-ribocytidylic acid; poly-rI and poly-dI, polyribo- and deoxyribo-inosinic acids; poly-(X+U), 1:1 complex of poly-X with poly-U, with similar connotations for other polymer complexes; T_m, temperature corresponding to mid-point of temperature profile; ΔT, measure of breadth of temperature profile, defined as temperature range over which the hyperchromicity of a given polymer or complex increases from 25% to 75% of the maximum hyperchromicity.

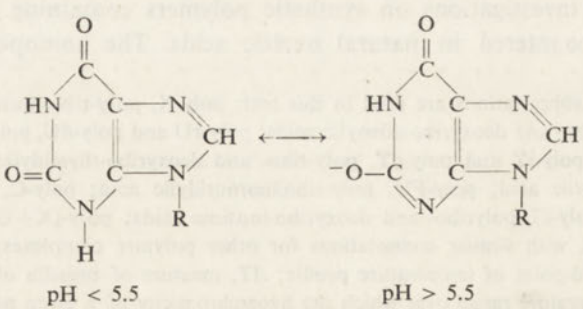
in which the base residue is the deamination product of guanine, is of special interest, and has already received some attention (Michelson & Monny, 1966).

It has been reported that nitrous acid deamination of guanine residues in DNA leads only to inactivation, and not to mutations (Schuster & Vielmetter, 1961); but this claim will probably require reinvestigation in view of the finding that the action of HNO_2 on guanine leads to appreciable formation of side products, including the 2-nitro and 8-nitro derivatives (Shapiro & Pohl, 1968). The same argument may be applicable to the observation that nitrous acid deamination of guanine-containing polynucleotides renders them inactive as messengers (Basilio, Wahba, Lengyel, Speyer & Ochoa, 1962).

Poly-X itself has been found to be inactive as a messenger in the *in vitro* amino acid incorporating system (Michelson & Grunberg-Manago, 1964), but this could be due to its failure to bind to ribosomes because of secondary structure (see below). Bessman *et al.* (1958) observed that dXTP cannot replace dGTP in the DNA polymerase system; on the other hand, dXTP is readily incorporated into polydeoxynucleotides by calf thymus deoxynucleotidyl transferase (F. J. Bollum, personal communication), although the relationship of this enzyme to DNA polymerase remains to be clarified. Furthermore XTP is unable to replace any of the natural substrates in the DNA-dependent RNA polymerase system; nor does it, at concentrations equivalent to those of a natural substrate, inhibit RNA synthesis when added to reaction mixtures containing the four natural substrates in this system (Kahan & Hurwitz, 1962).

While the weight of evidence apparently points to the absence of any significant role of xanthine in information transfer, this is difficult to reconcile with its demonstrated ability to pair with a wider variety of purine and pyrimidine bases than any other base or base analogue (Michelson & Monny, 1966), and lends additional interest to a study of the properties of poly-X.

Poly-X itself might also be expected to exhibit some rather unusual properties due to the fact that xanthosine possesses a pK_a of 5.5 (Cavalieri, Fox, Stone & Chang, 1954), due most likely to dissociation of the number 3 proton of the purine ring, as in Scheme 1, so that at neutral or slightly alkaline pH the base residues in the polymer are virtually in the ionized form. This has been taken account of in the planning of the experiments to be described below.



Scheme 1.

MATERIALS AND METHODS

Initial experiments were performed with a commercial sample of poly-X (Miles Chemical Co., Elkhart, U.S.A.), kindly provided by Dr. M. Laskowski Sr. These were subsequently continued with several samples prepared by polymerization of XDP (Sigma Chem. Co., St. Louis, Mo., U.S.A.) with polynucleotide phosphorylase from *Micrococcus lysodeikticus* (Matthaei *et al.*, 1967) and *E. coli* (Kimhi & Littauer, 1968), gifts of Dr. H. Matthaei and Dr. U. Z. Littauer, respectively. All preparations exhibited essentially the same properties.

Samples of poly-X were also kindly made available by Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.) and by Sigma Chemical Co. We are indebted to Dr. H. Matthaei and Dr. H. G. Zachau for samples of poly-rU, poly-rA and poly-rI; and to Dr. F. J. Bollum for poly-dA, poly-dI and poly-dT. Poly-FU was prepared according to Szer & Shugar (1963), poly-rT according to Świerkowski, Szer & Shugar (1965), and poly-EtU as elsewhere described (Świerkowski & Shugar, 1969). Poly-dU was prepared by Dr. Barbara Żmudzka by deamination of poly-dC (Żmudzka & Shugar, in preparation). Polymer concentrations were determined spectrally by alkaline or enzymic hydrolysis to mononucleotides.

Preparation of poly-X. (a), With polynucleotide phosphorylase from *E. coli.* The conditions used are described by Kimhi & Littauer (1968). The maximum yield of polymer, 15%, was attained with 1.5 hr. incubation at pH 8.2 and 37° and this value was unchanged on increasing the incubation period to 35 hr. No attempt was made to delineate the effect of pH on polymer yield.

(b), With polynucleotide phosphorylase from *M. lysodeikticus.* The conditions for polymerization were those described by Matthaei *et al.* (1967) and a 6 - 8 hr. incubation at pH 8.8 and 37° gave a 15% yield of polymer estimated by paper chromatography. Prolongation of the incubation time to 13 hr. did not increase the yield. Neither did a change in pH: at pH 5.4 and 5.9 only traces of polymer appeared after 20 hr. incubation. At pH 7.3 there was a 10% yield at 4 hr. and 15% at 20 hr. At pH 8.4 the maximum yield was about 15% after 20 hr. incubation, and this was unchanged on increasing the pH to 8.9.

Attempts to increase the yield of polymer were rather unrewarding. At best, the yield (estimated chromatographically) was raised to 20% by decreasing the incubation temperature to 32° and increasing the NaCl concentration to 3-fold that employed by Matthaei *et al.* (1967); or by increasing the pH to 10 at 32°. This latter modification permitted a decrease in incubation time to 2 - 3 hr. No improvement in yield was noted on increasing the Mg^{2+} concentration, using a 3′-hydroxyl terminated oligonucleotide primer, increasing the incubation temperature to 45°, or stepwise addition of enzyme during incubation. Following incubation, the polymer was isolated and freed of short chains by precipitation with ethanol and deproteinization with phenol, followed by dialysis against NaCl and EDTA. Maximum yields of isolated polymer samples did not exceed 10% with respect to substrate.

Aggregation of poly-X. Preparations of poly-X exhibit a marked tendency to form aggregates, evidenced by the following criteria: (a), an initial increase in

sedimentation constant and sharpening of the sedimentation boundary; (b), broadening of the temperature profile, with a concomitant decrease in temperature hyperchromicity; (c), increased resistance to hydrolysis by RNase T_1 (see below) and alkali; (d), decrease in solubility, leading eventually to precipitation in water or 0.1 M salt. This tendency to form aggregates is enhanced in acid medium and at low salt concentrations, and occurs also with lyophilized preparations if not stored under strictly anhydrous conditions. Once aggregation has occurred, it cannot be readily reversed by an increase in salt concentration or temperature, but can on prolonged storage at pH 7.5.

In most instances complex formation between two polymers was initially tested for in 1:1 mixtures of the two components, followed occasionally by 1:2 mixtures. In several instances, where necessary, one component was titrated continuously against the second.

A Radiometer type PHM 22-meter, with glass microelectrode, was employed for pH measurements. Absorption spectra and temperature profiles were run on a Unicam SP 500 spectrophotometer with a specially constructed heating block, the temperature of which was controlled by means of a circulating mixture of glycerol-water from a Hoeppler ultrathermostat. Temperature measurements were made by means of a thermistor located in a dummy cuvette; and several minutes were allowed to elapse for equilibrium to be attained at a given temperature. Cuvettes with 10-mm. path length were used throughout, the stoppers being either of ground quartz (Hilger) or Teflon (Unicam); in general the latter were the more convenient.

All temperature profiles were tested for reversibility, and reference to this will be made below, where necessary. Attention is drawn to this point because of the previously observed lack of reversibility of the profile of poly-A under certain conditions (Barszcz & Shugar, 1964).

RESULTS AND DISCUSSION

Properties of poly-X

At room temperature at pH 2, where the xanthosine residues are presumably in the neutral, diketo form, addition of 0.1 M-NaCl to a solution of poly-X results in the appearance of 2.5% and 10% hyperchromicity at the maxima of the two absorption bands, 238 mμ and 260 mμ, respectively. At pH 12, where all the residues are in the form of the monoanion, addition of 0.1 M-NaCl does not alter the absorption at the 250 mμ maximum, but leads to 8% hyperchromicity at 275 mμ.

In 0.3 M-KOH at about 20°, the half-time for alkaline hydrolysis of poly-X to mononucleotides is 60 min. Complete hydrolysis to monomers is accompanied by the appearance of 8% hyperchromicity at 250 mμ and 45% at 275 mμ, in 0.3 M-KOH. These figures vary slightly for different preparations of poly-X.

The temperature profiles of three commercial preparations of poly-X, as well as a typical one prepared as described above, at neutral pH, are exhibited in Fig. 1.

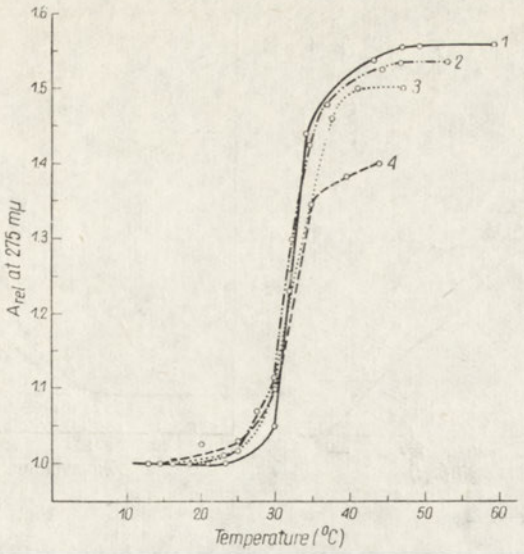


Fig. 1. Temperature profiles in 0.1 M-Na^+ and 0.025 M-phosphate buffer, pH 7.0, of different preparations of poly-X. Samples *1*, *2* and *4* are commercial preparations; sample *3* was polymerized from XDP as described in Text.

Note: In this and following figures, A_{rel} is the ratio of the absorbance at a given temperature to the absorbance of the fully structured form, both measured at the same wavelength.

Note that, while there is some variation in temperature hyperchromicity and form of the profiles, the T_m value is essentially the same for all preparations.

As might have been anticipated, poly-X was found to be resistant to pancreatic ribonuclease. However, in agreement with the observation of Egami, Takahashi & Uchida (1964), who showed that XpC is slowly hydrolysed by ribonuclease T_1, the latter enzyme hydrolysed poly-X rapidly to monomers at neutral pH at 37°. The resulting hyperchromicity for one of the commercial preparations under the foregoing conditions was 31% at 250 mμ and 86% at 275 mμ. For one of our own preparations the corresponding values were 23% and 80%. The changes in absorption accompanying enzymic hydrolysis are illustrated in Fig. 2; note, in particular, the appearance of the well-defined 275 mμ maximum of the monomer, masked in the polymer, on hydrolysis to mononucleotides.

The two maxima of xanthosine are clearly evident in the polymer, although that at 275 mμ is partially masked in the latter (Fig. 2). These two maxima in the quartz ultraviolet correspond to two different electronic transitions in the purine ring, each or both of which may be involved in the interactions between residues leading to formation of secondary structure. It is consequently of interest that each of these maxima exhibits temperature hyperchromicity (as well as residual hyperchromicity, see Fig. 4 below), more pronounced at the longer wavelength maximum. In a number of experiments, therefore, profiles were run simultaneously at both wavelength maxima. It was found that, irrespective of the pH, salt concentration, or presence of various cations, the breadth and T_m of the profile at 250 mμ were always identical with that for the longer (as well as other) wavelengths (Fig. 3).

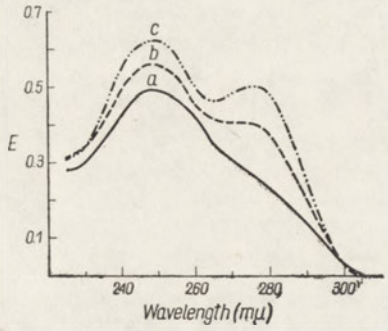


Fig. 2

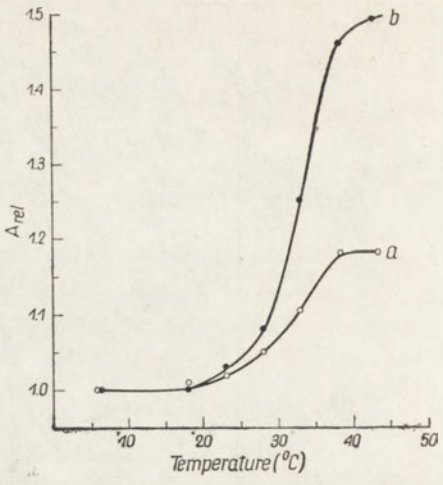


Fig. 3

Fig. 2. Change of absorption spectrum of poly-X on enzymic hydrolysis (RNase T_1) in 0.1 M-Na^+ and 0.025 M-phosphate buffer, pH 7.0, at 25°. (*a*), Control sample; (*b*), following 40 min. hydrolysis; (*c*), following complete hydrolysis to monomers.

Fig. 3. Typical profiles (*a*), at 250 mμ and (*b*), at 275 mμ for poly-X in 0.1 M-Na^+ and 0.025 M-phosphate buffer, pH 7.0.

The same was found to hold for complexes of poly-X with other homopolymers (see below), as illustrated for the complexes of poly-X with poly-rA and poly-dA in Figs. 13 and 15, below.

One additional fact is worthy of note, and is illustrated in Fig. 4. When a given preparation of poly-X at neutral pH was heated to the point of maximum hyperchromicity, the resulting absorption spectrum, corresponding to the so-called random coil form, is exhibited by curve *b* in Fig. 4. On hydrolysis to mononucleotides, this spectrum is transformed to that of the monomer, curve *c*. It will be seen that the random coil form of poly-X and the monomer exhibit identical absorption at 260 mμ. We shall refer to this further below.

The properties of the polymer were next examined by measurements of temperature profiles over a range of pH values, from 4.7 to 8.8, sufficiently broad to include both the neutral and fully ionized forms of the xanthosine residues, $pK \sim 5.5$, in the free state.

At pH 4.7 and low ionic strength (0.05 M-Na^+), the temperature profile of poly-X exhibits only 8% hyperchromicity at 260 mμ, while the profile breadth, ΔT, extends over a 17° range (Fig. 5). With increase in pH, a well-defined profile begins to appear at about pH 5.2 with a breadth, ΔT, of about 7 - 8°. The temperature hyperchromicity then increases stepwise with pH. At about neutrality, pH 6.6, the temperature profile resembles that for a co-operative helix-coil transition when the salt concentration is 0.05 M and higher. A similar co-operative transition is observed at pH 7.1. Notwithstanding the similarity of the profile at pH 7.1 to that at pH 6.6, the former exhibited both a lower T_m and higher temperature hyperchromicity. Above pH 7.5

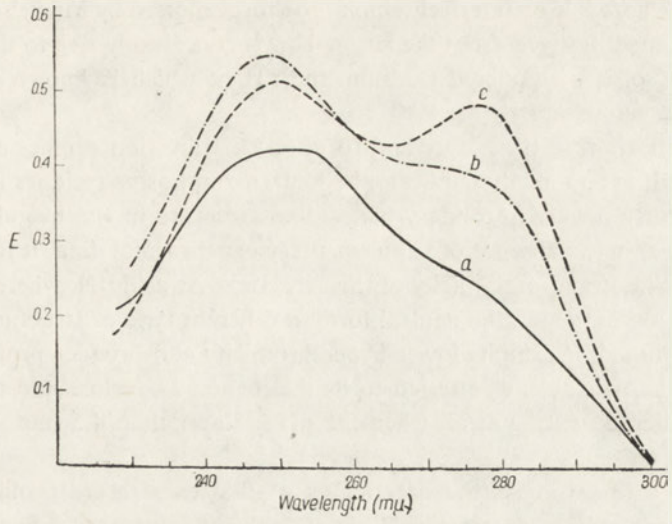


Fig. 4. Absorption spectra in 0.1 M-Na^+ and 0.025 M-phosphate buffer of (*a*), poly-X at 15° (helica form); (*b*), poly-X at 50° (random coil form); (*c*), poly-X hydrolysed by RNase T_1 to mononucleotides (and identical to spectrum of xanthosine-3′-phosphate).

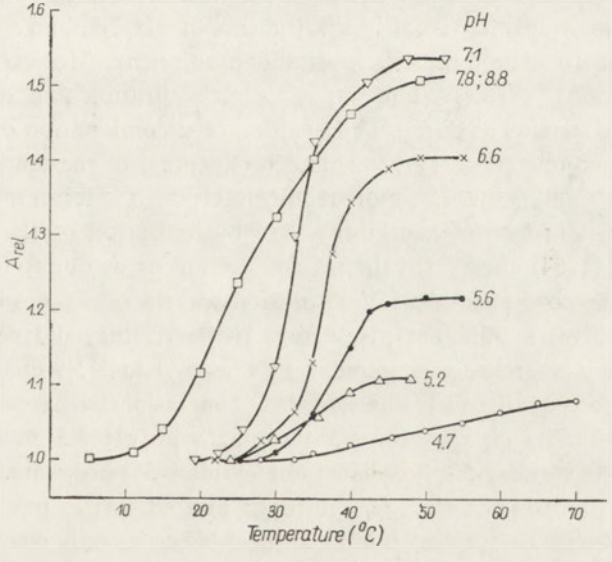


Fig. 5. Helix-coil transitions of poly-X at various pH values as indicated, in 0.05 M-Na^+ at pH 4.7, and in 0.1 M-Na^+ for other pH values. From pH 4.7 to 5.6 the profiles were run at 260 mμ and from pH 6.6 to 8.8 at 275 mμ.

the breadth of the profile increases to the extent that the transition no longer appears to be co-operative in nature.

These results, while qualitatively similar to those reported by Michelson & Monny (1966), differ quantitatively from the latter. This is conceivably due to the use by the latter authors of a tris-buffered medium, the pH of which is known to be highly dependent on temperature.

The modification of the nature and form of the transition profiles as a function of pH are such as to directly implicate the ionized xanthosine residues in the formation of a clearly defined secondary helical-like structure in the neighbourhood of neutrality. But, in the absence of supplementary experimental data, it is not feasible to determine the strand multiplicity of this structure. At acid pH, where the xanthosine residues are largely in the neutral form, a different type of structure is formed; this latter, although it exhibits lower hyperchromicity and broader profiles, is nonetheless remarkably stable, as attested to by the higher T_m values and the apparent failure to undergo melting at all when the pH is lower than 4.5 (not shown in Figure).

Even more surprising is the broadening of the temperature profile above pH 7.5, with a concomitant decrease in T_m, although the temperature hyperchromicity is unaltered. It should, however, be noted that at pH 7.8 (and 8.8) the profile, while less co-operative in nature than at neutral pH, is still unlike the broad, non-cooperative profile of neutral poly-A which is known to be due largely to base stacking in a single strand.

The relatively abrupt transition from a co-operative to a less co-operative profile between neutrality and pH 7.8 is obviously related to ionization of some of the xanthosine residues. Michelson & Monny (1966) have shown by spectrophotometric titration that this transition is fairly sharp at about pH 7.3, and we interpret it as most likely due to a modification in strand multiplicity. However, this problem can probably only be resolved by potentiometric titration and determination of sedimentation constants at different pH values, or a combination of both.

The loss in co-operative character, and broadening, of the temperature profiles in acid medium could perhaps be more readily interpreted in terms of the protonation of the $N_{(7)}$ ring nitrogen of xanthosine than by ionization of the $N_{(3)}$ hydrogen. Cavalieri *et al.* (1954) interpreted the pK_a of xanthosine as due to ionization of the $N_{(3)}$ hydrogen by comparing the UV absorption spectra of a series of *N*-methylated xanthine derivatives at different pH values. However, they did not have at their disposal two key reference compounds: 1,9-dimethyl and 3,9-dimethyl xanthines. We have therefore qualitatively checked their conclusion by paper electrophoresis, in which the mobility of xanthosine-5′-phosphate at pH 3.5 was tested against the mobilities of uridine-5′-phosphate and cytidine-5′-phosphate. At pH 3.5 the latter is protonated on the ring $N_{(3)}$ nitrogen and so carries one negative charge and one positive charge, whereas uridine at this pH carries only one negative charge. It was, in fact, found that xanthosine-5′-phosphate travelled along with uridine-5′-phosphate, in agreement with the existence of the base residue of the former as the neutral species at this pH.

A qualitative examination was made of the influence of concentration of monovalent sodium ions on the helix-coil transitions over a pH range of 4.7 to 8.8. At all pH values the T_m increased with increasing ionic strength, e.g. at pH 5.7 from 35° to 56° when the Na^+ concentration was raised from 0.05 M to 1.0 M. At pH 7.8 the corresponding increase in T_m was from 24° to 35°. At pH 6.6 the increase in going from 0.05 M to 0.2 M-Na^+ was from 35° to 37.5°. Somewhat significant was the fact that in 1.0 M-Na^+ the co-operative character of the profiles was diminished.

The influence of divalent cations, exemplified by Mg^{2+}, is somewhat exceptional. The influence of this cation on the temperature profiles was examined in 0.01 M-acetate buffer at pH 5.7 (where approximately half the base residues are ionized) and in 0.01 M-phosphate buffer at pH 7.8 (where all the base residues are certainly ionized). The results are illustrated in Fig. 6 which, for purposes of comparison, shows also the profiles of poly-X under the same conditions in 0.1 M-NaCl. These experiments were conducted with water doubly distilled in glass vessels.

At both pH values it is only at very low Mg^{2+} concentrations (about 10^{-5} M) that the profiles of poly-X resemble those in 0.1 M-NaCl. As the concentration of the divalent cation is increased, the temperature profiles are broadened at the higher pH value, while the hyperchromicity is appreciably reduced at the lower pH. Since at all Mg^{2+} concentrations the spectrum of poly-X is unaltered at the lower temperature (ordered form), it follows that the presence of this ion inhibits and reduces the co-operative nature of the melting process. This is to be contrasted with the observation of Michelson & Monny (1966), who found that the addition of 10^{-2} M-Mg^{2+} to a complex of poly-X and poly-U resulted in an enhancement of helix stability without modification of the co-operative nature of the helix-coil transition.

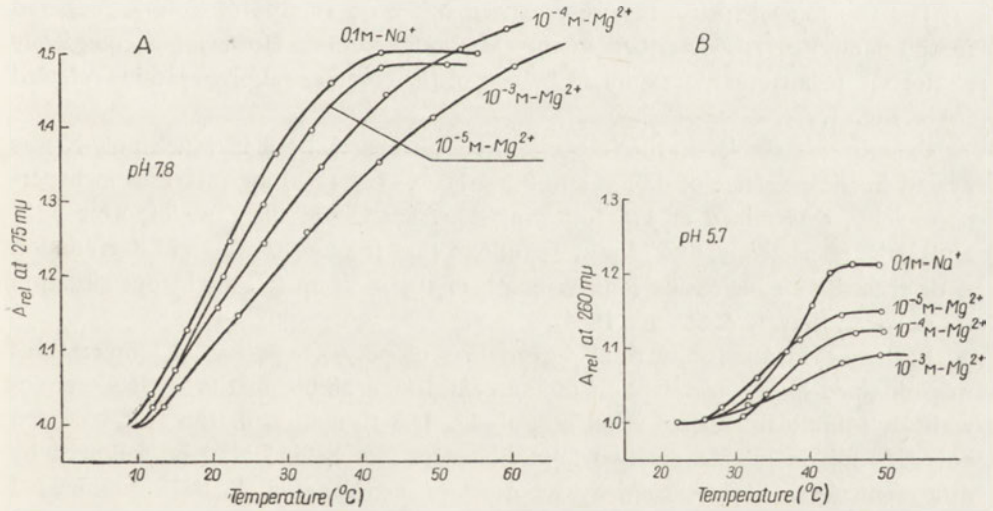


Fig. 6. Effect of various concentrations of Mg^{2+} on the helix-coil transition in poly-X (*A*), at pH 7.8 in 0.01 M-phosphate buffer, and (*B*), at pH 5.7, 0.01 M-acetate buffer. For comparison purposes each set of curves exhibits the profile in the absence of Mg^{2+} in 0.1 M-Na^+.

Thermal lability of poly-X

The thermal transitions described in the previous section were fully reversible. However, in subsequent experiments on the formation of complexes with other potentially complementary homopolynucleotides, to be described below, it proved necessary to follow some transitions to temperature above 60°. Under these conditions the subsequent cooling profiles were observed to depart appreciably from the initial heating curves; while reformation of the presumed complexes, on cooling to room temperature, was incomplete. By comparison with the analogous situation for poly-rA (Barszcz & Shugar, 1964), it was suspected that degradation of poly-X at elevated temperatures was involved.

Samples of poly-X, at a concentration of 5×10^{-5} M in 0.02 M-phosphate buffer, pH 7.0, were therefore heated in sealed ampoules at 90° for 0.5, 2, 3 and 4 hr. The samples were then cooled and Na^+ added to each to a final concentration of 0.1 M. As can be seen from Fig. 7B, heating at this elevated temperature led to a marked broadening of the temperature profile and a decrease in the T_m value. A similar behaviour was observed when poly-X was heated at pH 7.8 (Fig. 7C).

Attention should now be drawn to the fact that, whereas enzymic hydrolysis of poly-X leads to a uniform increase in absorbance of the entire spectrum, whether hydrolysis is carried out at 23° (helical form) or at 40° (random coil form) at pH 7, the foregoing thermal degradation produces appreciable increase in hyperchromicity of the helical form of poly-X principally at the 275 mμ peak and very little in the 250 mμ band (Fig. 7A, curves *b*, *c*, *d*). Irrespective of the time of heating the absorption spectra of all heated samples are almost identical following thermal melting (Fig. 7A, curve *e*); this is, of course, to be expected in the light of the spectrum which results from enzymic hydrolysis to monomers (cf. Fig. 7A, curves *e*, *f*).

The foregoing experiments were carried out on a sample of poly-X prepared in this laboratory as described in the Methods section. However, a completely analogous behaviour was exhibited by one of the commercial preparations referred to in Fig. 1.

Experiments similar to the foregoing were also carried out in which poly-X was heated in the presence of 0.05 M and 0.2 M-Na^+. The resulting increases in hyperchromicity, determined at 17°, following 4 hr. heating of the samples, were 32% and 21%, respectively, at 275 mμ. It follows that the rate of thermal degradation is decreased with increasing ionic strength of the medium (cf. analogous situation for poly-rA, Barszcz & Shugar, 1964).

Further evidence for thermal degradation of poly-X at elevated temperatures was obtained by an examination of the complexing ability of the heated samples with an equimolar concentration of poly-U. The formation of the twin-stranded poly-(X+U) on mixing the two homopolymers (see below) may be followed by measurements of hypochromicity at different wavelengths. With a non-heated control sample of poly-X, this is 6% at 250 mμ immediately after mixing and is unchanged with a poly-X sample heated for 30 min. With poly-X heated for 2 hr. at 90° it is 20%. Maximum complex formation is much slower at pH 7.1 than pH 7.8

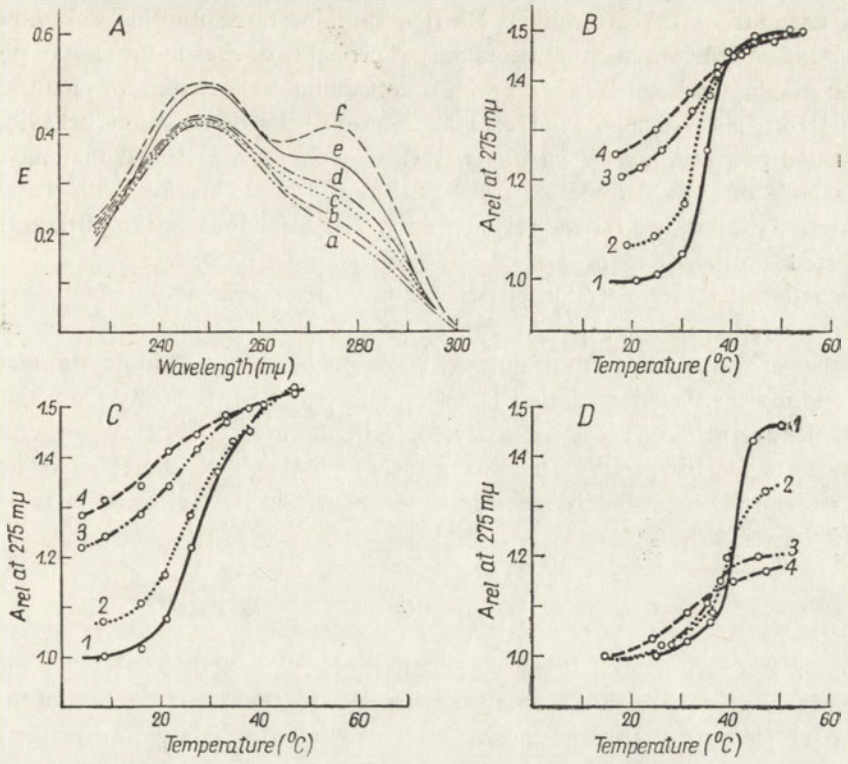


Fig. 7. Illustrating the thermal lability of poly-X.

A, Poly-X in 0.1 M-Na^+ and 0.02 M-phosphate buffer, pH 7.0: *a*, non-heated control; *b*, *c*, *d*, heated at 90° for 0.5, 2 and 3 hr., respectively, and spectra then determined at 17°; *e*, curves *a*, *b*, and *c* at 50°; *f*, curves *a*, *b* and *c* following enzymic hydrolysis to mononucleotides.

B, Temperature profiles of poly-X in 0.1 M-Na^+ and 0.02 M-phosphate buffer, pH 7.0, following heating at 90°: *1*, non-heated control; *2*, *3* and *4*, following heating for 0.4, 2 and 4 hr.

C, Temperature profiles of poly-X in 0.1 M-Na^+ and 0.02 M-phosphate buffer, pH 7.8, following heating at 90°: *1*, *2*, *3*, *4*, as in *B*.

D, Temperature profiles of the twin-stranded poly-(X+U) in 0.1 M-Na^+ and 0.02 M-phosphate buffer, pH 7.0, following heating at 90°: *1*, *2*, *3*, *4*, as in *B*.

(see below) and was allowed to proceed for 24 hr. at 4°. The temperature profiles, and T_m values, of the complexes were then measured, with results shown in Fig. 7D.

The thermal lability of poly-X recalls the previously observed lability to elevated temperatures of both the acid and neutral forms of poly-A, which was shown to be due to fragmentation of the polynucleotide chain (Barszcz & Shugar, 1964). That the same phenomenon occurs with poly-X was demonstrated by subjecting the heated samples to paper chromatography, using the solvent system *n*-butanol -glacial acetic acid - water (50:25:25, by vol.), in which the R_F values of xanthine and xanthosine are, respectively, 0.45 and 0.34. On heating for 4 hr. at pH 7.1 and 90°, the formation of shorter fragments was clearly placed in evidence by their small

displacement from the starting line. No free xanthine or xanthosine was, however, detected. Consequently the thermal lability is probably due, as in the case of poly-A, to phosphodiester bond breakage, with a concomitant shortening of chain length.

The foregoing suggested an examination of the sedimentation behaviour of the heated preparations. When this was carried out, it was found that the $S_{20,w}$ of a sample of poly-X decreased from 10 to 6 after 3 hr. heating, with the heated preparation exhibiting a broad polydispersity. The behaviour in the ultracentrifuge of heated preparations of poly-X (and of poly-A) is being studied in more detail and will be reported on elsewhere.

However, the thermal degradation of poly-X differs in one important respect from that of the *neutral* form of poly-A. When poly-A is heated in neutral medium, the resulting thermal degradation is discernible only with difficulty from the profile in neutral medium; it is necessary to form the acid twin-helical structure to demonstrate that degradation has taken place. With poly-X, the effect of heating at neutral pH is immediately reflected in the profile in neutral medium as well as at pH 7.8 (Fig. 7).

Complexes of poly-X with analogues of poly-U

Poly-X and poly-U. In 0.01 M-acetate buffer, pH 5.2, with 0.05 M-NaCl, a mixture of these two polymers exhibits an absorption spectrum which is the sum of the two components; and this situation prevails when the mixture is heated over the range 18° to 50°, due regard being paid to the temperature hyperchromicity of the poly-X component. Increasing the salt concentration to 0.1 M is without effect. Heating and cooling, followed by repeated heating, is also without effect. The same results were obtained at pH 5.75.

However, when a 1:1 mixture of the two polymers was prepared in 0.01 M-phosphate buffer, pH 7.8, in the presence of 0.05 M-NaCl, at 20°, a decrease in absorption of 20% at 255 mμ, the wavelength of the absorption maximum of the resulting complex, Fig. 8 (with respect to the arithmetical sum of the two compo-

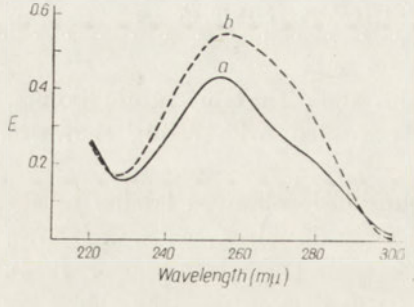


Fig. 8. Absorption spectra in 0.05 M-Na^+ and 0.01 M-phosphate buffer, pH 7.8, of (*a*), the helical form of the 1:1 complex of poly-X with poly-U (at 20°), and (*b*), the melted form (at 50°). T_m value, measured from profile at 275 mμ, is 42.5°.

nents) was observed immediately on mixing the two polymers. Heating of this solution gave a melting profile, measured at 270 mμ (at which wavelength the hyperchromicity is maximal) illustrated in Fig. 9 which also shows additional profiles under similar conditions, but with different NaCl concentrations. These results

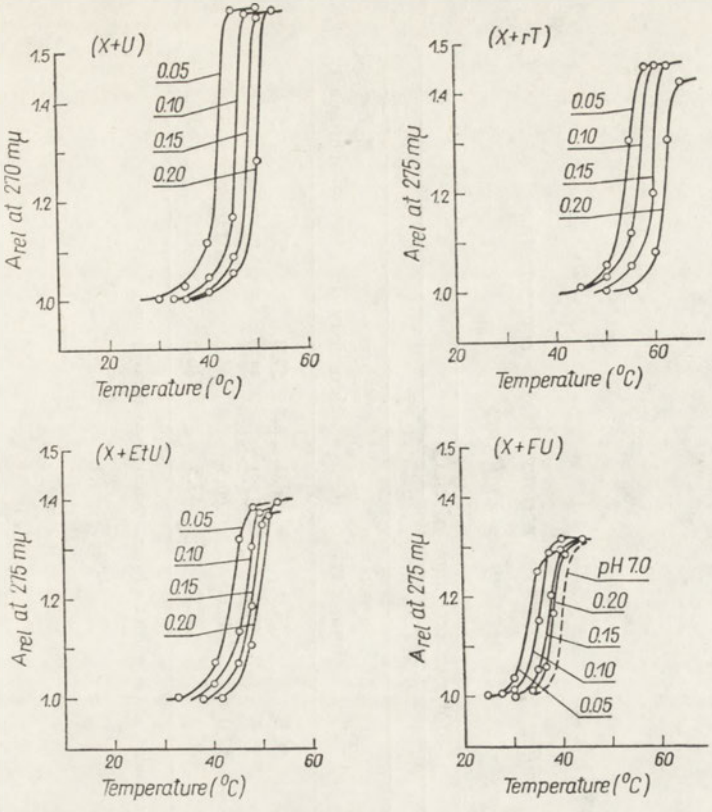


Fig. 9. Helix-coil transitions for the twin-stranded complexes of poly-X with poly-U, poly-rT, poly-EtU and poly-FU at various Na^+ concentrations as indicated, all in 0.01 M-phosphate buffer, pH 7.8.

are also presented quantitatively in Table 1. The absorption spectra of the complexes (at low temperatures), as well as of the melted mixtures, cf. Fig. 8 (at temperatures above the top of the profiles) were unaltered by modifications in the NaCl concentration.

Nature of complex of poly-X and poly-rU. The form of the profile of poly-X with poly-U shows clearly that the resulting complex is 1:1; and this situation prevails for complexes of poly-X with all the poly-U analogues described below.

If, in a 1:1 mixture of X and U, there were formed a (2U+1X) complex, then the excess free poly-X in solution would immediately reveal itself by its own temperature profile in the range 10° - 30° (cf. Fig. 5) where the (X+U) complex has not yet begun to melt. It is clear from Fig. 9 that this is not the case.

The foregoing was further confirmed by an examination of the profiles of mixtures containing different proportions of X and U, and illustrated in Fig. 10. A sample solution was prepared containing 1X + 1.2 U, and the temperature profile examined. To this same solution was then added poly-X so that the final ratio of the components was 1X:0.63U; and, finally, poly-U was added to give a ratio of 1X:2.2U. From

Table 1

Properties of temperature profiles, in 0.01 M*-phosphate buffer,* pH *7.8, at various* NaCl *concentrations, of the 1:1 complexes with poly-X of poly-U and several of its analogues*

Poly-U analogue	λ^a (mμ)	Molar concentration of NaCl								
		0.05			0.10		0.15		0.20	
		T_m^b	% hyperchromicity	ΔT	T_m	% hyperchromicity	T_m	% hyperchromicity	T_m	% hyperchromicity
Poly-U	270	43.0	58.0	1.5	46.5	57.0	48.0	57.0	50.0	57.0
Poly-FU	275	32.5	29.0	3.0	35.5	31.0	37.0	31.0	37.5	29.0
Poly-rT	275	54.5	45.0	2.0	57.0	45.0	60.5	45.0	62.0	42.0
Poly-EtU	275	43.0	38.5	3.5	46.0	37.5	47.5	36.5	49.0	39.5

[a] This is the wavelength at which the temperature profiles of the complexes were measured, and at which the hyperchromicity was at maximum.
[b] T_m values of the profiles were estimated to the nearest 0.5°.

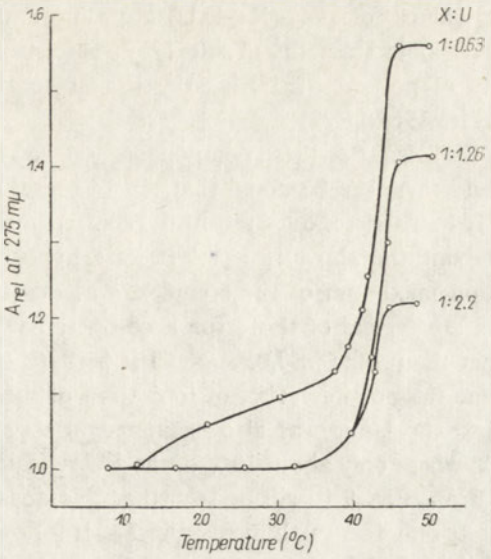


Fig. 10. Temperature profiles in 0.05 M-Na^+ and 0.01 M-phosphate buffer, pH 7.8, of mixtures of poly-X and poly-U in various proportions as indicated.

Fig. 10 it will be seen that, when there is an excess of X in the presence of 1X and 1U, the temperature profile of the excess poly-X (with its characteristic T_m) is at once placed in evidence. When there is an excess of poly-U in the solution, its presence is revealed by the apparent decrease in hyperchromicity of the complex following the addition to a 1X + 1U mixture of an additional equivalent of poly-U.

Finally, whatever the ratio of the two components, the thermal profile observed possesses the same T_m. This by itself does not prove that the complex formed is always 1:1 but, taken in conjunction with the observations outlined in the preceding paragraph, it may be considered as conclusive that the complex formed between poly-X and poly-U is 1:1, i.e. the complex formed is poly-(X+U). The same arguments apply to the complexes with analogues of U described below.

Poly-X and poly-rT. A 1:1 mixture of these two polymers in 0.01 M-phosphate buffer, pH 7.8, and 0.05 M-NaCl, exhibits 12% hypochromicity at 260 mμ, the absorption maximum of the complex, on mixing at 20°. This value is unchanged when the mixture is stored for 18 hr. at 4°. The thermal profiles under these conditions at various NaCl concentrations are represented in Fig. 9 and the corresponding values of T_m in Table 1. Note, in particular, that regardless of the NaCl concentration, poly-(X+rT) possesses a T_m value which is 12° above that for the corresponding poly-(X+U). We shall revert to this in the Concluding remarks.

Poly-X and poly-EtU. A 1:1 mixture of these two in 0.01 M-phosphate, pH 7.8, with 0.05 M-NaCl exhibits a hypochromicity at the wavelength of maximum absorption of the complex, 260 mμ, of 18%, unchanged after 18 hr. storage at 4°. The profiles of the resulting poly-(X+EtU) are exhibited in Fig. 9 and the corresponding T_m and ΔT values shown in Table 1.

Note that the T_m values for poly-(X+EtU) are almost identical to those for poly-(X+U) at various ionic strengths (Table 1), in agreement with the observation that the stability of poly-(A+EtU) is very similar to that of poly-(A+U) (Świerkowski & Shugar, 1969).

Poly-X and poly-FU. Mixing of these components was carried out at 23° at pH 7.8, as above. It should therefore be noted that the FU residues possess a pK_a of 7.8, so that at this pH half the residues are in the ionized form.

Immediately on mixing the above in a 1:1 ratio, a hypochromicity of 19% at 260 mμ, the absorption maximum of the complex, was observed. The profiles of the resulting complex are typical of those for a co-operative helix-coil transition, with the exception that the profile in 0.05 M-NaCl is perhaps slightly broader (Fig. 9). In order to examine the possible effect of ionization of the FU residues on the nature of the complex, the latter was also examined in 0.01 M-phosphate buffer, pH 7.0, in 0.2 M-NaCl, where only about 10% of the FU residues would be expected in the ionized form. From Fig. 9 it will be seen that this does not alter the shape of the profile, but results only in a small increase in T_m. It follows that the ionization of the FU residues is inhibited in the complex, and that the $N_{(3)}$ proton must be involved in the hydrogen bonds of the poly-(X+FU) complex. This in turn makes it possible to draw some conclusions as to the nature of the resulting complex (see below).

Under all the above conditions the T_m of poly-(X+FU) is about 10° below that of poly-(X+U). In this respect these two complexes differ from the corresponding poly-(A+FU) and poly-(A+U), which exhibit almost equal stabilities at neutral pH (Szer & Shugar, 1963).

Poly-X and poly-dT. A 1:1 mixture of these two was prepared initially under conditions identical to those used above for poly-X and poly-rT, i.e. in 0.01 M-phosphate buffer, pH 7.8, with 0.05 M-NaCl, at 8°. Possible complex formation was, however, examined at NaCl concentrations up to 0.7 M (at the latter salt concentration the pH of the solution shifted to about 7.2).

Under all conditions outlined in the preceding paragraph, no evidence for any complex formation could be observed. The thermal profiles at various salt concentrations demonstrated additivity of the components and the temperature hyperchromicity observed was due to the poly-X alone, as confirmed also by its T_m.

In view of this unexpected finding, it was deemed advisable to check the poly-dT preparation. This was done by complexing poly-dT with poly-A at a ratio of 1:1 in 0.1 M-NaCl, pH 7.8, as described by Riley, Maling & Chamberlin (1966). The spectral properties of the resulting complex were identical to those described by the previous authors, thus confirming the inability of poly-dT to complex with poly-X.

Poly-X and poly-dU. The absorption spectrum of a 1:1 mixture of these two components, in 0.1 M-NaCl at pH 7.8 and at a temperature of 10°, was fully additive. Heating to 60° and subsequent cooling did not alter this situation, pointing to the absence of complex formation; and the thermally induced changes in absorption corresponded to those expected from poly-X alone (poly-dU, like poly-dT, is unaffected by changes in temperature). These observations were duplicated at pH 7.1.

In the pH range 7 - 8, in 0.1 M-Na^+, the helical form of poly-X begins to dissociate at about 10° and dissociation is complete at about 50° (cf. Fig. 5). It may be anticipated that complexes of poly-X with poly-dU or dT would exhibit T_m values below those for complexes with poly-rU or rT, by possibly as much as 20° (cf. results below for complexes of poly-X with poly-rA and poly-dA). One might therefore have expected the presumed complexes of poly-X with poly-dU and poly-dT to possess T_m values of about 23° and 35°, respectively (cf. Table 1). This is the temperature range in which about 50% of poly-X under these conditions is in the helical form and this form is favoured thermodynamically relative to the complexes. The absence of complex formation here is obviously related in some way to the absence of the 2'-hydroxyl, regarding the role of which there is at present considerable speculation (Ts'o, Rapaport & Bollum, 1966), but no concrete evidence.

Complexes of poly-X with poly-I

Poly-X and poly-rI. No hypochromicity is exhibited by a 1:1 mixture of these two homopolymers at pH 4.6 (0.01 M-acetate buffer) in the presence of 0.1 M or 0.2 M-Na^+. Prolonged storage, or heating and cooling of the solution, were without effect.

Complex formation was observed in 0.01 M-phosphate buffer, pH 6.65, in the presence of 0.05 M to 0.2 M-Na^+; but the nature of the complex could not be readily characterized because at this pH its T_m was very close to that of poly-X. At approximately the same pH (pH 7), Michelson & Monny (1966) concluded that only a 1:1 complex is formed on the basis of mixing curves.

However, at pH 7.8 and in 0.1 M-Na^+, the profile of a 1:1 mixture consisted of two components with T_m values of 25° and 36°. Under these conditions poly-rI exhibits little temperature hyperchromicity, and poly-X a profile with a T_m of 25° (cf. Fig. 5).

An examination was therefore made of three mixtures, as follows (pH 7.8, 0.1 M-Na^+): (*a*) 1X + 1rI; (*b*) 1X + 2rI; (*c*) 2X + 1rI (Fig. 11A). The profiles for samples *a* and *c* were found to contain two components, one of which corresponded to free poly-X. Sample *b* exhibited a single profile corresponding to the triple-stranded (1X+2rI) with $T_m = 37°$.

Formation of the triple-stranded helix is rapid when the components are mixed at 50° (i.e. above the melting temperature) and then cooled. At 4° complex formation requires about 18 hr., and the spectral characteristics of the resulting helix are identical to those of the complex formed at elevated temperatures. Profiles for the (1X+2rI) helix as a function of Na^+ concentration are shown in Fig. 11B, from which it will be noted that the co-operative nature of the helix-coil transition increases with ionic strength. It should be emphasized that the absorption spectra of both the helical and coil forms were almost independent of the ionic strength of the medium.

Poly-X and poly-dI. An equimolar mixture of these two homopolymers was prepared at pH 7.85 in 0.05 M-Na^+. Under these conditions the T_m for poly-dI

is 5 - 8° (Inman, 1964a), while that for poly-X is ~ 21° (see above). Profile measurements were therefore made from 2°, at 275 mμ (where only poly-X exhibits temperature hyperchromicity, about 50%), and at 250 mμ (where poly-X exhibits 20%, and poly-dI 36%, hyperchromicity). The mixture was put through a heating and cooling cycle over the range of 2° - 50°, stored at 2° for 18 hr., and then put through a second heating and cooling cycle. The resulting profiles revealed the independent melting of all the X in the solution.

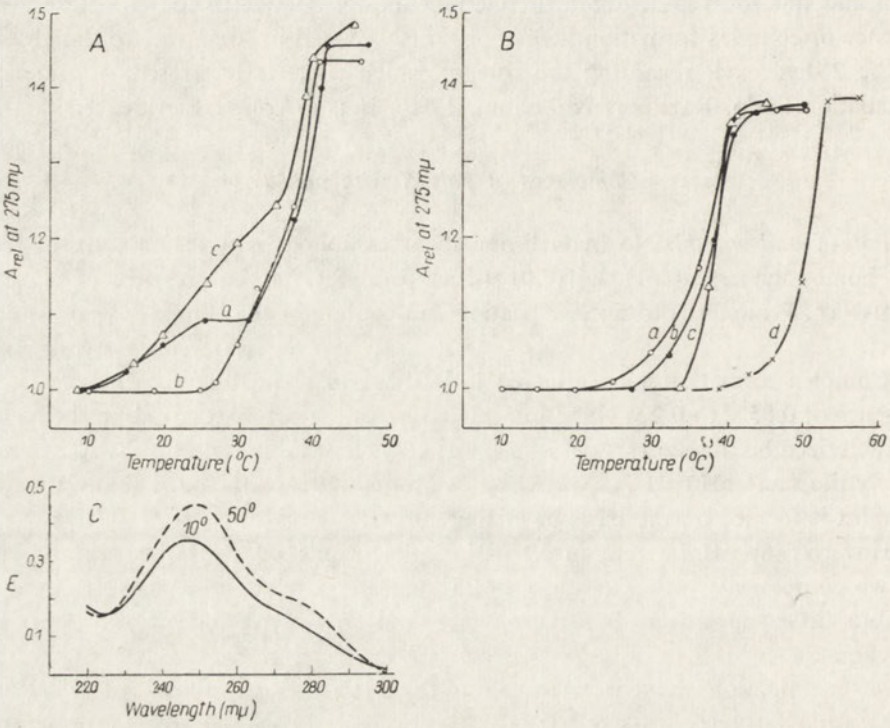


Fig. 11. Characteristics of complex of poly-X with poly-rI in 0.02 M-phosphate buffer, pH 7.8:

A, In presence of 0.1 M-Na^+: (*a*), 1X + 1rI; (*b*), 1X + 2rI; (*c*), 2X + 1rI.

B, Profiles of poly-(1X+2rI) measured at 275 mμ in (*a*), 0.1 M-Na^+; (*b*), 0.15 M-Na^+; (*c*), 0.2 M-Na^+; (*d*), 0.7 M-Na^+ (pH 7.2).

C, Absorption spectra of poly-(X+2rI) in 0.1 M-Na^+ at 10° (helical form) and 50° (melted form).

An increase in Na^+ concentration to 0.15 M (expected T_m for poly-dI, ~ 22°) led to the slow formation of helical poly-dI, which was complete after 3 days at 4°. Heating of such a solution demonstrated the independent melting of poly-X (T_m = 26°, followed at 275 mμ) and poly-dI (T_m = 20°, followed at 250 mμ).

It is consequently clear that, under conditions where the triple-stranded helix (1X+2rI) is readily formed, there is no complex formation between the corresponding X and dI.

Attempts at complex formation of poly-X with poly-rC

Various experiments performed at pH values of 4.6 and 7.8, in the presence of appropriate concentrations of Na^+, merely confirmed the findings of Michelson & Monny (1966), i.e. absence of any complex formation.

Complexing of poly-X with poly-A

Poly-X and poly-rA. When these two homopolymers were mixed at a ratio of 2X:1rA in 0.1 M-Na^+ at pH 7.0 at 20°, the resulting solution exhibited 10% hypochromicity at 250 mμ, initial complex formation being instantaneous. The hypochromicity subsequently increased slowly with time so that, at 4°, it attained 29% at 250 mμ after about 24 hr. Such behaviour is consistent with the formation of an initial complex, which then underwent some rearrangement.

This was further examined by heating the foregoing solution immediately after mixing and following changes in absorbance at 250 mμ (maximal for both the complex and free poly-A) and at 275 mμ (maximal for poly-X). From Fig. 12 it will be seen that an increase in temperature places in evidence the melting profile of some free poly-X at 275 mμ, with a simultaneous enhancement of hypochromicity at 250 mμ, up to 45 - 50° (transition I). This is followed by the melting of a new complex resulting from the rearrangement of the first (transition II).

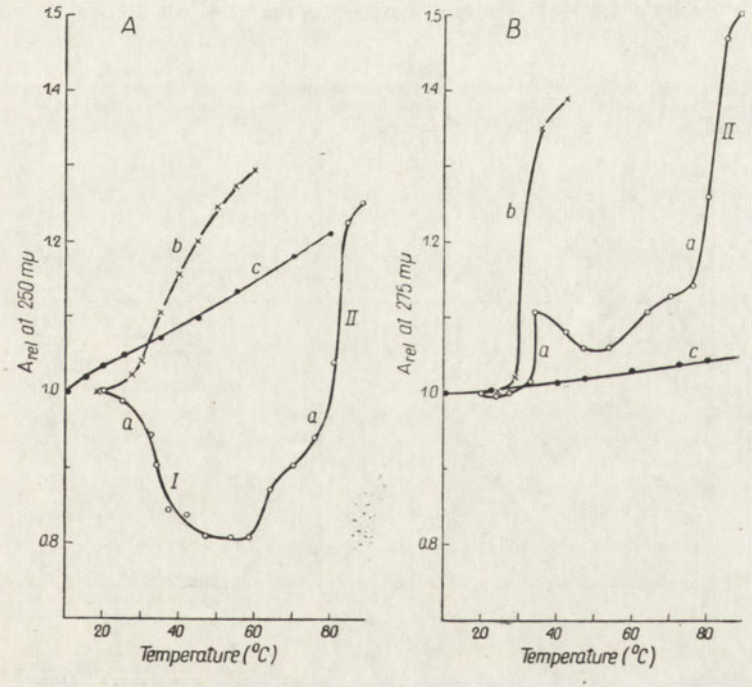


Fig. 12. Course of formation of triple-stranded poly-(X+2rA) in 0.1 M-Na^+ and 0.025 M-phosphate buffer, pH 7.0, by following hypo- and hyperchromicity *A*, at 250 mμ and *B*, at 275 mμ: (*a*), poly-(2X+1rA); (*b*), poly-X; (*c*), poly-rA.

It must therefore be concluded that at 275 mμ both the first and second complexes possess equivalent molar extinctions for the ordered forms. This was further confirmed by an examination of the absorption spectra of 2 mole equivalents of poly-X with one of poly-rA immediately following mixing of the two components (curve *a*, Fig. 13) and following transformation of the complex to the second form (curve *b*, Fig. 13).

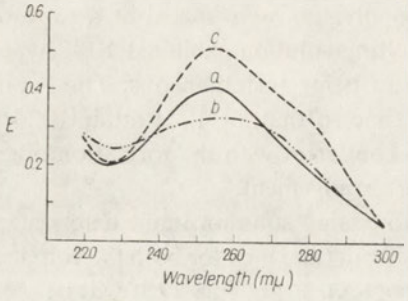


Fig. 13. Absorption spectra in 0.1 M-Na^+ and 0.025 M-phosphate buffer, pH 7.0, of poly-(2X+1rA): (*a*), immediately after mixing of the two components; (*b*), following final formation of complex, at 20°; (*c*), following melting of the complex, at 90°.

If the 2X + 1rA mixture, immediately after mixing, is warmed only to 50°, then cooled to 8° and subsequently heated to 90°, the resulting temperature profiles, both at 250 mμ and 275 mμ, correspond to transition II (Fig. 14). An identical profile is exhibited by a 2X + 1rA mixture after storage at 4° for 24 hr.

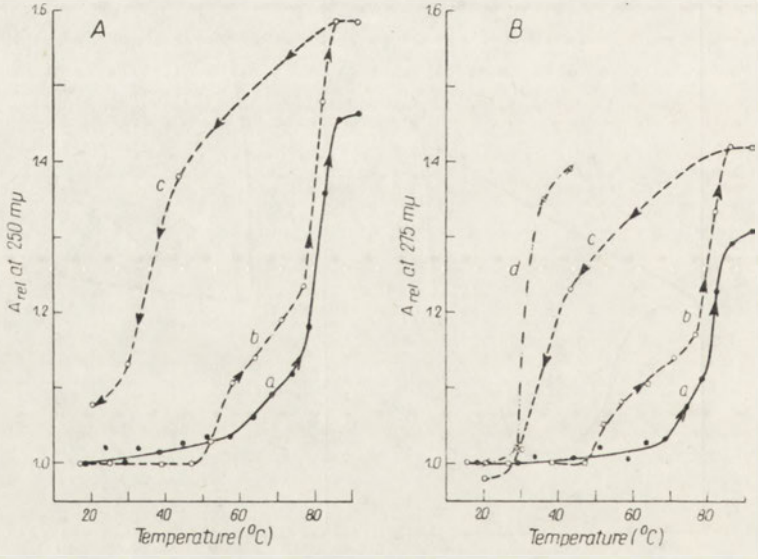


Fig. 14. Helix-coil transitions in 0.1 M-Na^+ and 0.025 M-phosphate buffer, pH 7.0, measured *A*, at 250 mμ and *B*, at 275 mμ, of complexes of poly-X with poly-rA: (*a*), 1X + 1rA, heating profile; (*b*), 2X + 1rA, heating profile; (*c*), 2X + 1rA, cooling profile; (*d*), poly-X alone (for comparison purposes).

It follows from the above that in a 2X + 1rA mixture there is initial formation of a twin-stranded complex, which then undergoes one of the two rearrangements shown in Scheme 2.

$$2X + 1rA \xrightarrow{(10°)} (1X+1rA)+1X \xrightarrow{(10°\text{-}50°)} (2X+1rA)$$

$$(2X+1rA) \begin{cases} \xrightarrow{(50°\text{-}90°)} 2X+1rA \\ \xrightarrow{(50°\text{-}70°)} (1X+1rA)+1X \xrightarrow{(70°\text{-}90°)} 2X+1rA \end{cases}$$

Scheme 2.

Still to be clarified is the nature of the complex formed in a 1:1 mixture of X and rA, as well as the effect of ionic strength on the rearrangement presented in the previous paragraph (Table 2). Over an Na^+ concentration range of 0.05 to 0.2 M, the formation of a (2X+1rA) helix proceeds as described above, with a somewhat enhanced rate at higher ionic strengths. Furthermore the profiles at 275 mμ demonstrate that, following complex formation both in 1X + 1rA and 2X + 1rA mixtures, no melting of free poly-X can be detected. In addition, over the temperature range 15 - 55°, the profile for the 1X + 1rA mixture exhibits a higher hyperchromicity than that for the 2X + 1rA mixture by an amount equal to that due to melting of free poly-rA. The complexes formed in both mixtures possess identical T_m values; and that arising in the 1:1 mixtures has a lower hyperchromicity, quantitatively in accordance with that expected for a (2X+1rA) complex plus free poly-rA. It may therefore be concluded that, irrespective of the ratio of the two components, it is only the three-stranded (2X+1rA) helix which is formed following attainment of equilibrium.

The cooling profiles for the complexes, from 95°, exhibit very pronounced hysteresis, as can be seen from Fig. 14, although leading eventually to reformation of the initial spectra of the complexes. But a second heating profile no longer coincided with the first, the T_m being decreased, and the initial portion of the profile lower, indicative of some irreversible modification of one or both of the components (cf. with thermal lability of poly-rA, Barszcz & Shugar, 1964).

Particular attention should be drawn to the fact that the T_m of the (2X+1rA) complex *decreases* with increasing ionic strength, as in the case of native DNA, and contrary to the behaviour of other synthetic polynucleotides with the exception of acid poly-rA. This is of obvious significance in relation to the nature of the forces responsible for the stability of the (2X+1rA) helix. The T_m of the latter is also reduced in the presence of Mg^{2+} ions (10^{-3} M) without modification of the form of the transition profile.

The stability of the (2X+1rA) helix is likewise dependent on the pH of the medium. No complex formation is detectable at pH 8, whereas the stability of the complex formed at pH 7 increases rapidly with decreasing pH. Furthermore complex formation is observed even at pH 5.1, i.e. under conditions normally leading to formation of the acid twin-stranded poly-rA helix. At this latter pH the solubility of the complex

Table 2

Characteristics of complexes between poly-X and poly-rA and poly-dA in 0.025 M-*phosphate buffer*, pH *7.0*

1X + 1rA				2X + 1rA			
Na^+ (M)	Hypochromicity (%) at 255 mμ immediately after mixing	Hypochromicity (%) at 255 mμ after attainment of equilibrium	T_m	Na^+ (M)	Hypochromicity (%) at 255 mμ immediately after mixing	Hypochromicity (%) at 255 mμ after attainment of equilibrium	T_m
0.05	10	33	84	0.05	6	37	84
0.10	9	28	80	0.10	10	29	80
0.20	9	29	74	0.20	6	29	74
1rX + 1dA				2rX + 1dA			
0.20	0	20	54	0.20	0	26	54

is very low and, in the presence of 0.05 - 0.2 M-Na^+, it precipitates out during storage. Under these conditions neither the complex, nor either of its components, melt out on heating. This is essentially in agreement with the observations of Michelson & Monny (1966).

Poly-X and poly-dA. Solutions of 1X + 1dA and 2X + 1dA in 0.2 M-Na^+ at pH 7 show no hypochromicity immediately after mixing, and heating to 50° places in evidence only the profile of free poly-X, both at 250 mμ and 275 mμ (transition I, Fig. 15). Following storage of the solutions at 4° for 2 - 3 days, by which

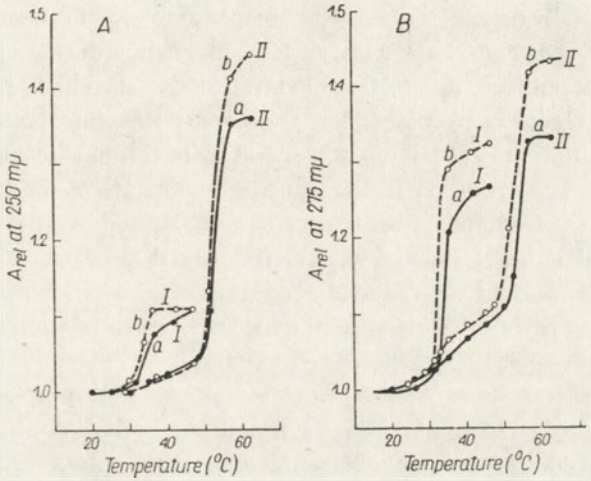


Fig. 15. Formation of complexes between poly-X and poly-dA in 0.2 M-Na^+ and 0.025 M-phosphate buffer, pH 7.0, followed by spectral methods *A*, at 250 mμ and *B*, at 275 mμ: (*a*), 1X + 1dA; (*b*), 2X + 1dA.

I, Heating profile immediately after mixing of components (corresponding to melting of poly-X alone, as seen also in Fig. 12).

II, Second heating profile, following cooling profile, and showing that complex reformed on cooling from first heating cycle.

time the hypochromic spectra have become stabilized, heating profiles demonstrated that, in both mixtures, all the poly-X is complexed with dA. The T_m is practically identical for both mixtures, but the temperature hyperchromicity for 1X + 1dA is lower, again suggestive of formation uniquely of three-stranded helices. The cooling curve exhibited appreciable hysteresis and a second heating cycle did not reproduce the original profile. In this case, there is little doubt but that the lack of reversibility is due to thermal degradation only of the poly-X component.

CONCLUDING REMARKS

The thermal lability of poly-X at elevated temperatures is deserving of some comment from two points of view. This lability apparently results in fragmentation to shorter chains, with no release of monomers. A similar lability to elevated tempera-

tures is exhibited by poly-rA, but not by a variety of other synthetic homopolynucleotides (Barszcz & Shugar, 1968). This thermal lability undoubtedly bears some relationship to the known thermal degradation of viral and transfer RNA, which has been shown to be due to phosphodiester bond breakage (Ginoza, Hoelle, Vessay & Carmack, 1964; Lindahl, 1967). The susceptibility to thermal degradation of poly-X and poly-A, as compared to other homopolymers, suggests that such degradation in high molecular weight RNA is not necessarily a random process, but proceeds *via* fission of linkages between specific bases.

Furthermore, the lack of appearance of appreciable hyperchromicity in the 250 mμ band of thermally degraded poly-X (as compared to the simultaneous appearance of hyperchromicity in both the 250 mμ and 275 mμ bands during enzymic degradation, which is undoubtedly due to random attack along the chain) points to the presence in shorter chains of poly-X of some secondary structure present also in high molecular weight specimens. A more detailed study of this phenomenon may possibly help to clarify the contribution of the transition moments of the two bands to the hypochromicity (and secondary structure) of poly-X, and is now under way with the use of circular dichroism and optical rotatory dispersion techniques.

The properties of the twin-stranded complexes of poly-X with poly-rU, poly-rT, poly-EtU and poly-FU (see Fig. 9) are of added interest in relation to the complexes formed by the poly-U analogues with poly-A (Barszcz & Shugar 1968; Szer & Shugar 1963; Świerkowski & Shugar, 1969); and by poly-C and poly-5-methyl cytidylic acid with poly-I (Szer & Shugar, 1966; Żmudzka, Bollum & Shugar, 1968). The data for the profiles of the poly-rU analogues with poly-X are summarized in Table 1. Note that in passing from poly-(X+U) to poly-(X+rT) there is an increase in T_m of about 12°, although the hyperchromicity is also reduced. A strict comparison with the analogous complexes of poly-A with poly-rU or poly-rT is not feasible, since the latter forms only the triple-stranded poly-(A+2rT) helix (Barszcz & Shugar, 1968). But a comparison of poly-(A+2rU) with poly-(A+2rT) shows that the replacement of rU by rT (i.e. 5MeU) leads to an increase in the T_m of about 23°, or about 12° for each rT strand (cf. Świerkowski & Shugar, 1969).

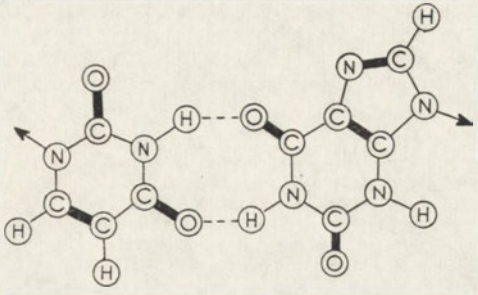
Furthermore, poly-C forms a twin strand with poly-I, and the increase in T_m in going from poly-(I+C) to poly-(I+5MeC) is 16° (Szer & Shugar, 1966). Hence the introduction of a pyrimidine 5-methyl substituent leads to an approximately similar increase in thermal stability of complementary twin strands involving either poly-A, poly-I or poly-X. The quantitative differences observed are most likely a reflection of some differences in base stacking or topography of the different types of complexes.

The foregoing analogy also prevails when we compare poly-(X+U) with poly-(X+EtU), for both of which the T_m values are the same (Table 1), i.e. the replacement of a pyrimidine 5-methyl substituent by a 5-ethyl essentially liquidates the enhanced stability conferred by the former. An almost identical situation exists for the complexes of poly-A with poly-rU and poly-EtU, for which the T_m values are likewise similar (Świerkowski & Shugar, 1969).

Similarly the reduction in T_m observed for poly-(X+FU) as compared to poly-

(X+U) (Table 1) is in reasonable accord with the decrease in T_m of poly-(A+FU) as compared to poly-(A+U) (Szer & Shugar, 1963).

The foregoing provides no clue as to the type of base-pairing involved in the complexes of poly-X with the poly-U analogues. But, if we accept the evidence of Cavalieri *et al.* (1954) that the pK_a of xanthosine is due to dissociation of the purine ring $N_{(3)}$ hydrogen, then a reasonable type of base-pairing, in which the glycosidic bonds are suitably orientated, would be as in Scheme 3.



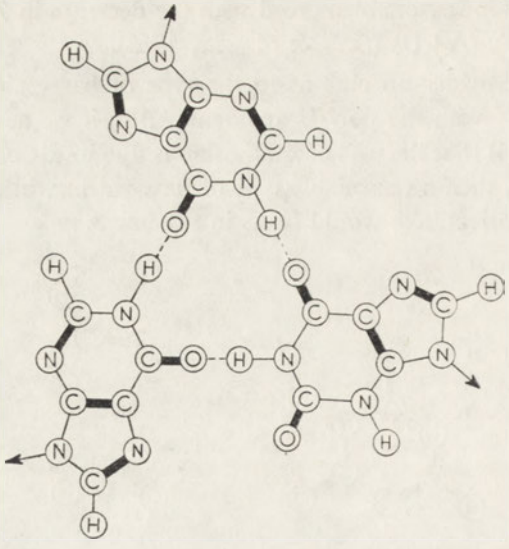
Scheme 3.

The involvement of the ring $N_{(3)}$ hydrogen and $C_{(4)}$ carbonyl of the uracil analogues in the above base-pairing scheme is supported by the fact that poly-FU complexes as readily as the other poly-U analogues at pH 7.8, where the FU residues normally undergo ionization of the ring $N_{(3)}$ hydrogen. Complex formation can occur only by inhibition of this ionization, as for poly-(A+FU) (Szer & Shugar, 1963).

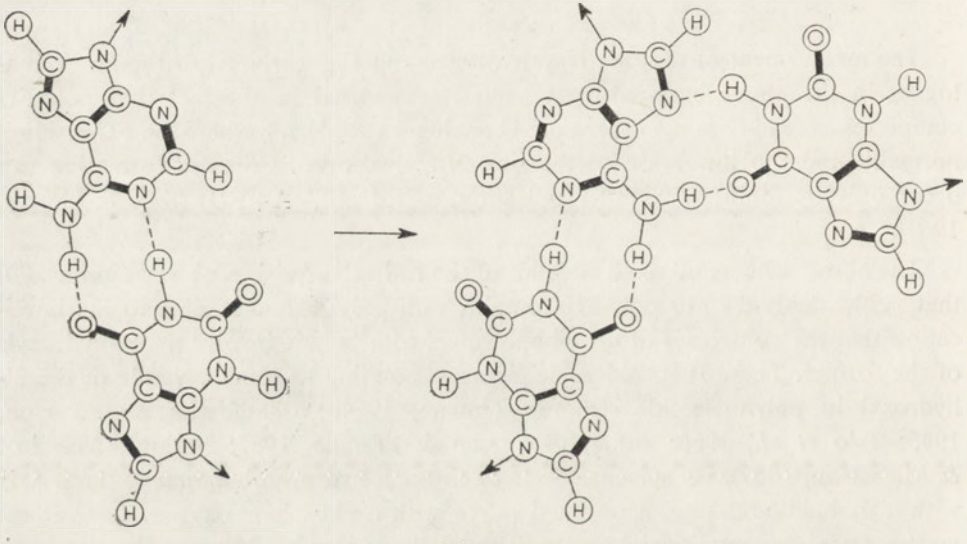
The above scheme takes no account of the rather surprising observation (p. 70) that neither poly-dT nor poly-dU complex with poly-X, and leading to the implication that the complexes of the ribopolymers with poly-X involve the 2′-hydroxyls of the former. There is considerable evidence pointing to some key role of the 2′-hydroxyl in polynucleotide structure (Inman, 1964b; Chamberlin & Patterson, 1965; Ts'o *et al.*, 1966; Adler, Grossman & Fasman, 1967; Brahms, Maurizot & Michelson, 1967), *via* solvent effects or through hydrogen bonding of the 2′-OH with a pyrimidine 2-keto, a purine $N_{(3)}$, or with a phosphate oxygen. The subject matter remains controversial and will most likely require X-ray diffraction data for resolution.

Our data do not enable us to draw any definitive conclusions regarding the type of base-pairing involved in the multi-stranded helices between poly-X on the one hand, and poly-rI and poly-rA (or dA) on the other. However, in the case of the triple-stranded poly-(X+2rI), where no intermediary formation of a twin strand could be detected, a reasonable model is illustrated in Scheme 4.

For the triple-stranded poly-(2X+1rA), which appears to form *via* the intermediary twin-stranded poly-(1X+1rA), we would propose for the twin- and triple strands the arrangements shown in Scheme 5.



Scheme 4.



Scheme 5.

The foregoing schemes do not take account of the possible participation of the 2′-hydroxyls of poly-rI, which must play some role since poly-X does not complex with poly-dI.

In agreement with Michelson & Monny (1966), poly-X appears to be the most versatile of the synthetic polyribonucleotides as regards ability to complex with other homopolyribonucleotides. It is, indeed, surprising that X, which pairs with so many bases, appears to be devoid of activity in biological systems; the single exception being its ability to serve as a substrate for deoxynucleotidyl transferase

(F. J. Bollum, personal communication), an enzyme the function of which is still not clear.

The apparent absence of messenger activity in poly-X has been taken to indicate that X is a "nonsense" base (Michelson & Grunberg-Manago, 1964), but such a conclusion is probably premature. It is equally conceivable that poly-X does not bind ribosomes under physiological conditions because of its highly ordered secondary structure, and this would have to be examined before drawing any conclusions.

The failure of poly-dU and poly-dT to complex with poly-X (see above) may be of significance also from a genetic point of view. Deamination of a G residue in a G-C base pair might be expected to result in two types of mutations, as follows:

$$\text{G-C} \rightarrow \text{X-C} \rightarrow \text{T-A} \quad \text{(X pairs with T)} \qquad (1)$$

$$\text{G-C} \rightarrow \text{X-C} \rightarrow \text{A-T} \quad \text{(X pairs with A)} \qquad (2)$$

The second of these appears reasonable, since poly-X pairs with poly-dA. The first was also proposed as acceptable by Michelson & Monny (1966) on the grounds that poly-X complexes with poly-rU. But this is now seen to be questionable, since neither poly-dT nor poly-dU complex with poly-X. In fact, an unequivocal conclusion here would require an examination of possible complex formation between poly-dX and poly-dT. More generally, this example underlines the need of using the appropriate ribo- or deoxyribo-polymers if one desires to extrapolate the results to biological or genetic systems. Strictly speaking, one should also employ appropriate co-polymers, e.g. a few scattered dT residues in a polymer of dA might well complex with poly-X. Such studies are now under way in these laboratories.

We are indebted to Prof. W. Jasiński (Director) and Dr. A. Michałowski (Head, Dept. of Radiobiology) of the Institute of Oncology for facilities made available during the initial stages of this work; to Prof. M. Laskowski Sr., Schwarz Bio-Research, and Sigma Chemical Co. for gifts of poly-X; to Dr. F. J. Bollum for samples of some of the deoxy polymers; and to the Wellcome Trust, the World Health Organization, the International Atomic Energy Agency, and the Agricultural Research Service, U.S.Dept. of Agriculture (UR-E21-(32)-30) for support of this and related investigations.

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WŁASNOŚCI POLIKSANTYLOWEGO KWASU I JEGO REAKCJE Z POTENCJALNIE KOMPLEMENTARNYMI HOMOPOLINUKLEOTYDAMI

Streszczenie

1. Opisano otrzymywanie kwasu poliryboksantylowego (poli-X) przy użyciu fosforylazy polinukleotydowej.

2. Otrzymany przez nas poli-X, jak również trzy preparaty handlowe wykazują podobne własności. Preparaty te badano w zakresie pH od 4,0 (w którym monomer istnieje w formie obojętnej) do 8,0 (monomer występuje jako monoanion). Opisano szczególny wpływ kationów Mg^{2+} na strukturę polimeru.

3. Poli-X jest odporny na działanie rybonukleazy trzustkowej, natomiast ulega hydrolizie do monomerów pod działaniem rybonukleazy T_1.

4. Poli-X ulega degradacji termicznej do krótszych łańcuchów, lecz bez wydzielenia monomerów, w temperaturach powyżej 60°. Mechanizm degradacji przypomina znane już zjawisko pękania łańcuchów poli-rA pod wpływem temperatury. Dyskutowane są biologiczne skutki tych zjawisk.

5. Poli-X tworzy łatwo podwójnopasmowe spirale z poli-rU i niektórymi analogami poli-rU. Zbadano trwałość tych kompleksów w różnych warunkach i przedyskutowano jej znaczenie.

6. Poli-X nie tworzy kompleksu z poli-dU ani z poli-dT. Rozważane są wnioski genetyczne i strukturalne płynące z powyższego faktu.

7. Poli-X nie tworzy kompleksu z poli-rC, poli-dC i poli-dI. Tworzy natomiast potrójnopasmowe struktury spiralne: poli-(1X+2rI), poli-(2X+1rA) i poli-(2X+1dA). Opisano przebieg procesu powstawania jak również własności tych struktur.

8. W świetle pozornej inaktywacji genetycznej guanozyny po jej dezaminacji w ksantozynę przedyskutowano godną uwagi zdolność ksantozyny do tworzenia par komplementarnych z innymi zasadami purynowymi i pirymidynowymi.

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INFLUENCE OF POLYNUCLEOTIDE SECONDARY STRUCTURE ON THYMINE PHOTODIMERIZATION

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1. Spectral techniques and helix-coil transitions have been applied to a study of the rate and extent of photodimerization and photodissociation of thymine residues in oligo-rT and poly-dT (which exhibit no secondary structure); in poly-rT (the secondary structure of which may be controlled by variations in ionic strength, temperature or solvent); and in twin- and triple-stranded helices of the foregoing with poly-A (rA and dA). 2. With 254 mμ irradiation, photodimerization in twin-stranded poly-rT is 4-fold faster than in the model dinucleotide TpTp; for single-stranded poly-rT the rate is only twice that for TpTp, and for single-stranded poly-dT three times that for TpTp. 3. At irradiation wavelengths to the red of 275 mμ the rates, relative to that for TpTp, are 7.5 for twin-stranded poly-rT and 4.0 for single-stranded poly-rT and poly-dT. 4. For the twin- and triple-stranded complexes of poly-T with poly-A, initiation of photodimerization is preceded by a "lag" which is accompanied by slight modifications in secondary structure. Following the lag phase, photodimerization proceeds at a reduced rate, by comparison with the homopolymers. The same phenomenon is reproduced to a certain degree in irradiated phage T_2 DNA. 5. Decreased dimerization rates in complexes are due in part to unfavourable orientation of thymine residues in the rigid helical structures of the complexes, and, perhaps, quenching of the appropriate excited states of thymine. 6. The pronounced influence of solvent and temperature, as well as the extent of photoreversibility under various conditions, have also been examined quantitatively. 7. In particular it has been shown that, in the presence of ribonuclease, which may hydrolyse internucleotide linkages during dimer photodissociation, the extent of photoreversibility of dimerization may exceed 90%. 8. The overall results are briefly discussed in relation to photodimerization and photodissociation of dimers in natural nucleic acids.

It might be anticipated that the photodimerization of thymine residues in DNA would be dependent on the secondary structure of the latter, which determines the relative orientation of two adjacent residues potentially capable of undergoing dimerization. And, in fact, at low radiation doses, which presumably do not appreciably disrupt secondary structure the rate of dimerization of thymine residues has been reported to be twice as high in denatured, as compared to native, DNA (Wacker, Dellweg & Jackerts, 1962). The effect of secondary structure is also illustrated by model experiments: e.g. the extent of dimerization of thymine in an ice matrix

is reduced 5-fold when a 1:1 mixture of adenine and thymine is frozen and irradiated (Smith, 1966).

Other illustrations, too numerous to cite here, qualitatively describe the influence of secondary structure on the photochemical transformation of nucleic acids. Perhaps one of the most striking of these is the observation that the replicative (i.e. twin-stranded) form of phage ØX174 DNA is 10-fold more resistant to UV inactivation than the single-stranded form. This fact has been profited from to study the intracellular replication of ØX174 DNA following infection (Sinsheimer, Starman, Nagler & Guthrie, 1962).

Although the dimerization reaction has been extensively studied in such model oligonucleotides as TpT (Sztumpf & Shugar, 1962a; Johns, Pearson, Le Blanc & Helleiner, 1964) and oligo-T with chain lengths of up to 10 - 15 residues (Wierzchowski & Shugar, 1960b; Deering & Setlow, 1963), it is clearly of greater interest to examine this reaction in longer polynucleotides capable of exhibiting secondary structure. The results of such a study are presented here, with the use of appropriate ribo- and deoxyribo-polynucleotides of thymine, and the helical complexes of these with the complementary poly-A[1].

During the initial phase of this study it was assumed, on the basis of previous findings (Szer, Świerkowski & Shugar, 1963) that the complex of poly-A with poly-rT was the twin-stranded poly-(A+rT), which would then correspond to the A-T base pairs in DNA. A more detailed investigation has now shown (Barszcz & Shugar, 1968) that poly-A and poly-rT form *only* the triple-stranded helix, poly-(A+2rT). This necessitated the use of an additional model system capable of providing a twin-stranded helix which, fortunately, is available from the corresponding deoxy polymers, which can be combined to give poly-(dA+dT) (Setlow, Carrier & Bollum, 1965). Reference is made to this point here for purposes of clarification, since most of the experiments to be described were carried out with poly-(A+2rT).

MATERIALS AND METHODS

Poly-A and poly-U were Miles (U.S.A.) products; the latter was further purified by phenol treatment in the presence of Macaloid adsorbent (Szer & Ochoa, 1964). Poly-dAT was isolated from *Cancer borealis* by Dr. H. Sierakowska. Poly-dT, poly-dA and poly-(dA+dT) were kindly made available by Dr. F. J. Bollum and TpTp by Dr. A. M. Michelson. The preparation and properties of poly-rT have been elsewhere described (Świerkowski, Szer & Shugar, 1965). Phage T_2 DNA was

[1] The following abbreviations are used in this text: DMU, 1,3-dimethyluracil; poly-U, polyribouridylic acid; poly-rT and poly-dT, poly-ribothymidylic and poly-deoxyribothymidylic acids; poly-A and poly-dA, poly-riboadenylic and poly-deoxyriboadenylic acids; poly-(A+U), twin-stranded complex of poly-A and poly-U; poly-(A+2rT), triple stranded complex of poly-A and 2·poly-rT, with similar connotations for other polymer complexes; poly-dAT, twin-stranded alternating co-polymer of dA and dT; TpT and TpTp, dinucleotide of thymidylic acid; ApA, dinucleotide of adenylic acid; T_m, melting temperature, or mid-point of temperature profile or helix-coil transition; E, einstein; RNase, pancreatic ribonuclease (EC 2.7.7.16).

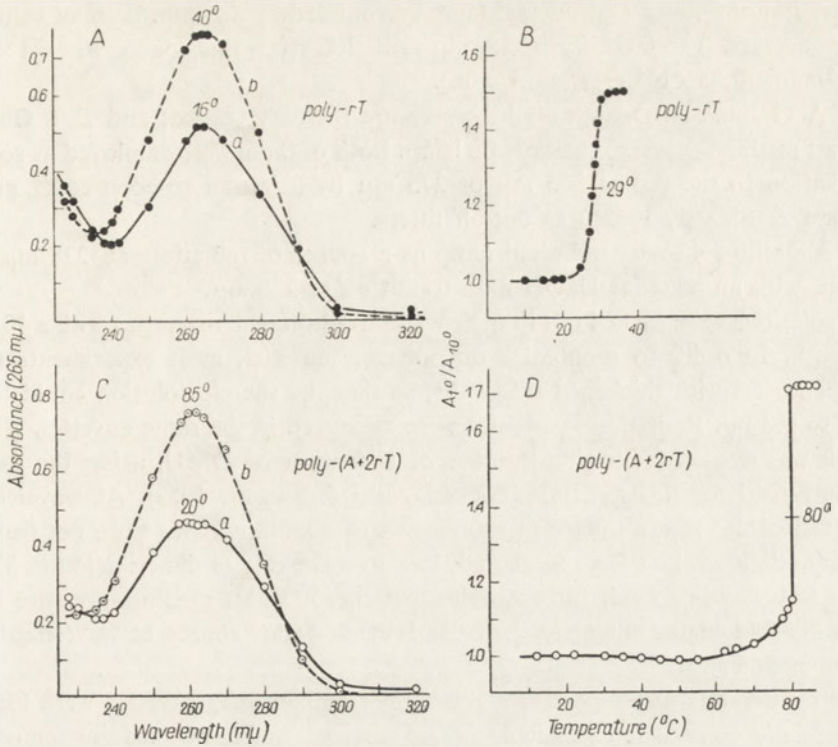

Fig. 1. Characteristics of poly-rT and its triple-stranded complex with poly-A in 0.15 M-NaCl and 0.015 M-phosphate buffer, pH 7.2.

A: Absorption spectra of poly-rT, (*a*) helical form, measured at 16°, and (*b*) random coil form, measured at 40°.

B: Helix-coil transition profile for poly-rT with T_m value as indicated.

C: Absorption spectra of poly-(A+2rT) in (*a*) triple-stranded helical form, at room temperature, and (*b*) melted, random coil form above 80°.

D: Helix-coil transition profile for poly-(A+2rT) with T_m value as indicated.

prepared by Drs. M. Piechowska and I. Pietrzykowska. RNase was a Worthington (U.S.A.) 2× crystalline preparation.

Figure 1A exhibits the absorption spectra of the helix and coil forms of the poly-rT sample used in this study. The corresponding temperature profile, or helix-coil transition, and T_m are shown in Fig. 1B.

The absorption spectra of the helical and dissociated forms of poly-(A+2rT) are shown in Fig. 1C, and the corresponding temperature profile and T_m value in Fig. 1D.

Combinations of sources and appropriate filters employed for irradiation in several different wavelength ranges were as follows:

A. Thermal Syndicate mercury resonance lamp, from which radiation below 240 mμ was filtered out by means of a 5-mm. layer of 75% aqueous acetic acid. The intensity of 254 mμ radiation of this source at the sample surface was 1.16×10^{-7} Einsteins/cm.^{2}/min. (5.45×10^3 ergs/mm.^{2}/min.).

B. A Phillips 40-watt germicidal lamp surrounded by a 2-mm. layer of saturated sodium acetate. The intensity of 254 mμ radiation from this source was 6.1×10^{-7} E/cm.^{2}/min. (2.85×10^4 ergs/mm.^{2}/min.).

C. A German HBO-200-watt high-pressure mercury source; and *D*, a German 700-watt medium-pressure therapeutic lamp. Both of these were employed as sources of radiation to the red of 265 mμ or 275 mμ by using microscope cover glasses (Sztumpf & Shugar, 1962b) as cut-off filters.

E. A Phillips 40-watt cadmium lamp as a source of radiation at 228 mμ and 225 mμ, with an aqueous NaOH filter to cut off at 220 mμ.

Irradiations were carried out in quartz spectrophotometer cuvettes with a 10-mm. light path. In order to economize on polymer material, most experiments made use of cuvettes with thickened side-walls, so that the sample solution could be reduced to 0.8 ml. Radiation intensities were measured in the same cuvettes. At 254 mμ this was done by following the rate of hydration of DMU, using the known quantum yield for this reaction (Wierzchowski & Shugar, 1959). At wavelengths to the red of 265 mμ, absolute measurements of quantum yields were not feasible, and only relative values were measured, based on the rate of dimerization of TpTp. In this way it was found, for example, that the 700-watt medium-pressure lamp gave a 2.5-fold higher intensity than the HBO-200-watt source at wavelengths to the red of 265 mμ.

For irradiations at temperatures other than ambient the cuvettes were located in a specially constructed jacketed copper carriage which was readily removable from the spectrophotometer. The entire carriage was brought to desired temperature by means of a water circulation system from a Hoeppler ultrathermostat. For temperatures higher than ambient, teflon stoppered cuvettes were employed.

Absorption spectra and temperature profiles were carried out with Beckman-DU and Unicam SP-500 spectrophotometers equipped with thermostated cell holders. Temperatures were measured by means of a thermistor in a dummy cuvette.

RESULTS

Photochemical transformation of poly-rT

Figures 2A and 2B exhibit the changes in UV absorption at 265 mμ (due principally to dimerization) of poly-rT, when irradiated in the random coil and helix forms, respectively, at wavelengths to the red of 275 mμ (source *C*). Figs. 2C and 2D present the accompanying modifications in secondary structure as estimated from the changes in temperature profiles of the irradiated polymers.

In order to follow these results, it should be recalled that, in 0.15 M salt solution, poly-rT is in the fully helical form at 16° and in the random coil form at 40°. The absorption spectrum of the latter, at the wavelength of the principal absorption maximum of 265 mμ, exhibits an extinction coefficient 50% higher than the helix, i.e. a hyperchromicity of 50% (see Fig. 1A).

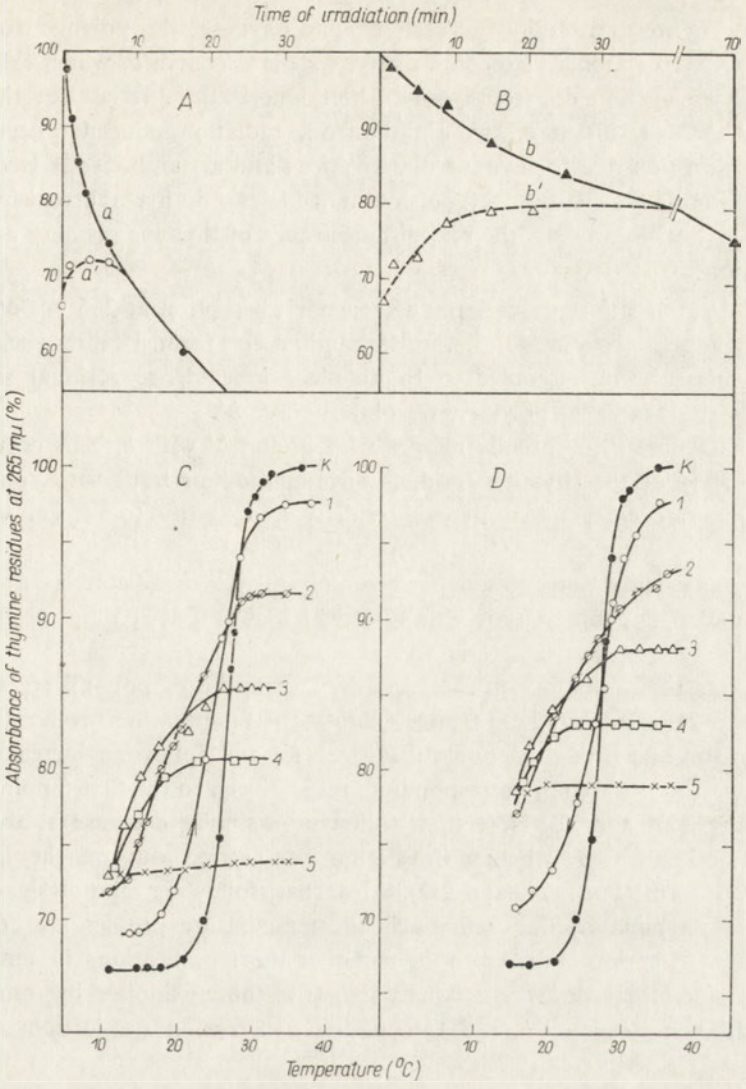

Fig. 2. Changes in absorbance of absorption maximum at 265 mμ, and in secondary structure, of poly-rT irradiated at wavelengths to the red of 275 mμ (source *C*) in 0.15 M-NaCl with 0.015 M-phosphate buffer, pH 7.2.

A: (*a'*), Polymer irradiated at 16° (helix form); (*a*), irradiated solution brought to 40° to give random coil and eliminate hypochromicity.

B: (*b*), Polymer irradiated at 40° (random coil form); (*b'*), irradiated solution brought to 16° to give helix form.

C: Temperature profiles following increasing doses of irradiation at 16° (in helix form).

D: Temperature profiles following increasing doses of irradiation at 40° (in melted form).

In Fig. 2A the polymer was irradiated at 16°, i.e. in the helical form. The resulting changes in extinction at the maximum of 265 mμ are shown by curve *a'*. Note that the absorption initially increases, and begins to decrease only after about 3 min.

irradiation. If, however, following each irradiation dose, the polymer solution is warmed to 40° to eliminate hypochromicity, we obtain curve *a*, which exhibits the real loss in absorption due to thymine photodimerization. It is clear that curve *a'* represents the resultant of two simultaneous radiation-induced processes: viz. a *loss* in absorption due to thymine dimerization, and a simultaneous *increase* due to loss of secondary structure with concomitant increase in hyperchromicity. Hence it is curve *a* which represents the real disappearance of thymine residues as a result of irradiation[2].

In Fig. 2B, curve *b* represents the decrease in absorption at 265 mμ of poly-rT when irradiated as above at 40°, i.e. in the random coil form. If, after each irradiation dose, the solution is cooled to 16° to place in evidence residual secondary structure in the irradiated polymer, we obtain curve *b'*.

Note that curve *a'* eventually merges with *a*, and *b'* with *b*. This occurs when about 20 - 30% of the thymine residues have photodimerized, with concomitant loss of temperature-dependent hyperchromicity and ability to reform secondary structure.

Analogous results were obtained when poly-rT was irradiated as above with sources *C* and *D* in conjunction with a filter which cut off at 265 mμ, or at 254 mμ with source *A*.

The effects of irradiation on the secondary structure of poly-rT are shown in Figs. 2C and 2D. Each of these figures exhibits the temperature profiles (helix-coil transitions) for non-irradiated poly-rT (curves *K*) and following increasing times of irradiation (curves *1* to *5*) corresponding, respectively, to the first 5 points on the curves in Figs. 2A and 2B. Note that, following a similar decrease in absorption, the profiles are the same whether irradiation was carried out on the helical or random coil form (Figs. 2C and 2D). Also that, following photolysis of about 25% of the thymine residues (curves *5*), the temperature profiles are completely flattened out. Attention is drawn to the fact that the modifications in temperature profile with increasing dose are strikingly similar to those exhibited by complexes of poly-A with a copolymer of A and U containing increasing proportions of A residues (Fresco & Alberts, 1960).

Note, in particular, that the rate of photodimerization of thymine residues is apparently considerably greater for the helical form of poly-rT (Fig. 2A, curve *a*) than for the random coil (Fig. 2B, curve *b*). This is not due to differences in absorption of the two forms of poly-rT, at least for irradiation at wavelengths to the red of 275 mμ, since in this wavelength range the absorption of both forms is almost identical.

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[2] The number of thymine residues which undergo dimerization as a result of irradiation may readily be estimated by treating the irradiated polymer with ribonuclease, to destroy the secondary structure of the polymer. This was the method adopted in initial experiments (see also Wierzchowski & Shugar, 1962). However, such a procedure was both uneconomical and tedious since it required a separate solution for each radiation dose. After several trial experiments had demonstrated that the same quantitative results were obtained by "melting" of the polymer after each radiation dose, the latter procedure was adopted in all subsequent experiments.

Influence of temperature and solvent on thymine photodimerization

In the foregoing experiments the temperature was maintained in turn at 16° and 40° during irradiation for the helix and coil forms, respectively, of poly-rT. It is therefore pertinent to inquire whether the observed difference in rates of photodimerization is due solely to the difference in structure at the two temperatures, or whether the difference in temperature itself is of any consequence.

It was, in fact, found that a further increase in temperature from 40° to 80°, over which range there is only a minimal change in the structure of poly-rT, was accompanied by an additional decrease in the rate of dimerization. An examination was therefore made of the rate of photodimerization at wavelengths to the red of 265 mμ in poly-dT, which exhibits virtually no secondary structure over the temperature range 10° - 80° (Riley, Maling & Chamberlin, 1966), as measured by UV absorption spectra. An appreciable change in structure is indicated by optical rotatory

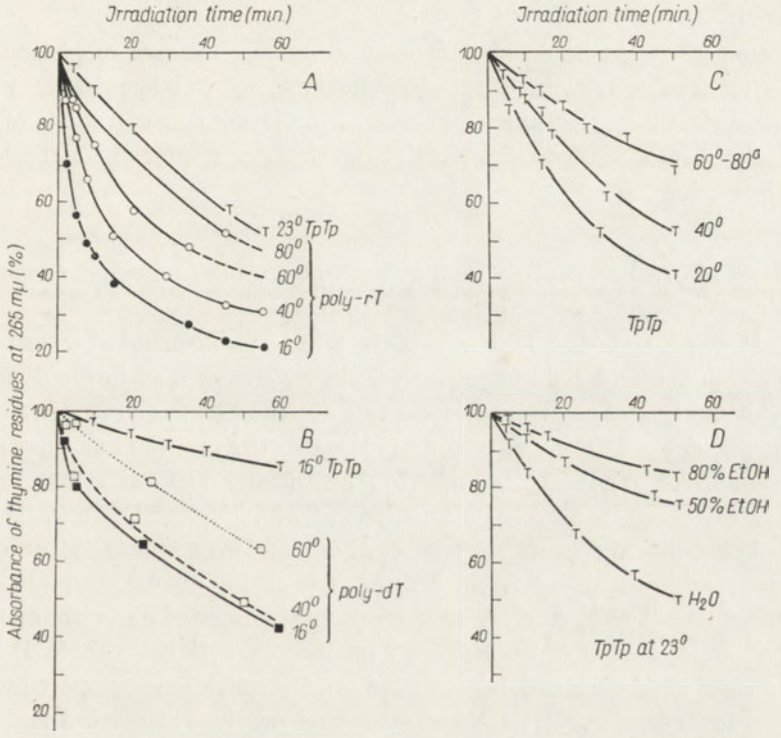

Fig. 3. Influence of temperature and solvent on rate of photochemical transformation of thymine residues in TpTp, poly-rT and poly-dT at neutral pH, at wavelengths to the red of 265 mμ (source *D*). *A*: Poly-rT in 0.01 M-Mg^{2+} and TpTp either in water or in 0.01 M-Mg^{2+}. *B*: Poly-dT and TpTp in 0.15 M-Na^{+}. *C*: TpTp in water at various temperatures. *D*: TpTp in aqueous ethanol as indicated.

The data for the individual series of experiments are not strictly comparable with each other, due to differences in irradiation intensities. However, for each series of experiments (i.e. *A*, *B*, *C* or *D*), the light doses were maintained constant and the corresponding curves represent a consistent picture.

dispersion measurements in the temperature range 22° to 80° (Ts'o, Rapaport & Bollum, 1966). From Fig. 3A it will be seen that whereas the dimerization rate for poly-rT decreased 2-fold between 16° and 40°, that for poly-dT was hardly affected in the same temperature range. A further increase in temperature to 60° resulted in a net decrease in dimerization rate (Fig. 3B), somewhat more pronounced than for poly-rT, but similar to that observed for TpTp (Fig. 3C).

From Fig. 3C it will be observed that the rate of photochemical transformation of TpTp, in which secondary structure is excluded, also decreases with increasing temperature. In fact the difference in rates between 20° and 40° is not very much different from that for poly-rT at the same temperatures. It follows that an increase in temperature decreases stacking of the bases, diminishing the probability of their encounter in a position suitable for dimerization. This interpretation is consistent with the observation of Ts'o *et al.* (1966) based on optical rotatory dispersion measurements, that the degree of stacking of pTpT decreases with increase in temperature by a factor of 1.3 over the range 22° to 81°, and for poly-dT by a factor of 1.55 over the same temperature range.

The same effect can be demonstrated by examining the rate of photodimerization of TpTp in solvents which reduce base stacking, e.g. in ethanol-water mixtures. Fig. 3D shows, in fact, that in the presence of increasing concentrations of ethanol in water, the rate of photodimerization of the thymine residues in TpTp is appreciably decreased.

Influence of secondary structure on rate of photodimerization in homopolymers

From Figs. 3A and 3B it is clear that the rate of photochemical transformation of thymine residues in both homopolymers is always greater than in the dinucleotide TpTp; even at elevated temperatures the rate for the homopolymers is more than twice that for TpTp. Deering & Setlow (1963) reported that the quantum yield for dimerization in oligo-dT (chain-length of 12 residues) was twice that for pTpT and this ratio was unchanged for irradiations at 240, 265 and 280 mμ, their interpretation being that the probability of collision of two residues in the oligomer was greater than in the dinucleotide. But this is probably not an adequate explanation in the case of the polymers. A comparison of the rates of dimerization in TpTp on the one hand, and in ordered poly-rT on the other shows (Table 1) a 4-fold increase in rate at 254 mμ and 7-fold at 265 mμ for poly-rT as compared to TpTp. For the random coiled poly-dT the corresponding relative rates are 3 and 4.4 (see Table 1). This large difference in dependence on irradiation wavelength of the initial rates of dimerization of both polymers, with respect to TpTp, disappears on irradiation at 40°, at which temperature the ratio for dimerization in the polymers to that for TpTp is about 3 for both of them (Table 1).

The quantum yield for dimerization of thymine residues in TpT has been reported to decrease with increasing wavelength of irradiation by 15% (Johns *et al.*, 1964) to 30% (Deering & Setlow, 1963). This serves to underline the fact that the *initial*

dimerization rate in ordered poly-rT increases, rather than decreases, at longer wavelengths. We would then expect the plateau level (i.e. equilibrium point between dimer formation and dissociation) to be lower in the polymers than in the dinucleotide; as will be seen subsequently, this is what is observed (see Fig. 4, part A, B).

Table 1

Quantum yields (at 254 mμ) and initial rates (at $\lambda > 265$) for photodimerization of thymine residues in TpTp, poly-rT and poly-dT (in 0.01 M-phosphate buffer, pH 7.4, and 0.1 M-Na^+)

	Irradiated at 254 mμ		Irradiated at $\lambda > 265$ mμ	
	$\Phi \times 10^{-2}$	$\frac{\Phi}{\Phi_{TpTp \text{ at } 16°}}$	Rate[a]	$\frac{\text{Rate}}{\text{Rate}_{TpT^p \text{ at } 16°}}$
TpTp at 16°	1.1	—	3.7	—
TpTp at 40°	0.8	0.7	3.1	0.8
Poly-dT at 16°	3.3	3.0	16.3	4.4
Poly-dT at 40°	2.4	3.0[b]	12.3	2.9[b]
Poly-rT at 16°	4.4	4.0	28.0	7.5
Poly-rT at 40°	2.2	2.8[b]	9.5	3.0[b]
Oligo-rT at 16°[c]	2.4	2.2	15.0	4.0

[a] Measured as % decrease in absorbance (after melting out of secondary structure, where necessary) at 265 mμ for 2.5 min. irradiation.

[b] In relation to TpTp at 40°.

[c] This is a sample of poly-rT thermally degraded to shorter chains which do not exhibit secondary structure (insert in Fig. 4A).

Figures 2 and 3A show that the rate of photodimerization is appreciably decreased in poly-rT at 40°, where its secondary structure is liquidated. As pointed out above, this effect is due in part to the influence of an increase in temperature. In order to eliminate the latter effect, a sample of poly-rT was thermally degraded to the point where it exhibited only minimal secondary structure (see insert in Fig. 4A). The rate of dimerization in this preparation was found to be similar to that for highly polymerized poly-rT at 40°.

Poly-rT in the presence of 10^{-2} M-Mg^{2+} exhibits a steeper temperature profile, with a T_m 6° higher, than in 0.15 M-Na^+ (insert in Fig. 4A). Note from Fig. 4A that the rate of dimerization in Mg^{2+} is 30% higher than in 0.15 M-Na^+, while the plateau value is unaffected. This enhancement of the initial rate of dimerization by magnesium ions was observable, although to a lesser degree, even in the case of the dinucleotide TpTp. The quantum yield for dimerization in poly-rT after 25% photolysis (disappearance of secondary structure) is close to the value for oligo-rT (2.44×10^{-2} as cf. to 2.4×10^{-2}), and comparable to that observed for oligo-dT, 2×10^{-2} (Deering & Setlow, 1963).

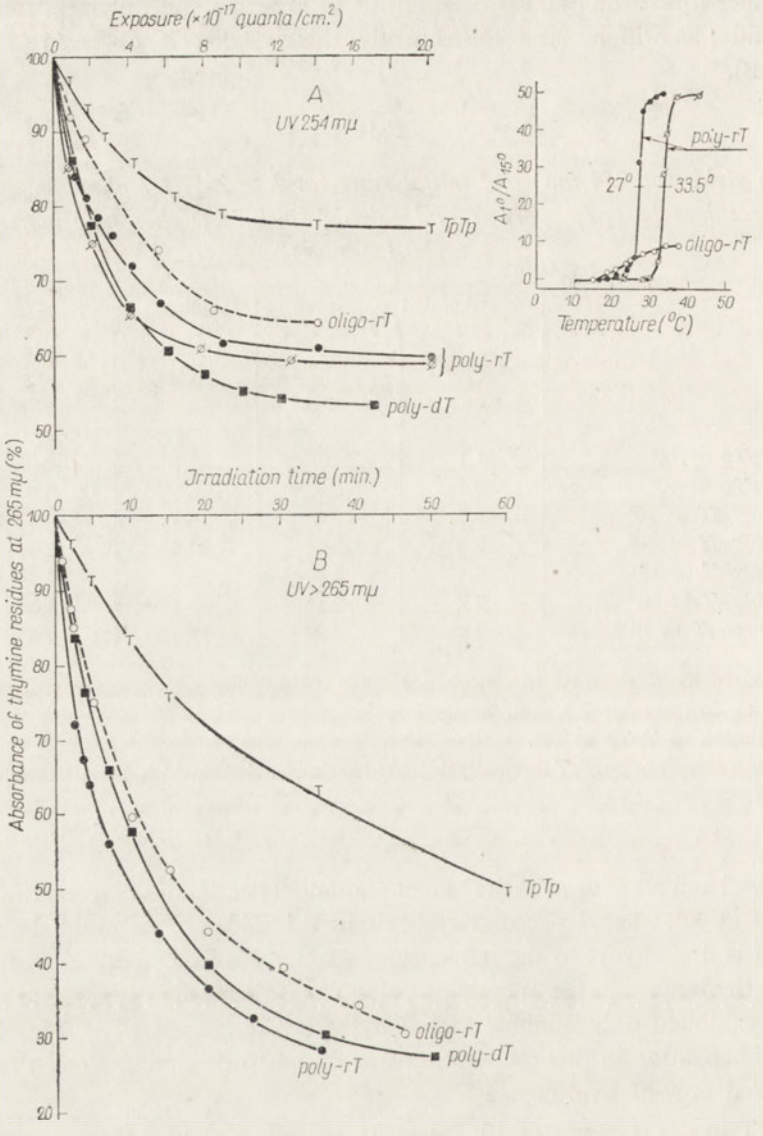

Fig. 4. Course of photochemical transformation of thymine residues in homopolymers at 16° in dilute salt solution ($<10^{-2}$ M-Na^+) at 16°, irradiated (*A*) at 254 mμ, and (*B*) at wavelengths to the red of 265 mμ (source *D*). The insert to part *A* shows the temperature profiles of poly-rT in dilute salt (●) and in the presence of 10^{-2} M-Mg^{2+} (Ø) (for comparison purposes), and of oligo-rT (thermally degraded poly-rT) (○).

Photochemical transformation of poly-(A+2rT)

The influence of irradiation at 254 mμ (source *A*) on the triple-stranded poly-(A+2rT) helix is illustrated in Fig. 5. Figure 5A exhibits the apparent and real changes in extinction of the complex with increasing doses of irradiation. It should be recalled that at room temperature, due to hypochromicity, the helix form exhibits only 65% of the absorption of the fully melted form. If the absorbance of the helical form is followed as a function of time of irradiation, we get curve α, from which it would appear that the polymer is hardly affected. However if, after each irradiation dose, the temperature is raised to melt out the helical structure and eliminate hypochromicity, we obtain curve β. This curve exhibits an initial lag, followed by a slow decrease due to photochemical transformation of thymine residues. If from curve β we subtract the absorption due to the unmodified poly-A, we obtain curve γ, which exhibits the rate of photochemical transformation of thymine residues in the irradiated helix.

Figure 5B exhibits the melting curves corresponding to the changes in secondary structure with increasing doses of irradiation. Curve *K* is for the non-irradiated control, and curves *a* to *f* following successive irradiation times correspond to the points on curve γ of Fig. 5A.

From curves β and γ in Fig. 5A it will be seen that, after 320 min. irradiation, the reaction still proceeds, slowly, as for poly-rT. At this point 72% of the thymine residues have undergone transformation, and the resulting temperature profile (curve *f* in Fig. 5B) is practically identical to that for poly-A alone, indicating that the radiation modified poly-rT has completely lost its ability to complex with poly-A.

By contrast, curve *d* in Fig. 5B, corresponding to 140 min. irradiation and transformation of about 46% of the thymine residues, shows that under these conditions there is still appreciable complexing, although with a lower T_m, 48°, as compared to 72° for the non-irradiated control. This result should be compared with that for poly-rT alone, where photochemical transformation of 25% of the thymine residues leads to complete loss of secondary structure (Fig. 2C and D, completely flat shape of curves *5*).

Irradiation of the complex to the point where 50% of the thymine residues have undergone photochemical transformation (Fig. 5B, curve *d*) modifies the temperature profile so that it is no longer affected by irradiation, and is similar to the profile of alkaline poly-A with a T_m about 47° [the T_m of poly-A under these conditions is 45° (Barszcz, 1967)]. It appears therefore that photolysis of 45% of the thymine residues in the complex abolishes the ability of poly-rT to complex with poly-A. These results may be compared with those of Fresco & Alberts (1960), who showed that, in a complex consisting of one strand of poly-A and another of a co-polymer of rA and rU, an increase of the number of rA residues in the latter to 31% resulted in a decrease in T_m of 36°, the resulting "complex" showing practically no hypochromicity. It is clear that the corresponding complex of rA and rT, following transformation of thymine residues, is considerably more stable; following transformation of 31% of the thymine residues, the T_m of the complex decreases by only 19°

(from 72° to 53°). This may, of course, be due to the fact that rA + rT base pairs are considerably more stable than rA + rU base pairs (Barszcz & Shugar, 1968), and that the rA + rT complex is triple-stranded.

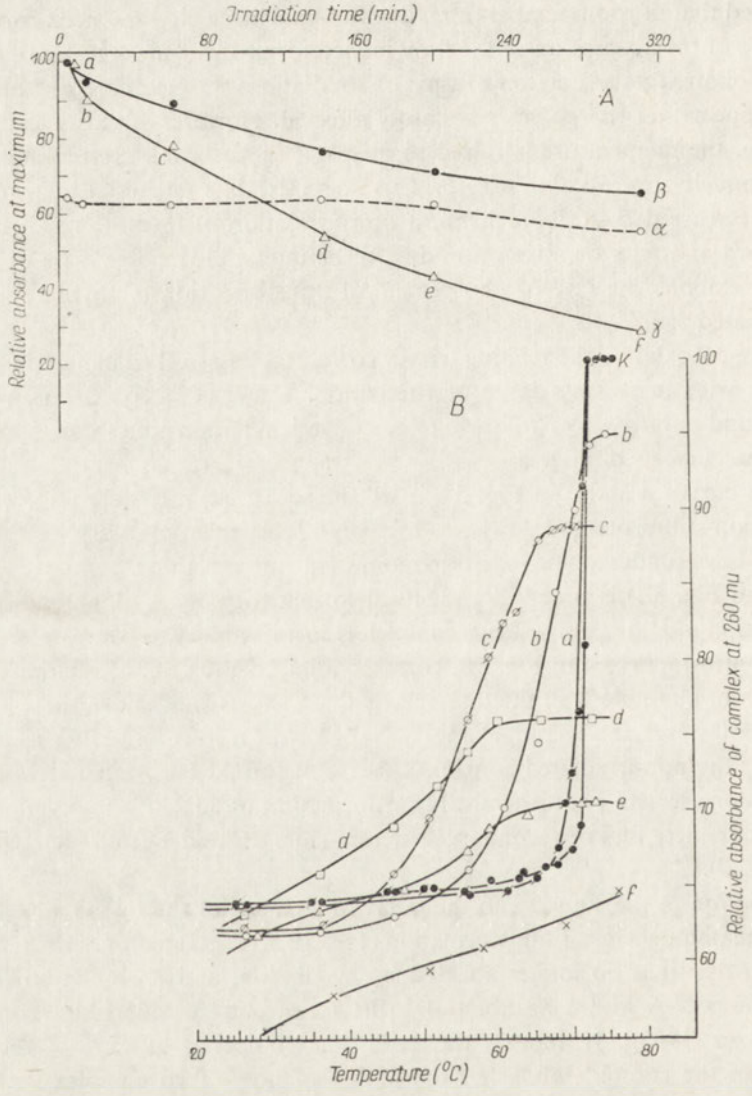

Fig. 5. Irradiation at 254 mμ (source *A*) of poly-(A+2rT) at room temperature (helical form): in 0.1 M-Na^+ and 0.01 M-phosphate buffer, pH 7.4.

A: (α), absorbance of UV absorption maximum as a function of dose (time of irradiation). (β), Irradiated solution heated to melt out helix, and absorbance of UV absorption maximum measured. (γ), Absorption of poly-A subtracted from curve β to give absorption corresponding to number of unchanged thymine residues.

B: Temperature profiles of control solution (*K*), and of irradiated solutions (*a* to *f*) corresponding to first 5 points in *A*, curve γ.

Note, in particular, that for small doses (up to about 4 min. irradiation in Fig. 5A, curves β or γ), there is no observable disappearance of thymine residues, whereas the temperature profile (curve *a* in Fig. 5B) shows that some modification of secondary structure has taken place. The absence of such a "lag" period in the photochemical transformation of poly-rT alone, whether in the random coil or helix forms (Fig. 2A and B) points to the high initial radiation resistance of thymine residues in the helical form of poly-(A+2rT).

The key role played by secondary structure in the above phenomenon was further testified to by the following observations: When a 1:1 molar mixture of TpTp and ApA was irradiated, the rate of photochemical transformation of thymine residues was identical with that for TpTp alone, with no observable lag period (Fig. 6B). Furthermore, when poly-(A+2rT) was first heated in the presence of 2% formaldehyde to react with the adenine groups and dissociate the triple strand (Fig. 6C), the resulting mixture exhibited no lag period, while the photochemical

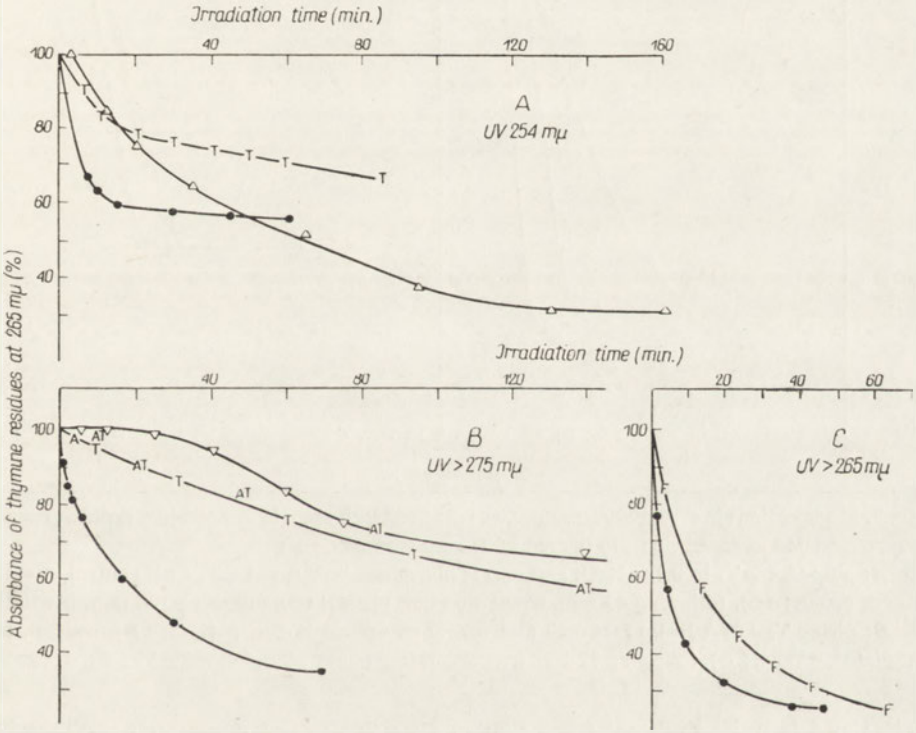

Fig. 6. Comparative rates of photodimerization of thymine residues in TpTp, poly-rT and poly-(A+2rT) in 0.15 M-Na^+ and 0.015 M-phosphate buffer, pH 7.3.

A: Irradiated at 254 mμ: (T), TpTp at 23°; (●), poly-rT at 16°; (△), poly-(A+2rT) at 20°.

B: Irradiated at wavelengths to the red of 275 mμ (source *C*): (T), TpTp at 23°; (AT), TpTp + ApA (1:1) at 23°; (●), poly-rT at 16°; (△), poly-(A+2rT) at 20°.

C: Irradiated at wavelengths to the red of 265 mμ (source *D*): (●), poly-rT at 16°; (F), poly-(A+2rT) treated with 2% formaldehyde at 60° and then irradiated at 20°.

transformation of thymine residues was similar to that in poly-rT alone at the same concentration[3].

When poly-(A+2rT) was irradiated at longer wavelength (above 275 mμ) the resulting behaviour was qualitatively similar to that for irradiation at 254 mμ, including the presence of the initial lag period (Fig. 5B, curve *e*).

It was established in these experiments that, during the entire lag period, the melting profiles were fully reversible and the T_m and maximum hyperchromicity unaltered; the only observable effect was a slight broadening of the temperature profile with increasing radiation dose. To some extent this behaviour is a reflection of the high degree of homogeneity, and ordered structure, of the poly-(A+2rT)

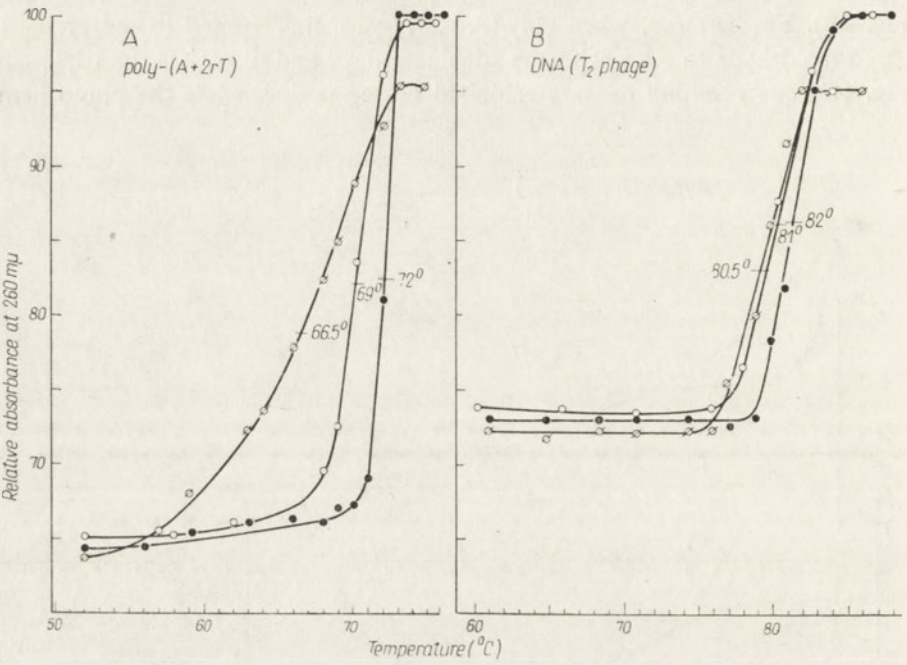

Fig. 7. Modifications in secondary structure, as reflected by changes in temperature profiles, resulting from irradiation at wavelengths to the red of 275 mμ (source *C*) of:

A: poly-(A+2rT) in 0.1 M-NaCl and 0.01 M-phosphate buffer, pH 7.2: (●), control, non-irradiated, profile; (○), following 43 min. irradiation (lag phase); (Ø), following 120 min. irradiation.

B: phage T_2 DNA in 0.15 M-NaCl and 0.02 M-phosphate buffer, pH 7.2: (●), control, non-irradiated, profile. (○), following 32 min. irradiation (lag phase); (Ø), following 75 min. irradiation.

helix; and is quite different from that of the twin-stranded poly-(dA+dT), where the mere operation of running a temperature profile on an irradiated solution led to inhibition of further photodimerization (see below).

The foregoing lag period was also observed with irradiated phage T_2 DNA (Fig. 7), which was selected for comparison purposes because of its high A-T content

[3] Formaldehyde was without effect on the photochemical transformation of thymine residues; this was established by examining the rate of phototransformation of TpTp in 2% formaldehyde.

(65%) and because, in place of cytosine, which may undergo hydration, it contains uniquely 5-hydroxymethylcytosine, which does not (Wierzchowski & Shugar, 1960a). The radiation-induced change in absorption of samples of such a DNA is due mainly to dimerization of thymine residues[4]. Furthermore in the wavelength range to the red of 275 mμ the absorption of the DNA is similar to that of the poly-(A+2rT). But, whereas 5% photolysis of the latter leads to an appreciable broadening of the profile and a decrease in T_m of 5.5°, the same degree of photolysis of the DNA sample hardly affects the breadth of the profile, and decreases the T_m by only 1.5°.

Photolysis of twin-stranded complexes

The photochemical behaviour of the complex between poly-rT and poly-A bears some resemblance to that of phage T_2 DNA, particularly as regards the initial lag period, which is common to both, although to different degrees. Since, however,

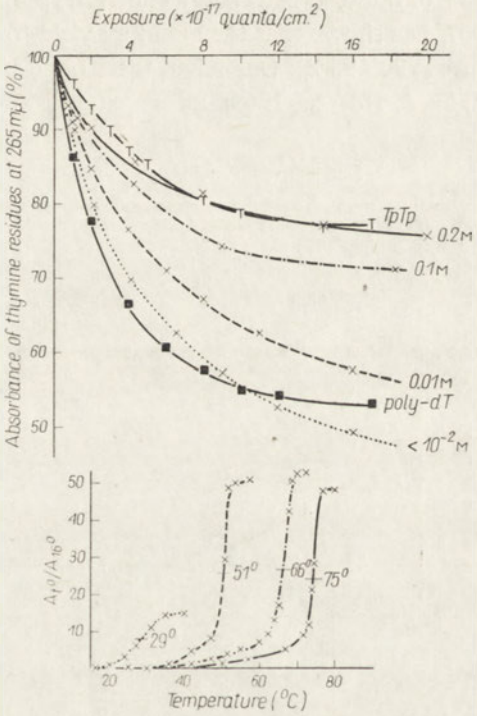

Fig. 8. Course of photochemical transformation of thymine residues in the twin-stranded poly(dA+dT), irradiated at 254 mμ at 18°, in the presence of different salt concentrations (as indicated for each curve); for comparison purposes, the figure shows the course of photolysis of thymine residues in TpTp and poly-dT in dilute salt solution. The insert shows the temperature profiles, which are a measure of the thermolability, of each sample of poly-(dA+dT) prior to irradiation.

the former exists only as the triple-stranded poly-(A+2rT), it was obviously desirable to extend the comparison to include a twin-stranded complex. Such a model is available, viz. poly-(dA+dT), which is a twin-stranded helix under defined conditions (Setlow *et al.*, 1965).

The course of the photochemical transformation at 254 mμ of thymine residues in poly-(dA+dT), as a function of the ionic strength of the medium, is illustrated

[4] There will, of course, be some dimerization of 5-hydroxymethylcytosine residues, but preliminary experiments with a polymer containing similar residues, poly-5-methyldeoxycytidylic acid have shown that this reaction is appreciably slower than thymine dimerization.

in Fig. 8. It will be observed that, as the Na^+ concentration is decreased from 0.2 M to 0.01 M, there is a parallel increase in the rate of photodimerization of thymine residues. This increase in rate of dimerization is strictly correlated with the decrease in T_m values (as can be seen from the temperature profiles in the insert to Fig. 8). Note in particular that, at the lowest Na^+ concentration, below 10^{-2} M, where the degree of secondary structure has been considerably reduced (as shown in the insert to Fig. 8 by the very broad temperature profile, low temperature hyperchromicity and low T_m), the dimerization rate almost attains the value for free poly-dT. Furthermore, the higher the thermal stability of the twin-stranded complex, the higher the plateau level for attainment of photochemical equilibrium, i.e. the lower the extent of dimerization at the equilibrium point.

Photodimerization of pyrimidine residues in an ordered polynucleotide requires utilization of energy to suitably orientate neighbouring residues. This implies that some of the absorbed energy undergoes radiationless transformation (in fact, Eisinger, 1967, has shown that the quantum yield for radiationless processes in diketopyrimidines is 90 - 98 %). One might therefore expect that, as for a decrease in temperature (Rahn & Hosszu, 1968), an increase in ionic strength should slow down the rate

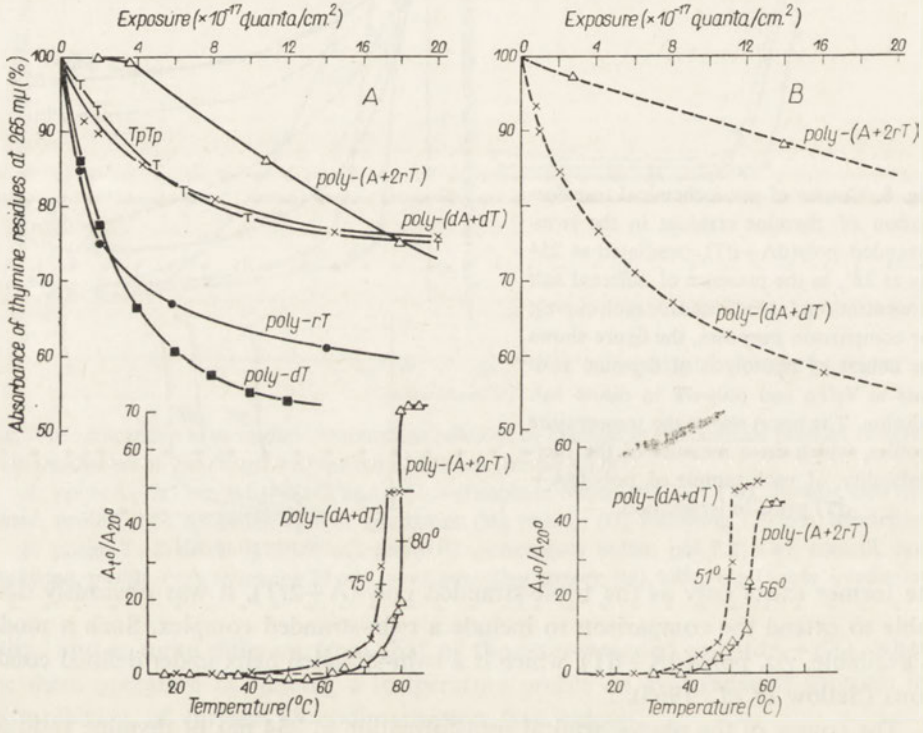

Fig. 9. Comparison of course of photochemical transformation of thymine residues in twin- and triple-stranded complexes irradiated at 254 mμ at 20°, in 0.01 M-phosphate, pH 7.2, (*A*) in 0.15 M-NaCl, and (*B*) in 0.01 M-NaCl: (△), triple-stranded poly-(A+2rT); (×), twin-stranded poly-(dA+dT); (●), poly-rT; (■), poly-dT included for comparison purposes; (T), TpTp. The inserts exhibit the temperature profiles of the complexes prior to irradiation.

of photodimerization, in agreement with the above results (Fig. 8; see also Fig. 9).

Figure 9 in turn exhibits the relative rates of thymine photodimerization in twin- and triple-stranded complexes as compared to the isolated thymine-containing homopolymers which are involved in the formation of these complexes. Undoubtedly the most striking characteristic is the extraordinarily high initial radiation resistance of the triple-stranded helix, poly-(A+2rT), as compared to the twin and single strands. In 0.15 M-NaCl at neutral pH, following "termination" of the initial lag period, the photolysis of thymine residues in the triple-stranded poly-(A+2rT) proceeds at a rate about one-fourth that for the twin-stranded poly-rT under the same conditions. If we include the radiation dose absorbed during the lag period by the thymine residues in the complex, it can be shown that photochemical transformation of 5% of the thymine residues requires, at 254 mμ, a 20-fold higher dose for the triple-stranded poly-(A+2rT) than for the twin-stranded poly-rT.

The foregoing findings demonstrate convincingly the relative stability to irradiation of the three-stranded, as compared to the two-stranded, helix; the former is more than 10 times as resistant if the comparison is made on the basis of absorbed dose leading to photolysis of 5% of the thymine residues. The higher radiation resistance of the triple-strand is clearly not due simply to its enhanced thermal stability, since its T_m is only 5° above that for the twin strand (see inserts in Fig. 9A and B).

The more marked radiation resistance of the triple-stranded complex is probably related to its more compact structure, due, conceivably, to the ribose 2′-hydroxyl, since poly-(dA+2rT) was found to exhibit a photosensitivity intermediate between that for poly-(A+2rT) and poly-(dA+dT).

In dilute salt solution (0.01 M), where the T_m of the triple-stranded helix is 24° lower, the lag period apparently disappears. Notwithstanding this, the radiation resistance of the triple strand is hardly affected (Fig. 9B), if one considers the total dose required to transform 5% of the thymine residues. By contrast, under these conditions, the twin-stranded poly-(dA+dT) is photolysed at a rate 50% higher than in 0.15 M-NaCl (cf. Fig. 9B and Fig. 8). That these modifications are due to differences in secondary structure of the complexes is best testified to by the fact that the photolysis of their poly-T components alone is hardly affected over a range of salt concentrations from 0.01 M to 0.15 M.

These results are of obvious interest in relation to the behaviour of irradiated DNA. The difference in extent of thymine photodimerization between native and denatured DNA is most marked in the inital stages of irradiation and decreases with increasing radiation dose (Wacker *et al.*, 1962; Hosszu & Rahn, 1967), and is probably due to the fact that in native DNA some of the absorbed energy is utilized to create localized areas with a less compact structure. A rough calculation from the results presented in Fig. 7 for the lag period in phage T_2 DNA, irradiated at wavelengths to the red of 275 mμ, showed that the dose required to overcome the lag period was about 10^4 ergs/mm.^{2}, which is close to the dose at which one observes the major difference in rates of dimerization between native and denatured DNA.

Photoreversal of dimerization

Up to the present we have been dealing with the influence of secondary structure on the rate and extent of photochemical transformation of thymine residues. But it should be recalled that the extent of dimerization at any given wavelength represents an equilibrium between photodimerization and dimer photodissociation, and that the equilibrium point is wavelength dependent. We shall now examine the conditions under which the equilibrium is shifted in the direction of dimer photodissociation. Photoreversal of dimers in irradiated biologically active DNA has been demonstrated (Setlow & Setlow, 1962; Setlow & Carrier, 1966), but the degree of reversibility is quite low. It is clearly desirable to establish whether this is due to formation in the irradiated DNA of products other than photodimers, or to other causes.

From Fig. 10, which presents the dependence of the T_m of poly-(A+2rT) on the extent of photochemical transformation of thymine residues, it will be seen that the resulting changes in secondary structure are identical for irradiation at 254 mμ

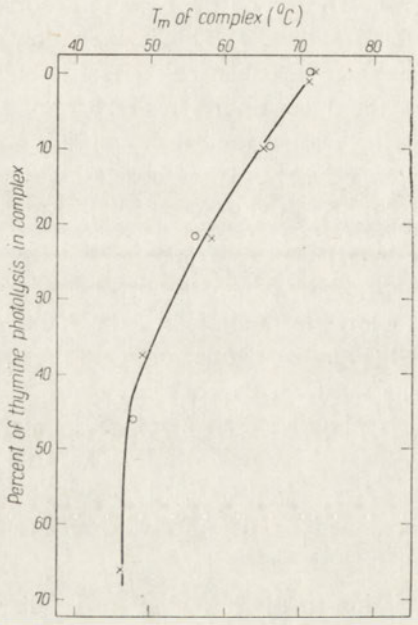

Fig. 10. Dependence of extent of photochemical transformation of thymine residues on the T_m of poly-(A+2rT) when irradiated (○), at 254 mμ and (×), at wavelengths to the red of 275 mμ (source *C*).

or $\lambda > 275$ mμ, if the same number of thymine residues has undergone photochemical transformation. By contrast, an examination of the reversibility of this reaction demonstrated in certain instances that, following irradiation at 254 mμ, the extent of reversibility was only one-half that following photolysis at wavelengths to the red of 275 mμ (Table 2 and Fig. 11).

The following three procedures were applied in attempts to obtain a high degree of reversal of photodimerization:

(a) Samples initially irradiated at wavelengths to the red of 265 mμ were sub-

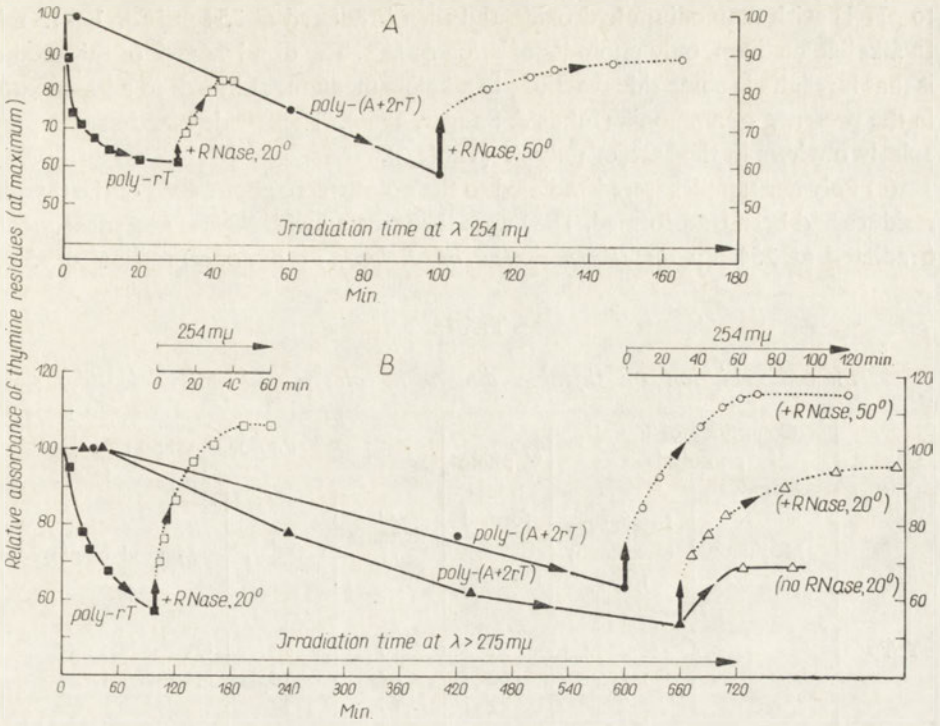

Fig. 11. Photochemical reversal, in the presence of RNase, of photodimers in poly-rT and poly-(A+2rT).

A: Photodimerization and photoreversal with 254 mμ: (■—■), poly-rT in 0.01 M-$MgCl_2$, photodimerization; (□···□), RNase added (5 μg./ml.) and irradiation continued at 20° to give photoreversal; (●—●), poly-(A+2rT) in 0.01 M-NaCl, pH 7.5, photodimerization; (○···○), RNase added (50 μg./ml.) and irradiation continued at 50° to give photoreversal.

B: Photodimerization with wavelengths to the red of 275 mμ (source *C*) and photoreversal in presence of RNase at 254 mμ: (■—■), poly-rT in 0.01 M-$MgCl_2$, photodimerization; (□···□), RNase added (10 μg./ml.) and irradiation continued at 20° to give photoreversal; (▲—▲), poly-(A+2rT) in 0.1 M-NaCl, pH 7.5, photodimerization; (△···△), RNase added (30 μg./ml.) and irradiation continued at 20° to give photoreversal; (△—△), no RNase added, and irradiation continued at 20° to give photoreversal; (●—●), poly-(A+2rT) in 0.01 M-NaCl, pH 7.5, photodimerization; (○···○), RNase added (30 μg./ml.) and irradiation continued at 50° to give photoreversal.

Note: 100% absorption refers to absorption of thymine residues in the melted polymer or complex (to eliminate hypochromicity). This does not include the residual hyperchromicity resulting from RNase hydrolysis (10 - 15%), which is shown by the dark vertical arrows.

sequently irradiated at 254 mμ or 228 mμ. The advantage of this method is that it makes it possible to follow dimer photodissociation as well as extent of reformation of polynucleotide structure, from reformation of thermal profiles (see Fig. 12). Its principal drawback is that it not only leads to formation of side products, but also results in supplementary formation of photodimers, the net result being a reduction in the degree of reversal.

(b) Samples irradiated to the equilibrium value for dimerization were brought

to pH 11 with ammonium hydroxide and then irradiated at 254 mμ for 10 - 15 min. In alkaline medium, only photodissociation occurs. The disadvantage of this method is that it results in some side reactions in alkaline medium, although to a lesser extent in the presence of ammonia (Fikus & Shugar, 1966). These side reactions are particularly obvious in the case of poly-rT (Table 2).

(c) Polymer samples were irradiated to the point where about 40% of the thymine residues had been transformed. They were then treated with RNase and subsequently irradiated at 254 mμ, *in the presence of RNase*, at a concentration of 5 - 30

Table 2

Photodissociation of thymine dimers in oligo- and polynucleotides

Polymer	Conditions of initial photolysis		% photo-transformed thymine residues[a]	Photodissociation		
	Medium[b]	Irradiation wavelength (mμ)		% thymine residues reformed	% reversal	Method employed[c]
TpTp		254	22	16	72	UV254-NH_4OH
		>275	62	47	76	UV254-NH_4OH
		>275	42	16	38	UV254
		>265	65	57	88	UV228
Poly-rT		254	15	9	60[d]	UV254-NH_4OH
		>275	71	54	76	UV254-NH_4OH
		>275	74	30	47	UV254
		>265	48	36	75	UV228
	0.01 M-Mg^{2+}	254	39	18	46	UV254-RNase 20°
	0.01 M-Mg^{2+}	>275	43	39	90	UV254-RNase 20°
Poly-dT		254	42	30	70	UV254-NH_4OH
		>275	71	53	74	UV254-NH_4OH
		>275	69	24	35	UV254
Poly-(A+2rT)	0.10 M-Na^+	>275	46	14	30	UV254
	0.10 M-Na^+	254	16	4	25	UV228
	0.15 M-Na^+	>265	38	18	47	UV228
	0.01 M-Na^+	254	41	16	39	UV254-RNase 50°
	0.01 M-Na^+	>275	37	35	95	UV254-RNase 50°

[a] Calculated from the decrease in absorption, following correction for thermal hyperchromicity and the absorption of the adenine residues in the complex.

[b] The nature of the irradiation medium is given only for those instances where it influenced the course of the reaction.

[c] UV254, UV228, irradiation at these wavelengths following prior irradiation at longer wavelengths. UV254-NH_4OH, irradiated about 15 min. under these conditions (pH 11). UV254-RNase 20° or 50°, irradiated 15 - 30 min. in presence of 5 - 30 μg. RNase, with appropriate correction for residual hyperchromicity following digestion with RNase.

[d] Prolonged irradiation led to irreversible transformation of photoproducts.

μg./ml., and under optimal conditions (in the presence of Mg^{2+} ions or 0.01 M-Na^+ at 50°, see Fig. 11) so that rapid hydrolysis of phosphodiester bonds occurred *during* photodissociation, thus preventing repeated dimerization. This procedure gave the highest degree of dimer reversal (about 95%).

The overall results are presented in Table 2. Fig. 11 exhibits the action of RNase during photodissociation; and Fig. 12 the reversal of temperature profiles following

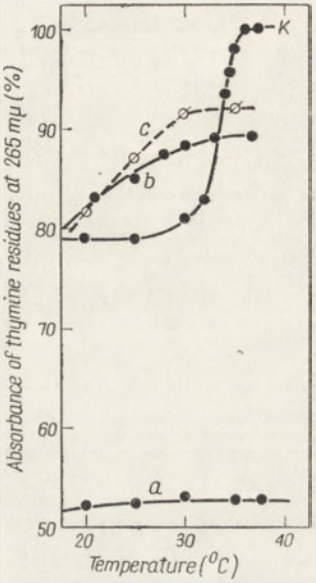

Fig. 12. Photochemical reversal at 228 mμ (source *E*) of photodimers in poly-rT: (*K*), temperature profile of poly-rT prior to irradiation in 0.004 M-$MgCl_2$; (*a*), profile following transformation of about 48% of the thymine residues by irradiation at wavelengths to the red of 265 mμ (source *D*); (*b*), profile following repeated irradiation at 228 mμ, and corresponding to (*c*), the profile which would be obtained following 11% photodimerization of thymine residues in poly-rT.

irradiation at 228 mμ. In the latter figure, curve *K* is the profile of non-irradiated poly-rT; curve *a* is the profile following photochemical transformation of 11% of the thymine residues by irradiation at wavelengths to the red of 265 mμ (cf. Fig. 2D, curve *3*); curve *b* is the "profile" following transformation of 47% of the thymine residues. At this point the solution was removed and re-irradiated with source *E* at 228 mμ, leading to extensive reversal of dimerization and partial reformation of secondary structure, placed in evidence by the profile *c*. The degree of reversal, while impressive, is not fully quantitative because at these short wavelengths other reactions occur simultaneously with dimer photodissociation.

Influence of citrate buffer on photochemical reaction

In the initial stages of this investigation, the photochemical transformation of helical complexes such as poly-(A+2rT) was studied in SSC solution (i.e. 0.15 M-NaCl and 0.015 M-sodium citrate). The use of this solvent has been widespread since its introduction by Marmur & Doty (1959), because it provides physiological conditions for formation of helical complexes and has been used as a standard solvent for estimating the G-C content of a given DNA preparation from its temperature profile.

When poly-(rA+rU) was submitted to *prolonged* irradiation at wavelengths

to the red of 275 mμ, the temperature profile of the irradiated complex was found to become very broad, while the absorbance rose 10% above that of the non-irradiated complex. Since it had previously been observed that sodium citrate itself exhibits a marked increase in UV absorption under the influence of ionizing radiations, (Barszcz, 1967), a solution of SSC was irradiated at wavelengths to the red of 275 mμ, and was found to lead to the appearance of a new band in the UV, which slowly disappeared on subsequent heating in the dark.

It is clear that citrate ion itself gives rise to thermolabile photoproducts, and the failure to take account of this in initial experiments led to fallacious results which necessitated a repetition of a number of experiments with the use of phosphate buffer.

In the light of the foregoing findings, it must be assumed that the extensive broadening of the temperature profile of UV-irradiated *Diplococcus pneumoniae* DNA observed by Marmur *et al.* (1961) was due in large part to the fact that the experiments were performed with SSC as solvent. It is strongly recommended that the use of this solvent in photochemical (and radiation chemical) investigations be discontinued.

CONCLUDING REMARKS

Some comment is called for with respect to the apparently high quantum yield for thymine photodimerization, 4.4×10^{-2}, in the ordered structural form of poly-rT. This value is to be compared with that for the dinucleotide TpTp, 1.1×10^{-2}; and with the value of about 2.2 for oligo-dT, neither of which is capable of assuming a secondary structural form. The implication is that in the ordered structural form of poly-rT, the thymine residues are more favourably orientated (or stacked) for dimerization. However, if this ordered form is a twin-strand with hydrogen bonds involving the $N_{(3)}$ ring hydrogens and the $C_{(4)}$ carbonyl (Green, Mathews & Rich, 1962), adjacent thymine residues in each strand would probably not be more favourably stacked with respect to each other than in native DNA. It is consequently possible that the proposed twin-stranded structure for poly-rT is not valid and, indeed, the failure to observe any secondary structure in poly-dT suggests that simple base-pairing is not the only factor responsible for the secondary structure of poly-rT. An alternative possibility is that in a highly ordered structure such as poly-rT, the reformation of dimers may be enhanced (as for model systems in matrices), thus leading to what appears to be an enhanced photodimerization rate.

The question also arises as to whether photodimerization of neighbouring thymine residues in a helical polynucleotide is at all possible without some prior reaction leading to "localized denaturation" which would then permit of a suitable time-dependent orientation of the 5,6 bonds of two adjacent thymine residues. In the case of poly-rT alone this point is rather difficult to establish by the techniques employed in this study. Phototransformation of even as much as 1% of the thymine residues in a poly-rT preparation would be difficult to measure accurately by spectral methods, although this would certainly be more than sufficient to produce local distortions

in the helix, facilitating further dimerization. An additional possibility is that the thymine residues in ordered poly-rT are so oriented as to favour dimerization between residues in complementary strands (cross-links).

For the complexes of poly-T with poly-A, where the secondary structures are better known, no doubts whatever prevail regarding the important role of steric hindrance on photodimerization of neighbouring thymine residues. One of the most striking observations in the present investigation is, in fact, the inhibitory effect of secondary structure on thymine photodimerization, in particular the initial lag phase in the triple-stranded poly-(A+2rT).

An interesting model for the dimerization reaction in polynucleotides is that of mixed crystals of adenine and thymine in ice (Smith, 1966) or KBr (Lisewski & Wierzchowski, 1969) matrices. For 1:1 mixed crystals of 1-methylthymine and 9-methyladenine in a KBr matrix the initial rate of dimerization of thymine is inhibited as compared to the rate of dimerization of 1-methylthymine alone, the inhibition being 3-fold for irradiation at 254 mμ and 7-fold at wavelengths to the red of 275 mμ. These are to be compared with the results reported above for polynucleotide complexes: viz. a 4-fold inhibition of dimerization at 254 mμ, and 11-fold on irradiation at wavelengths to the red of 275 mμ, as compared to the homopolymers.

An additional factor which may influence the initial rate of photodimerization of pyrimidine residues in a helical complex is the nature of the excited state(s) involved. No data are as yet available regarding the effect of complexing between poly-rT and poly-A on the nature of the emission of the complex components. In the case of the three-stranded poly-(A+2U), it has been demonstrated by Kleinwachter, Drobnik & Augenstein (1968) that there is practically complete quenching of the emission, both fluorescence and phosphorescence, as compared to the twin-stranded poly-(A+U). It is, consequently, conceivable that the inhibition of the initial rate of dimerization in poly-(A+2rT), as well as in DNA, is due not only to steric factors which hinder rotation of thymine residues to positions favourable for dimerization, but also to quenching of the appropriate excited state(s) of adenine-paired thymine.

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FOTODIMERYZACJA TYMINY W STRUKTURZE DRUGORZĘDOWEJ POLINUKLEOTYDU

Streszczenie

1. Zastosowano technikę badania widm w ultrafiolecie oraz śledzenie przejścia spirala - bezładny zwój celem zbadania szybkości i rozmiarów odwracalnej fotodimeryzacji reszt tyminowych w polinukleotydach. Zbadano: nie wykazujące struktury drugorzędowej oligo-rT i poli-dT oraz poli-rT, którego strukturę drugorzędową można kontrolować siłą jonową, temperaturą i rozpuszczalnikiem, jak również dwułańcuchowe i trójłańcuchowe kompleksy powyższych polimerów z poli-A (rA i dA).

2. Naświetlając promieniowaniem λ 254 mμ stwierdzono, że szybkość fotodimeryzacji w odniesieniu do wzorcowego dwunukleotydu TpTp jest 4-krotnie większa w dwupasmowym poli-rT, tylko dwukrotnie większa w jednopasmowym poli-rT, 3-krotnie większa w jednopasmowym poli-dT.

3. Przy użyciu promieniowania $\lambda \geqslant 265$ mμ mierzone szybkości dimeryzacji odpowiednio wynoszą: 7,5 razy szybciej dla dwupasmowego poli-rT i 4 razy szybciej dla jednopasmowego poli-rT i poli-dT.

4. Wykrywalną fotodimeryzację w dwu- i trój-pasmowych kompleksach poli-T z poli-A poprzedza okres „lagu", któremu towarzyszą nieznaczne zmiany w strukturze drugorzędowej. Po tym okresie fotodimeryzacja przebiega z mniejszą szybkością niż w homopolimerach. Podobne zjawisko zaobserwowano w DNA z faga T2.

5. Zmniejszenie szybkości dimeryzacji w kompleksach jest uwarunkowane po części nie sprzyjającą tej reakcji orientacją reszt tyminowych w sztywnej strukturze spirali kompleksów i być może wygaszaniem odpowiednich stanów wzbudzonych tyminy.

6. Zbadano w sposób ilościowy wpływ solwentu i temperatury oraz stopień odwracalności fotolizy w różnych warunkach.

7. W szczególności wykazano, że stopień fotoodwrócenia sięga do 90% w obecności RNazy, która jest zdolna do hydrolizy wiązań międzynukleotydowych w trakcie fotodysocjacji dimerów.

8. Powyższe wyniki omówiono pokrótce w odniesieniu do fotodimeryzacji i fotodysocjacji dimerów w naturalnych kwasach nukleinowych.

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