KOMITET BIOCHEMICZNY I BIOFIZYCZNY
POLSKIEJ AKADEMII NAUK

COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS
POLISH ACADEMY OF SCIENCES

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. 19
No. 4

WARSZAWA 1972
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ADDRESS

ul. Banacha 1, Warszawa 22
(Zakład Biochemii A.M.)
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 1772+148 egz. Ark. wyd. 9,75 ark. druk. 6,75+1,5 ark. wkł.
Papier druk. sat. kl. III, 90 g. 70×100
Oddano do składania 7.VIII.72 r. Podpisano do druku 1.XII. 72r.
Druk ukończono w grudniu 1972
Zam. 671 Cena zł 25,—

Warszawska Drukarnia Naukowa. Warszawa, Śniadeckich 8

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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ISOLATION AND CHARACTERIZATION OF BASIC PROTEINS (HISTONES) FROM SUBCELLULAR FRACTIONS OF HEART MUSCLE, KIDNEY AND LIVER**

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1. Sulphosalicylic acid (SSA) soluble proteins from subcellular fractions of beef tissues were compared with respect to their electrophoretic mobility, amino acid composition and chromatographic separation. 2. One protein fraction could be isolated by SSA extraction and chromatography on CM-cellulose column from each cellular fraction (cytoplasm, nuclei, mitochondria). These proteins were identical in three different beef tissues and were basic in character. 3. Nuclear protein extracted with SSA corresponds to homogeneous *F1* lysine-rich histone.

We have previously described extranuclear effects of very low concentrations of histones (Schwartz, Laseter & Ferguson, 1965; Johnson, Safer & Schwartz, 1966; Johnson, Mauritzen, Starbuck & Schwartz, 1967; Safer & Schwartz, 1967; Schwartz, 1965). These phenomena involved an interaction between the basic protein and mitochondria, cell membrane fragments containing Na^+,K^+-ATPase activity and actomyosin. The data from the mitochondrial studies suggested that an intermediate involved in ion transport is the locus of histone action (Johnson *et al.*, 1967). Histones isolated from heart muscle in a semipurified state, appeared to exert effects that suggested a possible modulating action on muscle contractility (Schwartz *et al.*, 1965).

Accordingly, further studies were undertaken to isolate and purify a basic protein from heart muscle and characterize some specific effects on an elementary process of membrane activity, ion transport.

This communication describes a procedure for the isolation of basic proteins from subcellular fractions from three tissues, heart muscle, kidney and liver, which involves extraction with sulphosalicylic acid and chromatography on CM-cellulose column. The proteins were characterized by their electrophoretic mobility and amino acid composition. The dynamic studies on potassium transport in mitochondria will be published elsewhere.

* Recipient of a Career Research and Development Award (K_3 HL 11,875).

** Supported by USPHS grants HL 05435, HL 07906, HL 05925 and HL 13837, NIH-71-2493 and American Heart Association, Texas Affiliate, Houston Chapter.

METHODS AND MATERIALS

Preparation of subcellular fractions from beef heart, kidney and liver. Total homogenate. Tissues (or heart ventricles) were trimmed of fat, passed through a meat grinder, and homogenized in KE medium (0.18 M-KCl, 10 mM-EDTA, pH 7.4) in a volume equivalent to a 10 to 20% homogenate, for 1 min in a Macro Waring blendor at medium speed.

Mitochondria. The homogenate was passed through four layers of cheesecloth and centrifuged at 600 *g* for 10 min. The supernatant was centrifuged at 8000 *g* for 10 min. The pellet (mitochondria) was washed twice in about five volumes of KE medium by resuspending with a Potter-Elvehjem homogenizer with Teflon pestle and centrifuged as above. The mitochondrial pellet was resuspended again in about five volumes of KE medium and centrifuged for 10 min at 600 *g* to remove contaminating nuclei. The supernatant from the last centrifugation was saved and contained mitochondrial suspension free of nuclei.

Mitochondria from beef heart muscle prepared on large scale were checked by sucrose density gradient centrifugation[1]. Mitochondria were centrifuged for 2 h in a linear gradient of 20 - 50% sucrose in KE medium at 112 000 *g*.

Nuclei. The pellet obtained after centrifugation of homogenate at 600 *g* was washed five times in one to two volumes of KE medium by resuspending in a Macro Waring blendor for 10 sec at medium speed and centrifuging for 10 min at 600 *g*. The final pellet was resuspended in about five volumes of KE medium.

Cytoplasm. The postmitochondrial supernatant after first centrifugation of the homogenate at 8000 *g*, was used as the cytoplasmic fraction.

Extraction of the homogenate and subcellular fractions with sulphosalicylic acid. The procedure was based upon that described by Rzeczycki, Grudzińska & Hillar (1962) and Rzeczycki, Grudzińska, Hillar & Wszelaki-Lass (1963). To the tissue preparations, 2 M-sulphosalicylic acid (SSA) was added in a 9:1 v/v ratio to obtain a final concentration of 0.2 M. The mixture was homogenized for 2 min in a Waring blendor at medium speed and centrifuged for 10 min at 2000 *g*. The sediment was discarded and the supernatant containing SSA-soluble proteins was used in the further steps.

Precipitation of proteins from the SSA extracts and concentration of their solution. The procedure was based upon that described by Mejbaum-Katzenellenbogen (1959). The SSA extracts were filtered through Whatman no. 1 filter paper and adjusted to pH 4.0 with concentrated NaOH. Protein from these extracts were precipitated with tannin solution (about 1 to 3%) added in a 1:20 ratio (protein: tannin, w/w) at 0 - 4°C and left for at least 30 min. The formed tannin-protein complex was centrifuged for 10 min at 2000 *g* at 0°C and washed once by stirring with 0.9% NaCl. Protein from the tannin-protein complex was regenerated with caffeine added as substance (protein : caffeine ratio 1:3, w/w). During this step the pellet was stirred occasionally for 10 - 30 min and allowed to stand overnight at 0 - 4°C. A small amount of 0.9% NaCl was added finally to dissolve the rege-

[1] Appreciation is extended to Dr. William Wood for these studies.

nerated protein and centrifuged for 10 min at 3000 *g*. The pellet obtained (tannin--caffeine complex) was discarded after one washing with 0.9% NaCl, and the washings were added to the supernatant (SSA-soluble protein solution) and dialysed against water for 48 h. During dialysis some residual tannin-caffeine complex precipitated and was centrifuged at 3000 *g* for 10 min. The supernatant contained SSA-extractable protein in concentrated solution. This protein was finally lyophilized and stored as a dry powder.

Chromatographic separation of the SSA extractable proteins. The chromatography procedure was based upon that described by Rzeczycki *et al.* (1963). The dry protein was dissolved in 0.05 M-sodium acetate buffer, pH 4.2 (about 50 - 100 mg in 5 ml of buffer) and applied to a CM-cellulose column (1 cm × 2 cm). Fractions were eluted with a gradient of increasing concentration of potassium chloride in acetate buffer (0 - 0.6 M in 0.05 M-sodium acetate buffer, pH 4.2). Fractions of about 5 ml were collected every 15 or 18 min. The peaks were collected, dialysed against water for 48 h and lyophilized.

Analytical procedures. Protein in the tissue preparations was measured by the biuret procedure (Jacobs, Jacob, Sanadi & Bradley, 1956). Protein in the SSA extracts and in the chromatography eluents was measured with the tannin turbidimetric method (Mejbaum-Katzenellenbogen, 1955).

RNase activity assay in the crude SSA extracts from tissue preparations was carried out by Dr. A. Bardoń by the procedure of Roth (1962).

Electrophoretic separation of the SSA extractable proteins was performed using a Gelman apparatus on Sepraphore III strip (15 cm long), in a sodium barbital buffer of 0.02 M, pH 8.8, for 1 h applying 1.4 mA/cm. *F1* histone fraction from calf thymus gland, applied for comparison, was supplied by Dr. C. M. Mauritzen; it was prepared according to the procedure of Johns (1964) with perchloric acid extraction and trichloroacetic acid precipitation.

Protein fractions obtained by chromatography on CM-cellulose column were hydrolysed in 0.6 N-HCl (0.4 ml/1 mg protein) for 24 h at 110°C in ignition tubes sealed under nitrogen.

Amino acid analysis of the hydrolysates was carried out using a Beckman 116 automatic amino acid analyser. Statistical elaboration of the results were performed by computer at the Common Computer Center of Baylor College of Medicine.

RESULTS AND DISCUSSION

Table 1 summarizes the quantitative data for the proteins extracted with SSA from cellular fractions of beef tissues. Heart nuclei, mitochondria and cytoplasm are relatively poor in SSA-extractable proteins. The high ratio for mitochondria is probably due to the contamination by other cellular fractions and is difficult to avoid in the large scale preparation.

RNase activity could be found only in the proteins extracted from homogenate and cytoplasm, and, to small extent, in the proteins from kidney and liver nuclei (Table 2).

Table 1

Amount of proteins extractable with SSA from subscellular fractions of beef tissues

Values are the means from five determinations.

	Liver	
	mg/100 g tissue	mg/1 g protein
Homogenate	60	
Nuclei	6.6	7.0
Mitochondria	1.6	4.3
Cytoplasm	21.1	3.0
	Kidney	
	mg/100 g tissue	mg/1 g protein
Homogenate	72.7	
Nuclei	11.8	14.8
Mitochondria	0.9	3.3
Cytoplasm	28.0	5.1
	Heart	
	mg/100 g tissue	mg/1 g protein
Homogenate	26.3	
Nuclei	2.1	1.1
Mitochondria	0.4	1.8
Cytoplasm	7.9	2.2

Table 2

RNase activity in the SSA-extractable proteins from subcellular fractions

The proteins extracted with SSA from subcellular fractions were lyophilized and RNase activity was determined according to Roth (1962) at pH 7.8. The activity is expressed as ΔE_{260}/mg protein/ /30 min.

Cell fraction	Kidney	Liver	Heart
Homogenate	840	30	35
Cytoplasm	630	30	50
Nuclei	36	10	4*

* Non-washed nuclei.

Electrophoretic patterns obtained for the extracted proteins from heart are presented in Fig. 1. The same pattern was obtained for beef kidney and liver.

SSA-extractable proteins from homogenate separate into seven fractions migrating under given conditions at pH 8.8 toward the cathode. The mitochondrial fraction corresponds to fraction no. 5 from the homogenate. Fraction 7 in the homogenate originates from nuclei and corresponds to the fastest moving band of thymus *F1* histone prepared by extraction with $HClO_4$. Only this histone can be extracted from nuclei using SSA.

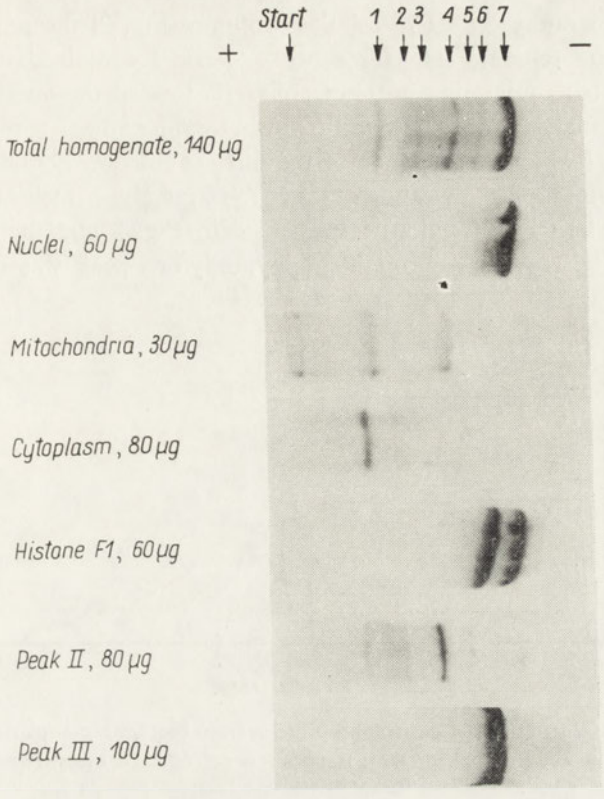

Fig. 1. Electrophoretogram of SSA-extractable proteins from beef heart. The electrophoresis was carried out for 1 h in 0.02 M-Na-barbital buffer, pH 8.8, at 1.4 mA/cm. The subcellular fractions from which the SSA-extractable proteins were obtained and the amount of protein, are indicated. Nuclei were washed 5 times; the mitochondrial preparation (undialysed sample) was contaminated with microsomes. Histone *F1* was from calf thymus gland. Peaks *II* and *III* were obtained from beef heart homogenate by CM-cellulose column chromatography (Fig. 2).

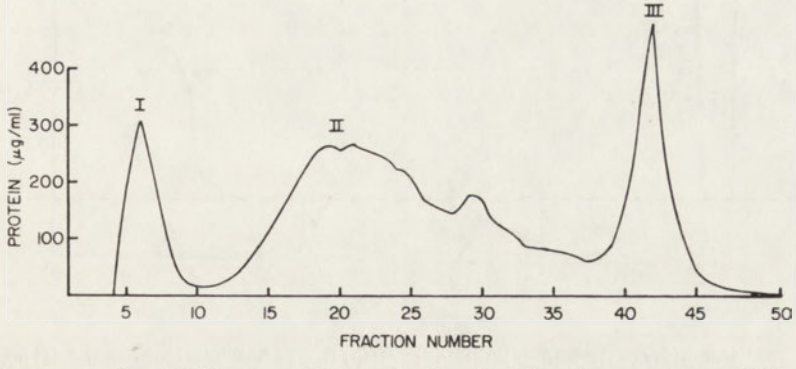

Fig. 2. Chromatography of SSA-extractable proteins from beef heart homogenate on CM-cellulose column (1 cm×20 cm). 110 mg protein were applied; 5 ml fractions were collected every 15 min. The same separation patterns were obtained also for beef kidney and liver.

On chromatography on a CM-cellulose column (Fig. 2) the proteins extracted from homogenate separate into three peaks. Peak *I* contained only very small amounts of proteins migrating on electrophoresis toward the anode (not shown). Electrophoretic analysis of peaks *II* and *III* from total homogenate revealed a homogeneous peak *III* which corresponds to the histone fraction obtained from nuclei (Fig. 1). Chromatographic separation of the SSA-extractable proteins from cytoplasm (postmitochondrial tissue fraction) is presented in Fig. 3 and the electrophoretic separation, in Fig. 1. After rechromatography, only one peak was obtained which

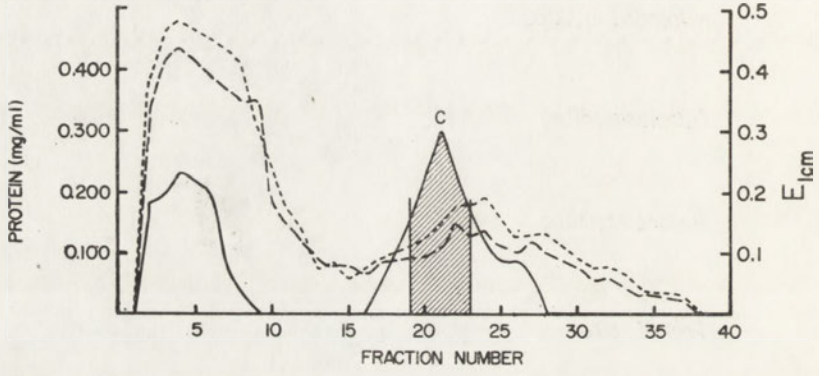

Fig. 3. Chromatography of the SSA-extractable proteins from beef heart cytoplasm on CM-cellulose column. 80 mg protein were applied; 4 ml fractions were collected every 18 min. ——, Protein determined with the tannin turbidimetric method; — — —, E_{260}; - - -, E_{280}.

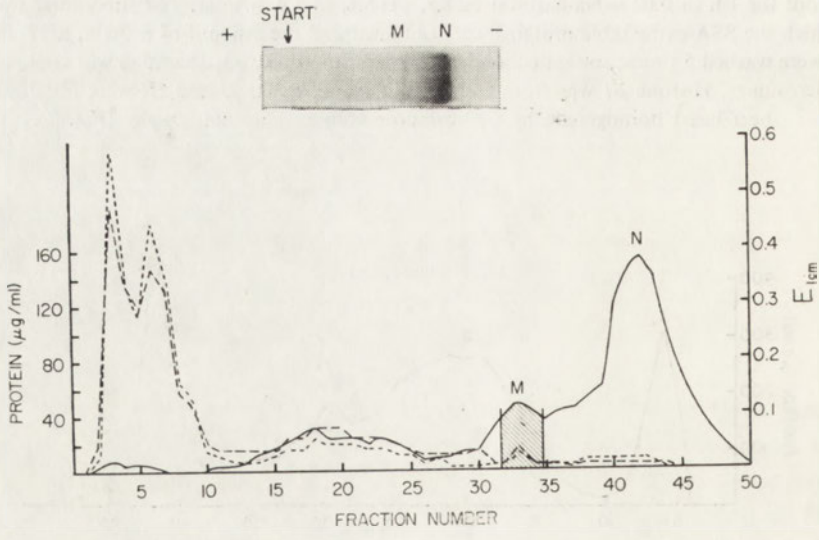

Fig. 4. Chromatography of the SSA-extractable proteins from a beef heart mitochondrial preparation. This preparation was contamined with nuclei (demonstrated by the presence of the nuclear protein fraction). — — —, E_{260}; - - -, E_{280}; ——, protein determined with the tannin-turbidimetric method. Insert, electrophoretogram of the SSA-extractable proteins from the same preparation (80 µg).

was electrophoretically homogeneous. This protein fraction was contaminated with nucleic acids (indicated by absorbancy at 260 nm; Fig. 3).

Heart mitochondria prepared on large scale displayed usually one peak on density gradient centrifugation with the slight contamination either by "microsomes" or by nuclei. After electrophoresis the SSA extract from mitochondria revealed cytoplasmic or nuclear bands (in Fig. 4 nuclear band). The largest protein peak (Fig. 4), *N* (free of nucleic acids), corresponds to the nuclear histone. The smaller peak, *M* (slightly contaminated with nucleic acids), represents a mitochondrial protein fraction extractable with SSA. This protein, in contrast to the other fractions, is only partly liberated from the tannin-protein complex.

The amino acid composition of the protein fractions obtained from heart, kidney and liver are identical for all three tissues examined (Tables 3 - 5). The use of SSA extraction and chromatography on CM-cellulose column yield homogeneous protein fractions one from each subcellular fraction. Nuclear protein is identical with the fastest moving band of very lysine-rich *F1* fraction obtained by Johns (1964). The other obtained fractions from cytoplasm (presumably from microsomes) or from mitochondria do not correspond to any known histone fraction. Of particular interest is the mitochondrial protein which contains a high percentage of the basic amino acids.

Studies in this laboratory suggest that basic nuclear proteins may exert some controlling extranuclear influence on cellular activity. It is believed that their

Table 3

Amino acid composition of the protein fractions obtained from beef heart

Values from three deteminations are presented and expressed as μmol/mg protein.

	Nuclear fraction (*F1*)			Cytoplasmic fraction (C)			Mitochondrial fraction (M)
	Mean	S.D.	(S.E.M.)	Mean	S.D.	(S.E.M.)	
Asp	2.02	0.07	0.04	7.47	0.40	0.23	5.53
Thr	5.26	0.40	0.23	5.32	0.05	0.03	5.95
Ser	6.02	0.20	0.12	8.40	0.58	0.33	15.81
Glu	3.84	0.03	0.02	15.98	0.28	0.16	13.83
Pro	10.52	0.33	0.19	11.70	0.16	0.09	0.0
Gly	6.30	1.47	0.85	7.13	0.08	0.05	15.81
Ala	26.50	0.93	0.54	10.08	0.03	0.02	10.67
Cys	0.00			0.91	0.08	0.05	0.0
Val	4.00	0.13	0.07	3.78	0.80	0.46	3.56
Met	0.00			1.03	0.09	0.05	0.0
Ile	0.91	0.06	0.03	3.29	0.06	0.03	2.37
Leu	4.18	0.41	0.29	6.08	0.10	0.06	4.74
Tyr	0.41	0.03	0.02	1.02	0.03	0.02	2.37
Phe	0.48	0.05	0.03	2.30	0.08	0.05	3.56
Lys	27.88	0.38	0.22	10.25	0.08	0.05	9.88
His	0.00			1.51	0.05	0.03	3.95
Arg	1.52	0.40	0.23	3.72	0.08	0.04	1.98
Ammonia	0.30	0.02	0.01	0.76	0.02	0.01	0.43

Table 4

Amino acid composition of the protein fractions obtained from beef kidney

Values from three determinations are presented and expressed as μmol/mg protein.

	Nuclear fraction (*F1*)			Cytoplasmic fraction (C)		
	Mean	S.D.	(S.E.M.)	Mean	S.D.	(S.E.M.)
Asp	2.44	0.09	0.05	8.05	0.18	0.11
Thr	5.08	0.21	0.12	5.78	0.46	0.27
Ser	5.90	0.19	0.11	7.81	0.15	0.08
Glu	4.64	0.17	0.10	12.88	0.62	0.36
Pro	9.36	1.65	0.95	6.66	0.29	0.17
Gly	7.18	0.26	0.15	8.45	0.11	0.06
Ala	25.14	0.82	0.47	13.47	0.63	0.37
Cys	0.00			0.86	0.06	0.04
Val	3.80	0.25	0.14	6.80	0.77	0.44
Met	0.00			0.60	0.08	0.05
Ile	0.99	0.01	0.00	4.87	0.67	0.39
Leu	3.94	0.09	0.05	7.08	0.38	0.22
Tyr	0.47	0.04	0.02	2.93	0.16	0.09
Phe	0.58	0.02	0.01	3.09	0.44	0.25
Lys	27.69	0.93	0.54	6.74	0.56	0.32
His	0.00			0.75	0.11	0.06
Arg	2.29	0.05	0.03	3.17	0.32	0.19
Ammonia	0.56	0.07	0.04	1.21	0.15	0.09

Table 5

Amino acid composition of the protein fractions obtained from beef liver

Values from three determinations are presented and expressed as μmol/mg protein.

	Nuclear fraction (*F1*)			Cytoplasmic fraction (C)		
	Mean	S.D.	(S.E.M.)	Mean	S.D.	(S.E.M.)
Asp	2.10	0.17	0.10	8.18	0.34	0.20
Thr	5.58	0.27	0.16	5.99	0.16	0.09
Ser	5.94	0.21	0.12	8.38	0.25	0.14
Glu	3.36	0.17	0.10	14.12	0.29	0.17
Pro	9.37	0.66	0.38	8.77	0.24	0.14
Gly	6.57	0.09	0.05	8.50	0.06	0.03
Ala	25.30	1.79	1.03	13.51	0.56	0.32
Cys	0.00			1.06	0.55	0.32
Val	3.40	0.59	0.34	6.36	0.17	0.10
Met	0.00			0.42	0.09	0.05
Ile	0.86	0.04	0.02	4.30	0.15	0.08
Leu	3.71	0.16	0.09	6.44	0.17	0.10
Tyr	0.41	0.02	0.01	1.86	0.09	0.05
Phe	0.59	0.05	0.03	2.21	0.03	0.01
Lys	31.28	2.99	1.73	5.65	0.18	0.10
His	0.00			0.98	0.21	0.12
Arg	1.57	0.16	0.09	3.28	1.10	0.64
Ammonia	0.52	0.11	0.07	1.19	0.09	0.05

primary effect is connected with potassium ion movement in mitochondria, although other organelles are not excluded. Of all three isolated fractions, only *F1* histone from nuclei displayed specific effects upon mitochondrial reactions. Furthermore, tissue specificity with respect to the histone action could be found. Detailed dynamic studies upon the mechanism of histone-produced potassium extrusion from mitochondria will be presented elsewhere.

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IZOLACJA I CHARAKTERYSTYKA BIAŁEK ZASADOWYCH (HISTONÓW) Z PODKOMÓRKOWYCH FRAKCJI MIĘŚNIA SERCOWEGO, NERKI I WĄTROBY

Streszczenie

1. Porównano ruchliwość elektroforetyczną, skład aminokwasowy i rozdział na kolumnie chromatograficznej białek rozpuszczalnych w kwasie sulfosalicylowym (SSA) ekstrahowanych z frakcji subkomórkowych tkanek wołu.

2. Z każdej frakcji subkomórkowej (cytoplazma, jądra, mitochondria) jedną frakcję białkową można było izolować przy pomocy ekstrakcji SSA oraz chromatografii na kolumnie z CM-celulozą. Białka te są identyczne w trzech różnych tkankach i mają charakter zasadowy.

3. Białko z jąder komórkowych ekstrahowane SSA odpowiada frakcji histonów *F1* bogatych w lizynę.

Received 1 December, 1971.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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INCORPORATION OF [^{14}C]PROLINE INTO PROTEINS OF RAT BLOOD SERUM, LIVER TISSUE AND AORTA

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1. After intraperitoneal administration of [^{14}C]proline, radioactive hydroxyproline, in addition to proline, was present in the proteins of studied tissues. 2. The increase in the radioactivity of hydroxyproline was most rapid in the serum euglobulin fraction. In the aorta it was much slower, especially in the insoluble protein fraction. 3. Comparison of specific activity of hydroxyproline present in serum proteins and connective tissue proteins seems to exclude involvement of "hypro-protein" in collagen metabolism.

The physiological role and properties of hydroxyproline-containing proteins (hypro-protein) of blood serum are not clear. Some authors (Frey, 1965; Sea, Neff & Block, 1968) suggested, on the basis of the immunological reaction of serum proteins with anti(soluble collagen) antibodies, that they are degradation products of collagen precursors or of mature forms of tissue collagen.

Taking into account the difficulties encountered in isolating from serum a homogeneous hypro-protein, Bańkowski, Gałasiński & Rogowski (1970) suggested that hydroxyproline is present in small amounts in various serum proteins.

Tomaszewski (1971) described a method for isolation and purification from human blood serum of high-molecular-weight fraction of hypro-protein, containing about 1.2% of hydroxyproline. The physico-chemical properties of this protein seemed to indicate some relation with the high-molecular-weight IgM immunoglobulins (Tomaszewski, in preparation).

Hydroxyproline-containing proteins have been found to occur also in blood sera of other species, e.g. in guinea pig, rabbit and rat (LeRoy, Kaplan, Udenfriend & Sjoerdsma, 1964). In rat and rabbit sera, similarly as in human serum, this protein is present in the euglobulin fraction (Tomaszewski, unpublished).

The conversion of proline to hydroxyproline present in collagen and elastin occurs after incorporation of proline into polypeptide chain (Udenfriend, 1966; Bentley & Hanson, 1969). The aim of the present work was to study the incorporation of [^{14}C]proline into blood serum and tissue proteins, and the formation of radioactive hydroxyproline.

MATERIALS AND METHODS

Wistar rats 2-months old (120 - 140 g) kept on a standard laboratory diet, were used. The animals were injected intraperitoneally with [U-^{14}C]proline (spec. act. 98 mCi/mmol; UVWR, Praha, Czechoslovakia) in physiological saline solution, in an amount corresponding to 10 μCi per 100 g body weight. The animals were killed by decapitation, four at a time, at different time intervals after the injection (from 1.5 to 24 h); blood was collected and liver and aorta were immediately isolated.

After blood clotting, serum was separated, and 2 ml of cold ethanol was added per 0.5 ml. The protein sediment was collected by centrifugation, washed three times with 80% ethanol, twice with ethyl ether, dried at room temperature, and submitted to hydrolysis. To isolate the euglobulin fraction containing high-molecular-weight hypro-proteins, 4 ml of serum was dialysed against 20 volumes of 1 mM-acetate buffer, pH 4.7. The formed sediment was centrifuged, washed three times with the same buffer, and hydrolysed. For determination of the total content of hydroxyproline and total radioactivity of [^{14}C]proline and [^{14}C]hydroxyproline in blood serum, 0.5 ml of serum was submitted to hydrolysis.

Liver tissue, 2 g, was homogenized in 20 ml of cold 10% trichloroacetic acid in Potter-Elvehjem all-glass homogenizer. The sediment was washed twice with 20 ml of 5% trichloroacetic acid, delipidated with acetone and ethyl ether, and dried to constant weight in air. From the obtained liver proteins, a 50 mg sample was hydrolysed.

The isolated aortas, after careful removal of the outer membrane, were washed with cold physiological saline solution, weighed and homogenized in cold 0.45 M-NaCl solution in a glass homogenizer. The proteins soluble in 0.45 M-NaCl, soluble in 0.5 M-acetic acid, and insoluble were separated as described by Heikkinen (1968). Due to the small amount of the available material, purification of the collagen fraction by precipitation with 10% NaCl was omitted. The obtained fractions were hydrolysed.

Analytical methods. Hydrolysis of all preparations was performed in 6 N-HCl, in sealed glass ampoules, for 16 h at 110°C. The hydrolysates were filtered through a 1 cm layer of a mixture of active charcoal with Dowex 50W X2, to remove humin (Prockop & Udenfriend, 1960). The adsorbent was washed twice with small volumes of 3 N-HCl. One part of the filtrate was neutralized with KOH, then hydroxyproline was determined by the method of Prockop & Udenfriend (1960) as modified by Tomaszewski & Hanzlik (1971), and total nitrogen according to Bürck (1960), the factor of 6.3 being taken to calculate the amount of protein.

The second part of the filtered hydrolysate was evaporated to dryness under vacuum. For radioactivity determinations, the sample was dissolved in a small volume of water, evaporated again and dissolved in a known volume of water. A part of the solution was applied to a disk of Whatman no. 3 paper, dried, and activity was measured. The second part was submitted to paper chromatography with butanol - acetic acid - water (4:1:1, by vol.) as solvent system. The spots of proline and hydroxyproline were located against standards stained with isatin, cut out,

and radioactivity was determined (Rubin, Kelmers & Goldstein, 1967) in USB-2 (IBJ, Poland) scintillation counter. The scintillation liquid contained 10 mg of POPOP (Koch-Light Lab., Colnbrook, Bucks., England) and 400 mg of PPO (Reanal, Budapest, Hungary) in 100 ml of redistilled toluene.

All determinations were made in duplicate. The activity of [^{14}C]proline and [^{14}C]hydroxyproline was expressed as counts per 1 ml of serum or 1 g of fresh tissue weight, per minute.

RESULTS

The content of hydroxyproline in the investigated proteins is presented in Table 1; in total serum proteins it was only 0.0126%, in the euglobulin fraction it was higher, 0.119%, and in liver proteins, 0.143%.

Table 1

Content of protein and protein-bound hydroxyproline in rat serum, liver and aorta

The results are mean values from 6 experiments, ±SD; they are expressed per 1 ml of serum or 1 g of fresh tissue weight.

	Protein (mg/g wet wt.)	Hydroxyproline (μg/g wet wt.)	Content of hydroxyproline in protein (%)
Serum, total proteins	59.30± 1.42*	7.40± 0.63*	0.0126±0.003
euglobulin fraction	1.80± 0.08*	2.14± 0.28*	0.119 ±0.004
Liver, total proteins	115.50±12.30	161.70±10.20	0.143 ±0.023
Aorta proteins			
soluble in 0.45 M-NaCl	17.50± 3.80	260.20± 22.04	1.495 ±0.168
soluble in 0.5 M-acetic acid	9.82± 0.66	127.80± 4.30	1.3 ±0.098
insoluble	191.00± 6.38	5815.0 ±426.0	3.0 ±0.143

* Per 1 ml of serum.

The protein of aorta contains a considerable percentage of collagen. Taking the factor 7.55 (13.2% of hydroxyproline in collagen) for calculation of the amount of collagen from the determined content of hydroxyproline (6.2 mg), the aorta of 8-week-old rat would contain about 47 mg of collagen per 1 g of wet weight. Collagen soluble in 0.45 M-NaCl corresponds to only 4% of this amount, and collagen soluble in 0.5 M-acetic acid, to 2%. It should be noted that the amount of hydroxyproline found in all aorta protein fractions was only 5.8% and not 13.2% as in purified collagen.

The activity of [^{14}C]proline and [^{14}C]hydroxyproline in serum taken from animals at different time intervals after administration of labelled proline, are presented in Table 2. In whole serum and in total serum proteins the ratio of hydroxyproline activity to proline activity was low. In the euglobulin fraction the radioactivity of proline per 1 mg of protein was practically the same as in total serum proteins, whereas the radioactivity of hydroxyproline was about 10 times higher and attained about 12% of the activity of incorporated [^{14}C]proline. In the euglobulin

Table 2

Radioactivity of proline and hydroxyproline in rat blood serum after intraperitoneal administration of [^{14}C]*proline*

	Time after injection (h)	Proline		Hydroxyproline			Activity (OH-Pro/Pro) ×100
		counts/min/ml	counts/min/mg protein	counts/min/ml	counts/min/mg protein	counts/min/μmol	
Whole serum	1.5	89 500		740		7 760	0.830
	3	83 200		1080		11 310	1.30
	6	78 300		1300		13 850	1.66
	12	63 000		950		11 620	1.50
	24	47 500		800		10 300	1.68
Total serum proteins	1.5	64 800	1120	740	13	11 530	1.13
	3	76 000	1300	1060	18	16 500	1.40
	6	72 400	1220	1280	22	22 000	1.78
	12	53 600	890	900	15	16 860	1.67
	24	45 500	760	750	13	15 850	1.65
Euglobulin fraction	1.5	2 760	1510	240	131	14 300	8.67
	3	3 220	1700	440	233	23 050	13.72
	6	2 800	1570	360	202	22 480	12.90
	12	2 600	1420	330	184	20 600	12.70
	24	1 450	880	180	108	13 100	12.30

fraction also the specific activity of hydroxyproline was higher. Three hours after the administration of [^{14}C]proline it was 23 000 counts/min/μmol as compared with 16 000 in total serum proteins.

The changes with time in percent distribution of [^{14}C]proline and [^{14}C]hydroxyproline in serum between free amino acids, those incorporated into proteins of euglobulin fraction, and the remaining serum proteins, are shown in Fig. 1. At variance with radioactive proline, after 1.5 h above 30% of the activity of the formed [^{14}C]hydroxyproline was present in the euglobulin fraction, and free [^{14}C]hydroxyproline appeared at the later hours of the experiment, probably as a result of degradation of newly-synthesized hydroxyproline-containing proteins.

In liver, the incorporation of labelled proline into proteins (Table 3) was the same as that observed with total serum proteins. The activity of hydroxyproline differed but little from the values obtained with serum. The only difference was observed in the specific activity which was much lower in liver proteins than in serum proteins, the maximum for liver being 3120 counts/min/μmol as compared with 22 000 for total serum proteins.

In aorta (Table 4) the content of hydroxyproline in the protein amounted only to 5.8% (see Table 1). Nevertheless it may be assumed that changes in the activity of hydroxyproline in aorta proteins reflect the metabolism of this scleroprotein. The amount of incorporated [^{14}C]proline was low. The activity was especially low in the insoluble protein fraction. On the other hand, as it appears from the ratio

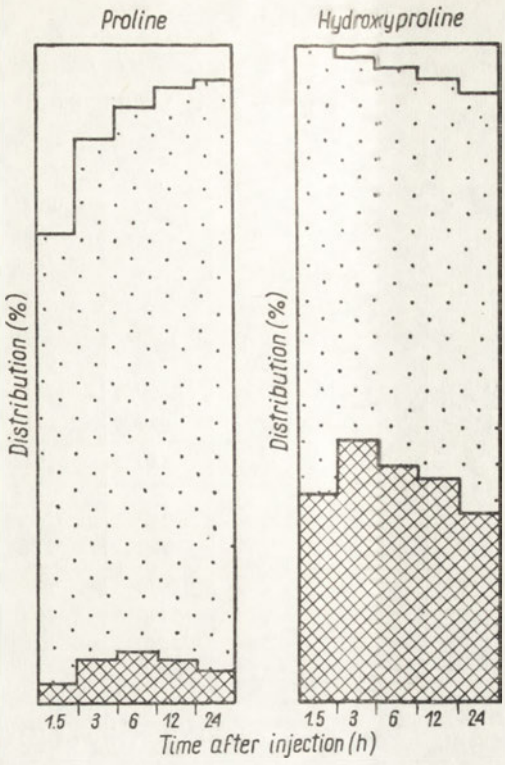

Fig. 1. Percentage distribution of [^{14}C]proline and [^{14}C]hydroxyproline in rat serum at different time intervals after intraperitoneal administration of labelled proline. Hatched area, activity in the euglobulin fraction; dotted area, activity in other serum proteins; outlined field, activity in the fraction of free amino acids and peptides.

Table 3

Radioactivity of proline and hydroxyproline in rat liver proteins after intraperitoneal administration of [^{14}C]*proline*

	Time after injection (h)	Proline		Hydroxyproline			Activity (OH-Pro/Pro) ×100
		counts/min/g fresh tissue	counts/min/mg protein	counts/min/g fresh tissue	counts/min/mg protein	counts/min/μmol	
Total liver protein	1.5	144 000	1270	1520	14	1230	1.06
	3	120 400	1150	2560	24	1990	2.12
	6	116 500	1130	3500	31	3120	3.00
	12	106 500	940	2300	20	1880	2.15
	24	96 200	730	1540	12	1170	1.60

of activities of [^{14}C]hydroxyproline and [^{14}C]proline, much more of the proline in this fraction became hydroxylated. After 24 h, the activity of hydroxyproline corresponded to 38% of the activity of proline.

Table 4

Radioactivity of proline and hydroxyproline in rat aorta proteins after intraperitoneal administration of [^{14}C]*proline*

Proteins soluble in	Time after injection (h)	Proline		Hydroxyproline			Activity (OH-Pro/ /Pro) ×100
		counts/ /min/g fresh tissue	counts/ min/mg protein	counts/ /min/g fresh tissue	counts/ /min/mg protein	counts/ /min/μmol	
0.45 M-NaCl	3	6 400	370	1030	63	570	16.0
	6	11 300	590	1860	98	890	16.6
	12	12 200	730	2100	125	1050	17.1
	24	6 300	360	1060	61	540	17.0
0.5 M-Acetic acid	3	2 100	220	180	19	190	8.8
	6	3 100	300	330	32	320	10.8
	12	3 400	360	380	39	400	11.0
	24	2 300	220	280	27	280	12.3
Insoluble	3	8 300	46	1800	10	43	21.5
	6	12 800	67	3740	20	88	29.2
	12	13 700	70	4340	22	98	31.4
	24	15 700	81	6020	31	135	38.2

The graphic representation of changes in the activity of [^{14}C]proline and [^{14}C]-hydroxyproline at different time intervals after intraperitoneal administration of labelled proline, is presented in Fig. 2. In whole serum, the activity of labelled proline was the highest at 1.5 h after the injection. In total serum proteins as well as in the euglobulin fraction, the maximum activity of proline was observed after 3 h. The activity of [^{14}C]hydroxyproline in whole serum and total serum proteins had a similar time course. The highest activities were observed 6 h after the injection. In the euglobulin fraction, the maximum of hydroxyproline activity appeared at 3 h and decreased somewhat more rapidly than in the total proteins.

In liver, where the protein metabolism is very high, the incorporation of labelled proline was the greatest at 1.5 h, but the maximum of hydroxyproline radioactivity was observed, similarly as in serum, 6 hours after administration of the label.

Connective tissue collagen is known to have a very low turn-over rate. The course of changes in the activity of [^{14}C]proline and [^{14}C]hydroxyproline in the proteins of aorta was consistent with the metabolism of connective tissue collagen (Jackson, 1957; Lindstedt & Prockop, 1961; Heikkinen, 1968). In the fraction soluble in 0.45 M-NaCl and in the acid-soluble fraction, the maximum activity of both amino acids was observed after 12 h. In the insoluble fraction both activities increased during the whole time of observation.

As it may be seen from the data presented in Tables 2, 3 and 4, the specific activity of hydroxyproline was the highest in serum euglobulins, 23 000 counts/min/μmol (after 3 h), and the lowest, 135 counts, in the insoluble proteins fraction of the aorta (after 24 h).

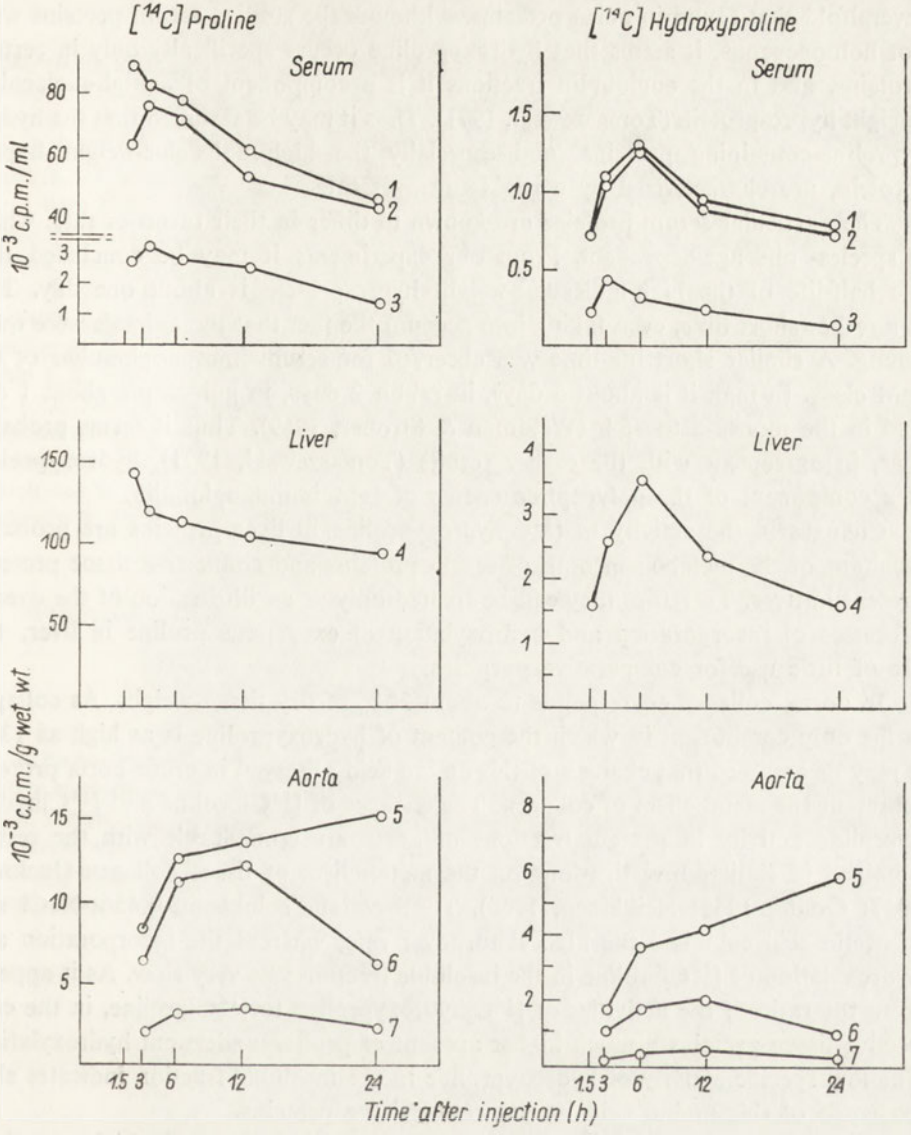

Fig. 2. Time-course of changes in the activity of [^{14}C]proline and [^{14}C]hydroxyproline in rat tissues after intraperitoneal administration of labelled proline. Serum: *1*, whole; *2*, total proteins; *3*, euglobulin fraction. Liver: *4*, total proteins. Aorta proteins: *5*, insoluble fraction; *6*, soluble in 0.45 M-NaCl; *7*, soluble in 0.5 M-acetic acid.

DISCUSSION

Significant differences were observed between serum proteins and connective tissue collagen in the rate of incorporation and hydroxylation of [^{14}C]proline. In serum proteins, especially in the euglobulin fraction, this process was much more rapid and the specific activity of hydroxyproline in serum proteins exceeded

severalfold that found in aorta proteins. Although the studied serum proteins were not homogeneous, it seems that hydroxyproline occurs specifically only in certain proteins, and in the euglobulin fractions it is a component of a high-molecular-weight hypro-protein (Tomaszewski, 1971). Thus it may be assumed that the hydroxyproline-containing proteins, and especially the high-molecular-weight hypro-protein, are characterized by a rapid turnover rate.

The particular serum proteins are known to differ in their turnover rate, which is species- and age-dependent. From our experiments it may be concluded that the half-life of the high-molecular-weight hypro-protein is about one day. This is a rather short time, even taking into account the fact that the animals were quite young. A similar short life-time was observed for serum immunoglobulins of the IgM class. In man it is about 5 days, in rabbit 2 days, in guinea pig about 1 day and in the mouse 5 to 15 h (Waldman & Strober, 1969). Thus it seems probable that, in agreement with the earlier results (Tomaszewski, 1971), hydroxyproline is a component of the polypeptide chains of IgM immunoglobulin.

Changes in the activity of [^{14}C]hydroxyproline in liver proteins are probably resultant of the metabolism both of serum proteins and connective tissue proteins present in liver. Therefore they can be treated only as an illustration of the overall processes of incorporation and hydroxylation of exogenous proline in liver, but are of little use for comparative purposes.

In aorta, collagen corresponds to about 30% of dry tissue weight. As collagen is the only component in which the content of hydroxyproline is as high as 13%, it may be assumed that changes of this amino acid observed in crude aorta proteins represent the metabolism of collagen. The changes of [^{14}C]proline and [^{14}C]hydroxyproline activity in protein fractions of aorta are comparable with the results obtained in similar investigations on the metabolism of tissue collagen (Jackson, 1957; Gould, 1968; Heikkinen, 1968). The fractions soluble in 0.45 M-NaCl and in acetic acid exhibited the highest turnover rate, whereas the incorporation and hydroxylation of [^{14}C]proline in the insoluble fraction was very slow. As it appears from the ratio of the activities of [^{14}C]hydroxyproline to [^{14}C]proline, in the connective tissue proteins a much higher amount of proline underwent hydroxylation. The low specific activity of hydroxyproline in the insoluble fraction indicates slow exchange of this amino acid in connective tissue proteins.

The fact that even in the newly synthesized soluble collagen the highest activity of [^{14}C]hydroxyproline appeared later than in serum proteins, and the specific activities were at least by an order of magnitude lower, seems to indicate that hypro-proteins are neither degradation products of mature collagen nor products of partial degradation of its precursors.

The authors wish to express their thanks to Prof. Dr. Tomasz Borkowski for the opportunity of performing the labelling experiments at the Department of Physiological Chemistry of the Medical School in Lublin, as well as for his interest and helpful discussion.

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WBUDOWYWANIE [^{14}C]PROLINY DO BIAŁEK SUROWICY KRWI, TKANKI WĄTROBOWEJ I ŚCIANY AORTY SZCZURA

Streszczenie

1. Po dootrzewnowym podaniu [^{14}C]proliny, w białkach badanych tkanek obok radioaktywnej proliny stwierdzono pojawianie się [^{14}C]hydroksyproliny.

2. Wzrost radioaktywności hydroksyproliny był najszybszy we frakcji euglobulinowej surowicy, a znacznie wolniejszy w białkach aorty, szczególnie we frakcji nierozpuszczalnej.

3. Porównanie aktywności właściwej [^{14}C]hydroksyproliny białek surowicy i białek tkanki łącznej wydaje się wykluczać udział hyproprotein w metabolizmie kolagenu.

Received 17 February, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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THE DEVELOPMENT OF HEPATIC 7-DEHYDROCHOLESTEROL REDUCTASE IN THE NEWBORN RAT

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1. 7-Dehydrocholesterol reductase activity in liver supernatant fractions from foetal and newborn rat was several times lower as compared with adult animal. 2. A sharp increase of the enzyme activity from the 19th day after birth was observed, adult values being achieved from the 22nd day of age onwards. This increase was completely arrested by actinomycin D, actinomycin K, puromycine and ethionine administered *in vivo* from the 19th to 22nd day of life. 3. Microsomal 7-dehydrocholesterol reductase from 18-day-old rat, in contrast to the enzyme from adult animal, was not activated by 105 000 *g* supernatant fraction of adult rat liver.

The final stage in hepatic biosynthesis of cholesterol is the Δ^7-reductase-catalysed conversion of 7-dehydrocholesterol (cholest-5,7-dien-3β-ol) into a 5-ene cholesterol system (Dempsey, 1965; Akhtar, Wilton & Munday, 1966; Dvornik, Kraml & Bagli, 1966; Wilton *et al.*, 1968). Some findings suggest age and tissue-dependent differences in the pattern of cholesterol precursors and in the activity of enzymic systems involved in the conversion of lanosterol to cholesterol (Fumagalli & Paoletti, 1963; Fumagalli, Niemiro & Paoletti, 1965). However, the role of 7-dehydrocholesterol as the immediate cholesterol precursor in newborn rat liver seems to be well documented (Fumagalli *et al.*, 1965). Although cholesterol synthesis from acetate in liver of foetal (Ballard & Hanson, 1967) and newborn rats (Givner & Dvornik, 1965) proceeds at a high rate, hepatic 7-dehydrocholesterol reductase activity in newborn rat has been found to be low (Wróbel & Niemiro, 1966).

The present paper reports on the developmental increase of hepatic 7-dehydrocholesterol reductase in the newborn rat; some of the factors controlling this increase were also investigated. Evidence is presented that the increased activity of the enzyme is related to synthesis of new protein.

MATERIALS AND METHODS

Reagents. DL-Ethionine, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were products of Koch-Light Lab. Ltd (Colnbrook, Bucks, England). NAD^+, $NADP^+$, NADH, NADPH were from C. F. Boehringer und Soehne GmbH (Mann-

heim, German Federal Republic). Puromycine dihydrochloride and 7-dehydrocholesterol were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Actinomycin K was from Instytut Antybiotyków (Warszawa, Poland). Actinomycin D was a generous gift from Merck, Sharp and Dohme Research Lab. (Rathway, N.J., U.S.A.) through the courtesy of Mr. W. B. Gall. All other reagents were analytical grade products, supplied by Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland).

Animals. Rats of both sexes of the Wistar albino strain were used for experiments. Birth dates of all litters were carefully recorded after daily inspection. For all the studies, litters of rats consisting of ten to 12 animals were taken. Age of foetuses was determined by establishing the onset of pregnancy from a fixed mating day. All experiments were started at the same time of day.

Preparation of homogenates and centrifugal fractions. The livers were excised as soon as possible after decapitation, and chilled on ice. All further steps were carried out at 0 - 2°C. In experiments with foetuses and young animals the livers from more than one rat were pooled. The tissue was homogenized by hand with 2 vol. of medium in a glass Potter-Elvehjem type homogenizer fitted with a loose teflon pestle. Three different media were used. The 1000 *g* supernatant fraction was obtained by homogenization of the tissue with Bucher's medium consisting of 0.1 M-phosphate buffer (pH 7.4), 30 mM-nicotinamide and 4 mM-$MgCl_2$ (Bucher & Mc Garrahan, 1956) and centrifugation for 10 min. The 13 000 *g* supernatant fraction was obtained by homogenization with the medium consisting of 0.03 M-nicotinamide - 0.1 M-tris-maleate buffer, pH 7.2 and centrifugation for 10 min. Microsomes and 105 000 *g* supernatant fraction were prepared from liver homogenate in 0.1 M-potassium phosphate buffer, pH 7.3, according to Dempsey (1969) except that the time of final centrifugation at 105 000 *g* was 45 min. For enzyme assays in the post-mitochondrial fraction, the upper half of the supernatant was taken.

7-Dehydrocholesterol reductase assay. The decrease of 7-dehydrocholesterol was measured by the spectrophotometric method of Kandutsch (1962). 7-Dehydrocholesterol (freshly crystallized from acetone-water) was emulsified in a glass homogenizer, by homogenizing 4.4 mg of the sterol in 0.2 ml of absolute ethanol with 1.8 ml of 5% human serum albumin in 0.9% NaCl solution. The emulsified 7-dehydrocholesterol was incubated in different media and under different conditions, depending on the fraction used. The 1000 *g* supernatant, equivalent to 0.2 g of fresh liver, was incubated with 0.56 µmol of 7-dehydrocholesterol in 0.8 ml total volume for 45 min. The 13 000 *g* supernatant, equivalent to 0.33 g of fresh liver, was incubated with 1.2 µmol of 7-dehydrocholesterol, 1 µmol of $NADP^+$, 16 µmol of glucose 6-phosphate and 0.15 unit of glucose 6-phosphate dehydrogenase in 1.5 ml total volume for 90 min. About 6 mg of microsomal protein and 24 mg of the 105 000 *g* supernatant protein were incubated with 1.2 µmol of 7-dehydrocholesterol, 1.0 µmol of NADP, 16 µmol of glucose 6-phosphate and 0.25 unit of glucose 6-phosphate dehydrogenase in 1.5 ml total volume for 90 min. All the incubations were carried out in 5 ml flasks in a shaker under a constant flow of oxygen-free nitrogen at 37°C. The incubations were stopped by the addition of 2 pellets of KOH and 2 ml of ethanol and saponification was carried out for 40 min at 70°C;

then the mixture was transferred to a test tube and shaken for 5 min with 10 ml of cyclohexane. After centrifugation at 400 *g* for 5 min, the concentration of 7-dehydrocholesterol in the cyclohexane layer at 281.5 nm was determined against a blank consisting of all components of the mixture except 7-dehydrocholesterol.

Treatment of animals with inhibitors of protein synthesis. Each experiment was performed on litter-mate young rats. Animals were divided into 3 - 4 groups by earmarking and left with their mothers. The rats of the first group were killed at the beginning of experiment to establish normal enzyme activity values for the litter. The second group remained as control and the animals received vehicule only. The rest of animals were injected intraperitoneally with solution of inhibitor of protein synthesis every day for 3 - 4 days, and were killed together with the control group on the day after the last injection. Actinomycin D, actinomycin K and ethionine were dissolved in 0.9% NaCl, puromycine was dissolved in 1.39% $NaHCO_3$. In general, the young animals tolerated these treatments well, and the few animals which did appear to be very sick were excluded from the experiments.

Analytical procedures. Total nitrogen was determined by the method of Kjeldahl with the Markham all-glass micro-apparatus. Protein was determined by the biuret method (Layne, 1957).

RESULTS

In preliminary experiments the activity of 7-dehydrocholesterol reductase during development of newborn rats was assayed in the 1000 *g* supernatant fraction (Fig. 1). Enzyme activity expressed on the basis both of wet weight of liver and nitrogen content in the extract, was low during the first 12 days after birth. Later the enzyme activity increased, and adult values were recorded from about the 23rd day ownwards.

It should be noted that the rate of 7-dehydrocholesterol conversion in the incubation system used in experiments with the 1000 *g* supernatant fraction was dependent only on the supply of endogenous NADPH. As reported by Niemiro & Fumagalli (1965), in adult rats the addition of NADPH was not essential in determination of hepatic enzyme activity in 1000 *g* supernatant fraction. It could be expected that formation of NADPH in newborn rat liver would be lower than in the adult one (Ballard & Hanson, 1967; Yeung & Oliver, 1967), and might affect the enzyme activity. Therefore, experiments with the 13 000 *g* supernatant fraction supplemented with NADPH-regenerating system were performed. The optimum conditions for enzyme assay were established in experiments on adult liver system. A broad optimum pH was between 6 and 7.3 (Fig. 2) and the optimum substrate concentration at 0.8 mM (Fig. 3). The rate of the reaction with 0.8 mM substrate was linear for at least 90 min, and increased linearly with the amount of the fraction. The effect of pyridine dinucleotides on the enzyme activity in the 13 000 *g* supernatant is shown in Table 1. $NADP^+$, NADPH and NADPH-regenerating system increased the activity about 5 times, NAD^+ and NADH being half as effective. Therefore in further experiments the NADPH-regenerating system was always introduced.

Figure 4 shows the age-dependent changes of hepatic 7-dehydrocholesterol reductase activity measured in the 13 000 *g* supernatant fraction and presented

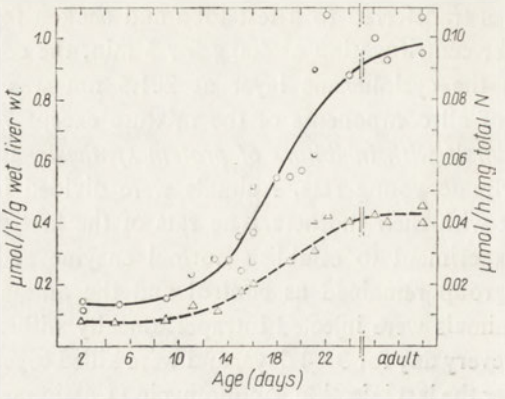

Fig. 1. Activity of 7-dehydrocholesterol reductase in the 1000 *g* supernatant fraction of rat liver, as a function of age. Each point represents one independent determination. The values are expressed on the basis of: ○, wet liver weight; △, total nitrogen content of the supernatant fraction. The incubation mixture, 0.8 ml, consisted of the 1000 *g* supernatant fraction equivalent to 0.2 g of wet liver and 0.56 µmol of 7-dehydrocholesterol.

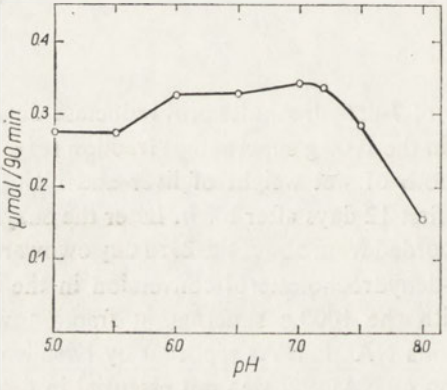

Fig. 2

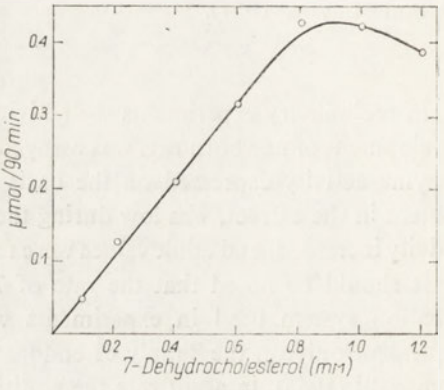

Fig. 3

Fig. 2. Effect of pH on 7-dehydrocholesterol reductase activity in adult rat liver. The composition of the incubation mixture was as described in the methods section, except that 2 µmol of NADPH was added instead of NADPH-regenerating system, and the mixture contained 100 µmol of tris buffer of appropriate pH.

Fig. 3. Effect of substrate concentration on 7-dehydrocholesterol reductase activity in the 13 000 *g* supernatant fraction from adult rat liver. Conditions as described in methods section, pH 7.2.

both on the basis of wet liver weight and on the basis of protein content. The enzyme activity was low in foetal and newborn rat liver and remained essentially unchanged during the first 18 days of postnatal life. Beginning with the 19th day, a sharp increase of 7-dehydrocholesterol reductase activity was observed, adult values being achieved from the 22nd day of age onwards.

To check whether in the liver of newborn rat an inhibitor of 7-dehydrocholesterol reductase activity is present, the 13 000 *g* supernatant fractions obtained

Table 1

Effect of pyridine dinucleotides and NADPH-regenerating system on the reduction of 7-dehydrocholesterol by adult rat liver

The incubation mixture consisted of 13 000 *g* supernatant fraction equivalent to 0.33 g of wet liver weight, 1.2 µmol of 7-dehydrocholesterol and the indicated additions in a total volume of 1.5 ml.

Additions	Reduction of 7-dehydrocholesterol (% of control)
None, control	100
G-6-P, 16 µmol G-6-P dehydrogenase, 0.15 unit $NADP^+$, 1 µmol	500
G-6-P, 16 µmol G-6-P dehydrogenase, 0.15 unit $NADP^+$, 2 µmol	500
NADPH, 1 µmol	500
$NADP^+$, 1 µmol	500
G-6-P, 16 µmol G-6-P dehydrogenase, 0.15 unit NAD^+, 2 µmol	250
NADH, 2 µmol	250
G-6-P, 16 µmol G-6-P dehydrogenase, 0.15 unit	100

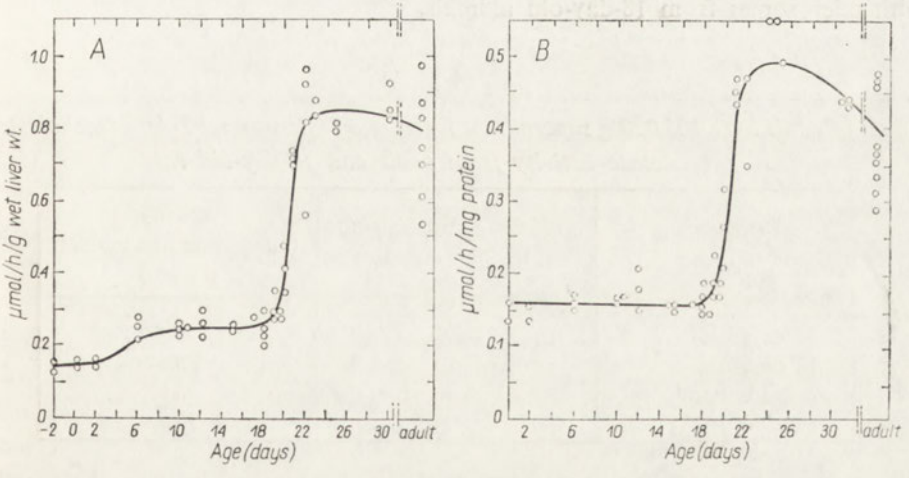

Fig. 4. Activity of 7-dehydrocholesterol reductase in the 13 000 *g* supernatant fraction of rat liver, as a function of age. Each point represents one independent determination. The values are expressed: *A*, per 1 g wet liver weight; *B*, per 1 mg of protein. The incubation mixture was as described in methods section (with NADPH-regenerating system).

from adult and 8-day-old animals were incubated together (Table 2). 7-Dehydrocholesterol reductase activities of mixed samples were always equal to the calculated sum of the activities of the component preparations within the limits of experimental error, and no activation or inhibition was observed.

Table 2

Activity of 7-dehydrocholesterol reductase of mixed 13 000 g *supernatant fractions from adult and 8-day-old rats*

Liver preparation from rats	Activity (μmol/h/g wet liver wt.)	
	found	calculated
Young	0.22	—
Adult	0.83	—
Young + adult, 1:1 (v/v)	0.56	0.52
Young + adult, 1:2 (v/v)	0.68	0.63
Young + adult, 3:2 (v/v)	0.47	0.46

As demonstrated by Kandutsch (1962), 7-dehydrocholesterol reductase is localized in the microsomal fraction, however, addition of the 105 000 *g* supernatant fraction enhanced the enzyme activity. Recently the presence of a specific sterol-activating protein responsible for this phenomenon was reported by Ritter & Dempsey (1970) and Scallen, Schuster & Dhar (1971). Therefore the effect of adult liver 105 000 *g* supernatant fraction on the microsomal 7-dehydrocholesterol reductase activity of 18-day-old animals was studied. As shown in Table 3, the addition of 105 000 *g* supernatant fraction increased about 6-fold the microsomal 7-dehydrocholesterol reductase activity of adult rat liver, but no activation was observed with microsomes from 18-day-old animals.

Table 3

Effect of adult liver 105 000 g *supernatant fraction on microsomal 7-dehydrocholesterol reductase activity from adult and 18-day-old rat*

Microsomes from rats	105 000 *g* supernatant fraction added	Activity (nmol/h/mg microsomal protein)
18 days old	None	5.3
18 days old	1 ml	5.3
Adult	None	5.8
Adult	1 ml	30.8

Agents known to inhibit protein synthesis as actinomycin D and K, puromycine and ethionine completely arrested the age-dependent increase of 7-dehydrocholesterol reductase activity when administered *in vivo* for 3 or 4 days beginning with

Table 4

Effect of actinomycins D and K, puromycine and ethionine on the developmental increase of 7-dehydrocholesterol reductase

Enzyme activity was determined in the 13 000 *g* supernatant fraction as described in Methods. Results of a typical experiment are presented.

Treatment	Daily dosis per g body weight	Age (days)	Activity (nmol/h/mg protein)	Increase of activity during development	Inhibition of increase of activity (%)
None, control		19	1.9	—	—
None, control		22	4.15	2.25	—
Actinomycin K, 3 days	0.15 μg	22	1.95	0.05	98
None, control		19	2.3	—	—
None, control		22	6.5	4.2	—
Actinomycin D, 3 days	0.16 μg	22	2.8	0.5	90
None, control		19	1.7	—	—
None, control		23	3.6	1.9	—
Puromycine, 4 days	0.11 mg	23	1.9	0.2	90
None, control		18	1.6	—	—
None, control		22	4.3	2.7	—
Ethionine, 4 days	0.5 mg	22	1.7	0.1	97

the 18th or 19th day of life (Table 4). The results obtained suggest that the developmental increase of 7-dehydrocholesterol reductase activity depends on the net synthesis of the enzyme protein.

DISCUSSION

Within the last few years considerable attention was paid to developmental changes in the activity of enzymes of metabolic pathways, e.g. those of glycolysis (Vernon & Walker, 1968), gluconeogenesis (Yeung & Oliver, 1967), urea cycle (Räihä & Suihkonen, 1968) and of the Krebs cycle (Hommes, Luit-De-Haan & Richters, 1971). No data are, however, available on the development of enzymes of cholesterol biosynthesis.

As demonstrated in the present work, hepatic activity of 7-dehydrocholesterol reductase, the enzyme catalysing in liver the final stage of cholesterol biosynthesis, is low in foetal and newborn rat until the 18th day of life with sharp increase to adult level between the 19th and 22nd day after birth. It could be expected that these age-dependent differences reflect either a temporary inhibition of enzyme activity, its developmental activation, or an increase in the amount of enzyme. No inhibitor was found to be present, as preparations from 8-day-old animals did not affect the activity of the adult enzyme. The microsomal enzyme from 18--day-old animals was not activated by the 105 000 *g* supernatant fraction from

adult rat (Table 3) which is known to contain a specific sterol-activating protein (Ritter & Dempsey, 1970; Scallen *et al.*, 1971). Actinomycins D and K, puromycine and ethionine, which inhibit protein synthesis, completely arrested the age-dependent increase of 7-dehydrocholesterol reductase activity when administered *in vivo* between the 19th and 22nd day of life. Thus it appears that the developmental increase in the enzyme activity may be due to enhanced synthesis of the microsomal enzyme. However, it should be noted that in the interpretation of experiments that employ inhibitors of protein synthesis as evidence that an increase in enzymic activity depends on synthesis of enzyme protein, it is always assumed that the rate of breakdown of the enzyme protein remains constant. Data obtained by Kuriyama, Omura, Siekevitz & Palade (1969) indicate that such an assumption is not always valid as some induction processes may be accompanied by marked changes in the rate of enzyme degradation. Although rates of 7-dehydrocholesterol reductase breakdown have not been measured, in view of the very low initial reductase activity it seems unlikely that the observed developmental increase of the activity could be due to a marked decrease in the rate of enzyme degradation.

The physiological significance of the observed developmental increase of enzyme activity is not clear. Previous evidence that no accumulation of 7-dehydrocholesterol occurs in newborn rat liver (Fumagalli *et al.*, 1965), indicates that the low activity of 7-dehydrocholesterol reductase in this period of development is not a rate-limiting step in cholesterol biosynthesis. It should be also noted that the increase of 7-dehydrocholesterol reductase at a comparatively late stage of development of the animal represents one of the final stages of liver maturation and coincides both with developmental increase of other enzymes involved in lipogenesis, as ATP--citrate lyase or NADP-malate dehydrogenase (Ballard & Hanson, 1967) and with the time at which rats begin to eat solid foods.

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AKTYWNOŚĆ REDUKTAZY 7-DEHYDROCHOLESTEROLU WĄTROBY SZCZURA W OKRESIE ROZWOJU

Streszczenie

1. U szczurów aktywność reduktazy 7-dehydrocholesterolu w supernatantach homogenatów wątroby płodów oraz noworodków była kilkakrotnie niższa niż aktywność u zwierząt dorosłych.

2. Pomiędzy 19-tym a 22-gim dniem życia następował gwałtowny wzrost aktywności enzymu do poziomu charakterystycznego dla osobników dorosłych. Podawanie *in vivo* aktynomycyny D, aktynomycyny K, puromycyny oraz etioniny od 19-go do 22-go dnia życia hamowało całkowicie zwiększanie aktywności enzymu.

3. Supernatant 105 000 *g* homogenatu wątroby dorosłego szczura, który aktywował mikrosomalną reduktazę 7-dehydrocholesterolu u szczurów dorosłych, nie miał wpływu na aktywność enzymu szczura 18-dniowego.

Received 2 March, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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RIBOFLAVIN CARRIER PROTEIN FROM EGG YOLK. SPECTRAL AND OTHER PROPERTIES OBSERVED UPON BINDING OF FLAVIN TO APOPROTEIN

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1. Apoprotein isolated from egg-yolk riboflavin flavoprotein bound riboflavin more strongly than FMN or FAD. Riboflavin flavoprotein underwent photoreduction in the presence of EDTA as electron donor to the semiquinone form, exhibiting electron spin resonance signal, which is characteristic of the flavin radical. 2. Riboflavin bound with apoprotein became more resistant to photolytic degradation. 3. The fluorescence spectra of apoprotein and flavoprotein showed that tryptophan residues were mainly responsible for fluorescence of apoprotein. Splitting off riboflavin exposed the tryptophan residues to the solvent and caused about twofold increase in intensity of fluorescence of apoprotein. 4. Analysis of ORD and CD spectra showed that conformational changes occurring on binding of apoprotein with various flavins were greater in the case of riboflavin than with FMN or FAD. Riboflavin flavoprotein possessed the highest content of ordered structure, and apoprotein about 30 - 50% lower content of α-structure. Conformational changes in the polypeptide chain during binding of riboflavin with apoprotein were confirmed also by immunochemical methods (C′ fixation test).

Most of the flavoproteins found in various organisms contain FMN or FAD as their prosthetic group. However, proteins specifically binding riboflavin have also been isolated from egg-white (Rhodes, Bennett & Feeney, 1959; Phillips, 1969; Clagett, 1971), egg yolk (Ostrowski, Skarżyński & Żak, 1962; Żak & Ostrowski, 1963; Ostrowski, Żak & Krawczyk, 1968) and chicken blood serum (Blum, 1967), and partially characterized. Due to the lack in the flavin component of phosphoric or adenylic acid, these flavoproteins are preferred in the study of the mechanism of interaction of the isoalloxazine ring with specific apoprotein.

The aim of this investigation was to study the interaction of riboflavin with apoprotein from egg yolk, by optical, immunological and other methods.

MATERIAL AND METHODS

Riboflavin flavoprotein was obtained from hen egg yolk by the method previously described (Ostrowski *et al.*, 1962; Żak & Ostrowski, 1963) which gives a preparation homogeneous on ultracentrifugation, free electrophoresis, disk electrophoresis and immunoelectrophoresis. As demonstrated by Ostrowski *et al.* (1968), the flavoprotein is a glycoprotein with molecular weight 36 000, composed of 297 amino acid residues and 21 residues of carbohydrates, combined with one molecule of riboflavin per molecule of protein.

The apoprotein was obtained by filtration of flavoprotein on a column with Sephadex G-25 at pH 3.0 (Ostrowski & Krawczyk, 1963) and after dialysis against 0.1 M-sodium phosphate buffer of pH 7.0 was stored in solution at 3°C. The capacity of apoprotein to bind riboflavin was determined by spectrofluorimetric titration (Ostrowski & Krawczyk, 1963).

Riboflavin was obtained from B.D.H. Ltd. (London, England). FMN and FAD were products of Sigma Biochem. Corp. (St. Louis, Mo., U.S.A.). Riboflavin monosulphate (FMS) was a gift of Dr. Kunio Yagi. EDTA was a product of Merck, Darmstadt, and dithionite of Schuchardt, Münich (West Germany). Sephadex G-25 was from Pharmacia (Uppsala, Sweden) and silica gel G from Wöhlm Co. (Eschwege, West Germany). The remaining reagents were analytic grade products of P.O.Ch. (Gliwice, Poland).

Photoreduction of riboflavin in flavoprotein. The photoreduction was carried out in spectrophotometric quartz cuvettes of 0.1-cm light-path, adapted to maintain anaerobic conditions. The solution of flavoprotein, 1 μmol in 1 ml of 0.1 M-phosphate buffer of pH 7.0 containing 0.15 M-EDTA, was placed in the cuvette, deaerated in vacuum, and washed with analytical pure nitrogen. This procedure was repeated four times, and then the solution was irradiated by a 1600 W xenon lamp from a distance of 50 cm for about 7 h at 0°C. Every 15 min the extinction at 570 nm was examined and when the maximum was obtained, usually after about 6 h of irradiation, the spectrum of the reduced flavoprotein was recorded with a recording spectrophotometer (Unicam SP-800).

Photolytic decomposition of riboflavin. Photolysis was carried out in Thunberg tubes in an atmosphere of nitrogen. The flavoprotein solution, 1.38×10^{-4} M in 0.1 M-borate buffer of pH 8.45 (10 ml), was irradiated with a 300 W mercury lamp from a distance of 33 cm for about 2 h. The photolysis products were separated by thin-layer chromatography on silica gel G in solvent systems: *n*-butanol - acetic acid - water (4:1:5, by vol.) and amyl alcohol - acetic acid - water (3:1:3, by vol.), (Treadwell, Cairns & Metzler, 1968). The spots were localized under an UV lamp (Hanau, Fluotest GmbH, type 5201, West Germany) and identified by spectral analysis.

Electron spin resonance measurements. ESR measurements were made on an X-Band spectrometer, Varian E-3 (Palo Alto, Calif., U.S.A.) at microwave frequency 9.5 GHz and frequency of modulation 100 kHz. For these determinations a $1.08 \times \times 10^{-4}$ M solution of flavoprotein in 0.1 M-sodium phosphate buffer of pH 7.0

was prepared, and measurements were made at room temperature in special flat quartz cuvettes with a light-path of 1 mm adapted to aqueous solution. During ESR analysis the flavoprotein solutions were reduced to semiquinone form by adding an equivalent amount of sodium dithionite.

Fluorescence. Excitation and emission fluorescence spectra were obtained in a spectrofluorimeter, Fluorispec SF-1 (Beard Atomic, Mass., U.S.A.). The measurements were made in quartz cuvettes with a light-path of 1 cm at 22°C. For these experiments, 2.2 to 3.0×10^{-6} M solutions of apoprotein in 0.1 M-sodium phosphate buffer of pH 7.0 and 1.4×10^{-4} M solutions of flavins in 0.1 M-phosphate buffer of pH 7.0 were used. Addition of equivalent amounts of flavin to apoprotein solution was accomplished by means of an "Agla" microburette (Burroughs Wellcome Co., London, England).

Optical rotation and circular dichroism. Optical rotatory dispersion and circular dichroism spectra were recorded on a Jasco Model ORD/UV-5 spectropolarimeter (Tokyo, Japan) with circular dichroism attachment. The sample compartment was maintained at 23.5°C, and 1.0-cm (320 - 500 nm) and 0.1-cm (190 - 320 nm) fused silica cells were used. The protein solutions (0.14 to 4.9 mg/ml) were prepared in 0.1 M-sodium phosphate buffer of pH 7.0.

Optical rotatory dispersion spectra for the estimation of Moffitt parameters a_0 and b_0 (Moffitt, 1956; Fasman, 1963) were recorded from 300 to 370 nm and calculated from the equation:

$$[m'](\lambda^2-\lambda_0^2)=a_0\lambda_0^2+\frac{b_0\lambda_0^4}{\lambda^2-\lambda_0^2}$$

where $\lambda_0=212$ nm.

Mean residue rotations $[m']$ were calculated as a function of wavelength:

$$[m']=\frac{\mathrm{MRW}}{100}\times\frac{3}{n^2+2}[a]_\lambda$$

where $[a]_\lambda$ is the specific rotation at a particular wavelength, MRW is the mean residue weight which was taken as 115 (Jirgensons, 1965), and n is the refractive index of the solvent, which was taken as 1.3344.

Residual ellipticities were calculated from circular dichroism measurements, taking $[\theta]=3300\,\Delta\mathrm{E}$ (Moscowitz, 1960) in the range of wavelengths from 205 to 500 nm:

$$[\theta]=\frac{3300}{l\times[c]}\times\Delta\mathrm{E}\times\frac{3}{n^2+2}$$

where ΔE is the circular dichroism observed, l is the cell light-path in cm, and $[c]$ is molar concentration of the analysed protein. Refractive index, n, of the solvent was not corrected, and was taken as 1.3344. The measurements were carried out at 28°C.

Complement fixation experiments. Antiserum against pure flavoprotein was prepared in rabbits as described previously (Weber, Żak & Ostrowski, 1966). The C′ in the antiserum was inactivated by heating at 56°C for 30 min. Reactivity of the

antiserum with flavo- and apoprotein and its titer were checked by the double diffusion test according to Ouchterlony (1948) and by immunoelectrophoresis (Grabar & Williams, 1955).

Binding of C′ complement and elaboration of the results were carried out by the routine technique in the modification of Levine (1967), running controls of antigen, antiserum and complement together with the sample. All the examinations and dilutions of antigens and antiserum were carried out in veronal-NaCl buffer of pH 7.4 containing Mg^{2+} and Ca^{2+} ions (Fulton & Dumbell, 1949).

RESULTS

Photoreduction of flavoprotein. As demonstrated previously (Żak, Steczko & Ostrowski, 1969), flavoprotein from egg yolk was reversibly reduced by dithionite and hydrosulphite, but not on the dropping electrode (Ostrowski & Krawczyk, 1963). Attempts to reduce it by irradiation under the conditions described by Massey & Palmer (1966) were unsuccessful. When, however, flavoprotein was irradiated with a light source of much higher intensity and for a longer time, the flavoprotein underwent reduction.

After about 7 hours of irradiation under anaerobic conditions at pH 7.0 in the presence of EDTA, the flavoprotein was reduced to the semiquinone form, as indicated by the appearance of absorption at 570 nm and a gradually decreasing shoulder in the region of 620 nm (Fig. 1) which are characteristic of the semiquinone form. About 70% of the flavoprotein was transformed to the semiquinone form, of brown colour.

The semiquinone form of flavoprotein obtained by photoreduction in the presence of EDTA or by the action of dithionite, in aqueous solution at pH 7.0 gave detectable signals of free radicals with g value of 2.003. An example of the ESR spectrum of half-reduced flavoprotein with sodium dithionite is shown in Fig. 2. The amplitude

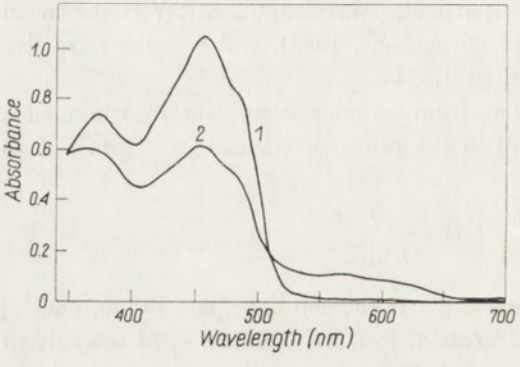

Fig. 1

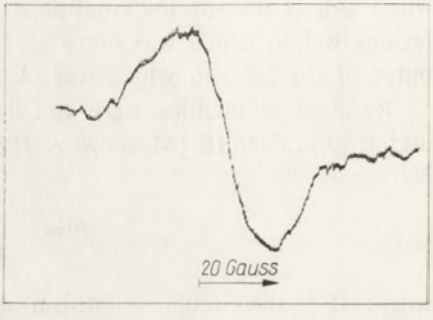

Fig. 2

Fig. 1. Absorption spectra of egg-yolk flavoprotein: *1*, before irradiation (quinone form) and *2*, after photoreduction in the presence of EDTA (semiquinone form).

Fig. 2. Electron spin resonance spectrum of riboflavin radical in half-reduced flavoprotein solution. The solution of protein was 1.08×10^{-4} M in 0.1 M-borate buffer, pH 7.0.

and shape of the signal resemble the signals of free radicals in the case of non-metalloflavoproteins with FMN (Ehrenberg & Ludwig, 1958) or FAD (Massey *et al.*, 1966) as prosthetic group. Reoxidation of the semiquinone form by bubbling oxygen through the solution led to the quinone form of flavoprotein with spectrum identical with the spectrum before reduction.

Photolytic degradation of the prosthetic group of flavoprotein. Free riboflavin irradiated under anaerobic conditions in the absence of an electron donor is converted to a number of derivatives, which were chromatographically separated and partly identified by Hais & Pećaková (1949) and Treadwell *et al.* (1968).

The protective effect of apoprotein on photolysis of riboflavin was studied by irradiating flavoprotein and free riboflavin under anaerobic conditions at pH 8.45, and separating the photolysis products by thin-layer chromatography.

Irradiation of free riboflavin for 30 min gave rise to a number of derivatives, of which seven or eight gave fluorescent spots on the chromatogram (Fig. 3). On

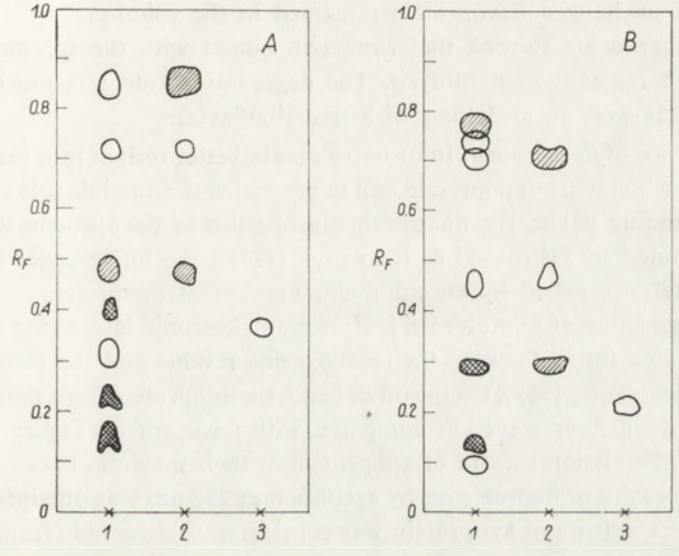

Fig. 3. Thin-layer chromatography of photolytic degradation products of *1*, free riboflavin and *2*, riboflavin-apoprotein complex. *3*, Non-irradiated riboflavin. 1.4×10^{-4} M-riboflavin and 1.38×10^{-4} M-flavoprotein were used, the irradiation time being, respectively, 30 min and 2 h. Solvents: *A*, *n*-butanol - acetic acid - water; *B*, amyl alcohol - acetic acid - water. The intensity of the fluorescence spots is shown by hatching.

the other hand, irradiation of flavoprotein even for two or more hours gave only three spots, which, judging from their R_F values and spectral properties, are probably flavin derivatives with modified side chains as a result of oxidation (Treadwell *et al.*, 1968). The spectrum of flavoprotein after photolysis exhibited a characteristic drop of extinction at 458 nm and increase at 320 nm (Fig. 4). The far-ultraviolet spectrum resembled that of denatured protein, due to photolytic decomposition of water in the presence of riboflavin (Holmström & Oster, 1961) and oxidation

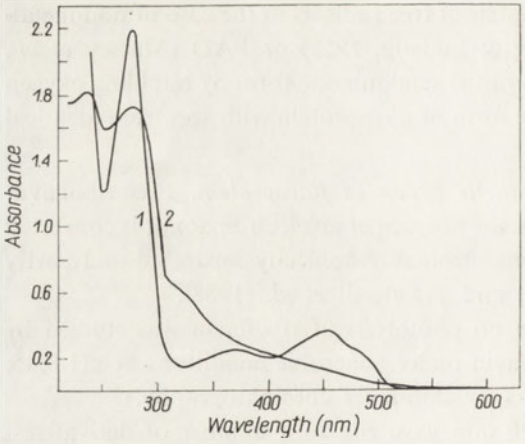

Fig. 4. Absorption spectra of *1*, native flavoprotein, and *2*, flavoprotein modified by the photolytic process. The spectra were measured in 0.1 M-borate buffer, pH 8.45, after 2 h irradiation. 1.08×10^{-4} M-solution of flavoprotein was used.

of aromatic amino acid residues (Penzer & Radda, 1968). After irradiation for 2 h, 40% of unchanged flavoprotein remained in the solution.

These experiments showed that riboflavin bound with the specific apoprotein became more resistant to photolysis. The degradation rate was much lower and fewer products were formed than with free riboflavin.

Fluorescence of flavoprotein. In order to obtain better insight into the mechanism of binding of riboflavin by apoprotein and to determine the possible role of apoprotein groups in binding flavin, the fluorescence properties of flavoprotein were studied. As demonstrated by Ostrowski & Krawczyk (1963), the fluorescence of riboflavin was completely quenched by an equimolar amount of apoprotein.

Rapid quenching of fluorescence indicates an electronic interaction and suggests multiple binding forces between the isoalloxazine residue and the protein (Rhodes *et al.*, 1959; Swoboda, 1969). On the other hand, the apoprotein itself shows a strongly enhanced protein fluorescence as compared with flavoprotein. Figure 5 shows the excitation and emission spectra of apoprotein at their maxima. It can be seen that maximum intensity of fluorescence by excitation at 292 nm was obtained at 366 nm. The emission spectrum of apoprotein was considered to arise only from tryptophan

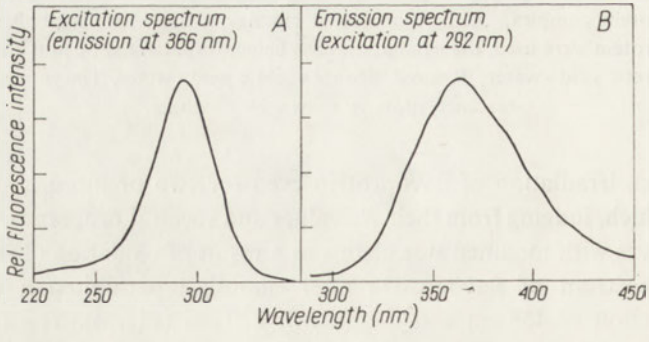

Fig. 5. Fluorescence excitation and emission spectra of apoprotein in 0.05 M-Na-phosphate buffer, pH 7.0. Protein concentration 2.2×10^{-6} M.

residues because the same spectrum was observed when an equimolar sample of tryptophan was excited under the same conditions (Fig. 6); the shape and intensity of the fluorescence band of apoprotein was very close to that of free tryptophan in 0.1 M-phosphate buffer solution of pH 7.0. This is in agreement with the observations of other authors (Teale, 1960; Bridges & Williams, 1968) that at pH 7.0 protein fluorescence is mainly due to excitation of tryptophan residues and not of tyrosine, which under these conditions plays a small role (Teale, 1960).

Addition of an equimolar amount of riboflavin to apoprotein quenched fluorescence of the protein. In the emission spectrum of flavoprotein at 366 nm, only residual fluorescence remained and an additional small peak at 540 nm appeared (Fig. 7), the latter being caused by riboflavin as a result of weak dissociation of

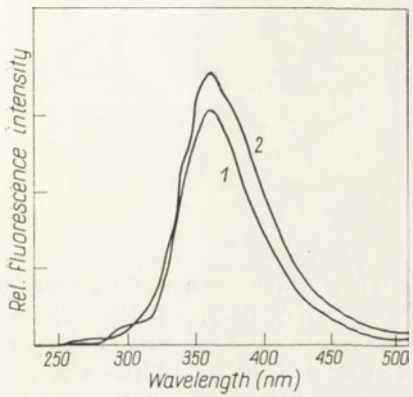

Fig. 6

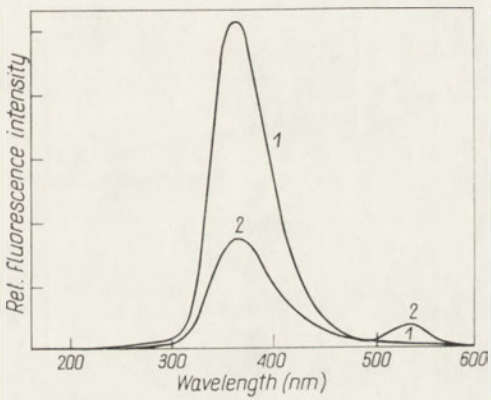

Fig. 7

Fig. 6. Fluorescence emission spectra of *1*, apoprotein and *2*, tryptophan. Apoprotein concentration 2.2×10^{-6} M, tryptophan concentration 1.8×10^{-5} M. Excitation at 292 nm.

Fig. 7. Fluorescence emission spectra in 0.05 M-Na-phosphate buffer, pH 7.0 of *1*, apoprotein alone and *2*, after addition of riboflavin at an equimolar amount. Protein concentration 1.3×10^{-7} M. Excitation at 280 nm.

the complex at pH 7.0 (Ostrowski & Krawczyk, 1963). If, instead of riboflavin, an equimolar amount of FMN or FAD was added, quenching of protein fluorescence was much smaller. The quenching of apoprotein fluorescence in the presence of different flavins is shown in Fig. 8. The average decrease in intensity of apoprotein fluorescence at equimolar concentrations of riboflavin, FMN and FAD was 65, 20 and 15%, respectively.

These results agree with the values of dissociation constants of complexes with riboflavin and its derivatives, indicating that riboflavin is the most tightly-bound flavin for apoprotein from egg yolk. Dissociation constants (Table 1) determined by the spectrofluorimetric method (Ostrowski & Krawczyk, 1963) show a hundredfold higher affinity of apoprotein for riboflavin compared with FMN and FMS, and one thousandfold higher compared with FAD.

It follows from these experiments that fluorescence of the apoprotein is caused mainly by tryptophan, of which 8 residues are present in protein molecule (Ostrowski

Table 1

Dissociation constants of riboflavin and flavin nucleotide complexes with egg-yolk apoprotein

The fraction of free flavin was determined by spectrofluorimetric method in 0.05 M-Na-phosphate buffer, pH 7.0, at 20°C. Figures represent mean values of three determinations.

Flavin	Dissociation constant
Riboflavin	2.6×10^{-9} M
FMN	6.3×10^{-7} M
FMS	5.6×10^{-7} M
FAD	2.5×10^{-6} M

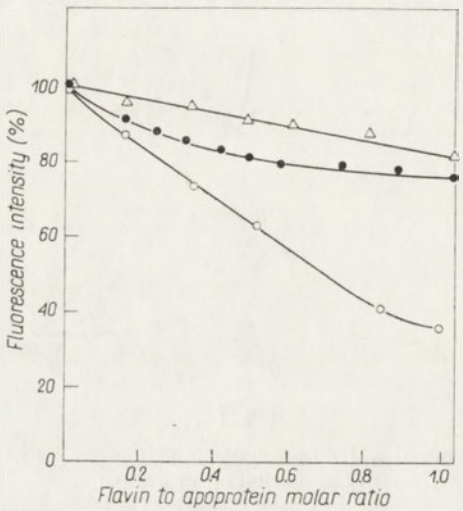

Fig. 8. Effect of the amount of ○, riboflavin; ●, FMN; and △, FAD, on the fluorescence intensity of apoprotein. Apoprotein at concentration 1.4×10^{-6} M was titrated with the appropriate flavin solutions. All measurements were carried out in 0.1 M-Na-phosphate buffer, pH 7.0, at 22°C. Excitation at 292 nm.

et al., 1968). Tryptophan in flavoprotein is buried and probably takes part in hydrophobic interactions of the polypeptide chain. It may also be involved in binding of riboflavin with apoprotein in view of the low intensity of fluorescence of the complex.

Optical rotatory dispersion of flavoprotein complexes. ORD spectra of the apoprotein and its complexes with riboflavin, FMN and FAD were measured in the range from 195 to 550 nm. In Table 2 the characteristic data of the spectra in the range of peptide transitions are summarized, as well as the values of Moffitt constants b_0 and a_0. Values of α-helix content of the apoprotein and flavin complexes calculated from the amplitude of the Cotton effect and from the values of b_0 are also included.

The ORD spectra showed that the riboflavin flavoprotein had in the far-ultraviolet wavelength range a Cotton effect with a maximum at 197 nm, and a minimum at 228 nm (Fig. 9); additional Cotton effects can be seen in the aromatic range: one centered at 295 nm with a maximum at 292 nm and a minimum at 298 nm (Fig. 10) and another one in the range of 350 to 520 nm (Fig. 11). The amplitudes

Table 2

Optical rotatory dispersion properties of apoprotein and its complexes with flavins in the wavelength range 195 and 370 nm

The $[m']$ values for 100% α-helix in synthetic polypeptides are in the region of minimum Cotton effect $-12\,700°$ and for maximum Cotton effect $+80\,000°$ (Fasman, 1963).

Protein	Moffitt parameters		Values for minimum Cotton effect			Values for maximum Cotton effect		
	b_0	a_0	λ	$[m']$	α-helix (%)	λ	$[m']$	α-helix (%)
Apoprotein	−188	−283	235	−3061	24	200	+15000	18
Riboflavin--apoprotein	−337	−185	228	−3755	30	197	+24600	30
FMN-apoprotein	−228	−248	230	−3037	24	197	+17240	21
FAD-apoprotein	−238	−238	233	−3050	24	198	+20170	25

of all these Cotton effects were the highest in the case of riboflavin flavoprotein as compared with the amplitudes of the complexes of apoprotein with FMN and FAD.

The apoprotein did not show any Cotton effects other than those in the range of peptide transitions with a maximum at 200 nm and the minimum at 235 nm (Table 2, Figs. 9, 10 and 11). The α-helix content calculated from the amplitude of the Cotton effects was the lowest for the apoprotein, and the highest for the

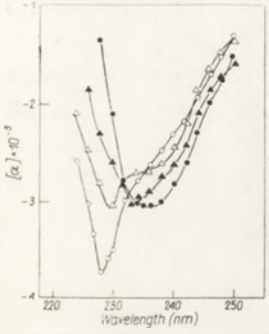

Fig. 9

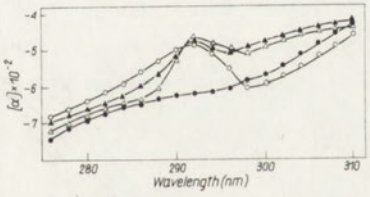

Fig. 10

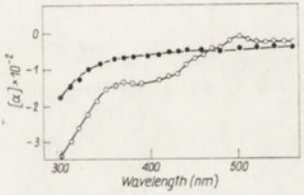

Fig. 11

Fig. 9. Far-ultraviolet optical rotatory dispersion spectra of ●, apoprotein, and of apoprotein complexes with ○, riboflavin; △, FMN and ▲, FAD. Protein concentrations were 0.14 mg/ml in 0.1 M-Na-phosphate buffer, pH 7.0.

Fig. 10. Near-ultraviolet optical rotatory dispersion spectra of ●, apoprotein, and of apoprotein complexes with ○, riboflavin; △, FMN; and ▲, FAD. Protein concentrations were 4.9 mg/ml in 0.1 M-Na-phosphate buffer, pH 7.0.

Fig. 11. Visible optical rotatory dispersion spectra of ●, apoprotein, and ○, riboflavin flavoprotein. Protein concentrations were 4.9 mg/ml in 0.1 M-Na-phosphate buffer, pH 7.0

riboflavin flavoprotein (Table 2). From the slope of Moffitt's plots in Fig. 12 it can be also seen that the riboflavin flavoprotein complex has the highest content of α-structure. It should be pointed out that the percentages of the α-helix structure for all studied complexes calculated from b_0 values are higher than those obtained from Cotton effects. This is probably due to the fact that the wavelength range in which the Moffitt analysis was performed is influenced by the optical activity of the flavin moiety.

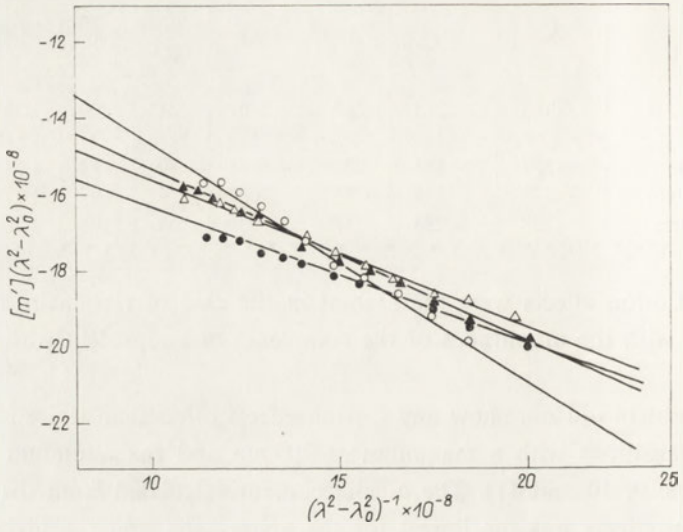

Fig. 12. Moffitt plots for ●, apoprotein, and apoprotein complexes with ○, riboflavin; △, FMN; and ▲, FAD at λ_0=212 nm.

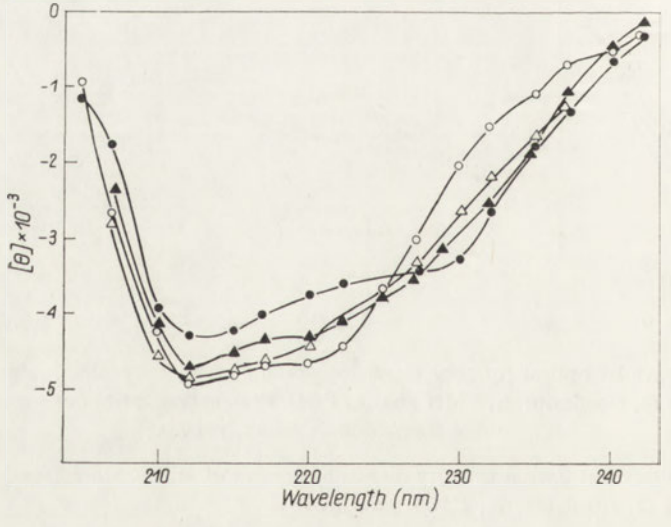

Fig. 13. Far-ultraviolet circular dichroism spectra of ●, apoprotein, and of apoprotein complexes with ○, riboflavin; △, FMN; and ▲, FAD. Protein concentrations were 4.73 mg/ml in 0.1 M-Na-phosphate buffer, pH 7.0.

Circular dichroism of flavoprotein complexes. Circular dichroism measurements showed differences in the ellipticities between the apoprotein and the flavin complexes in the range of peptide chromophores (200 - 250 nm), aromatic amino acid residues (240 - 300 nm) and flavin group (300 - 500 nm). Figure 13 shows the residual ellipticities of apoprotein and flavin complexes in the range of 200 to 240 nm. All the spectra exhibited minima at 212 nm and had a shoulder at 220 - 223 nm, which for the apoprotein was shifted to higher wavelengths.

In Table 3 the characteristic values of residual ellipticities are summarized, and the percentages of α-helix content calculated from the ellipticities in the range

Table 3

Circular dichroism properties of apoprotein and its complexes with flavins in the wavelength range of 200 to 250 nm

Values of θ are in degrees $\times$ cm^2 $\times$ decimol^{-1}. The ellipticity value for 100% α-helix in synthetic polypeptides θ_{223} is $-39\,000$ (Carver, Shechtel & Blout, 1966).

Protein	Minimum ellipticity (nm)	θ_{212}	Shoulder (nm)	$\theta_{220-229}$	α-helix (%)
Apoprotein	212	−4300	229	−3400	8
Riboflavin-apoprotein	212	−4950	220	−4700	13
FMN-apoprotein	212	−4850	220	−4400	12
FAD-apoprotein	212	−4700	220	−4300	11

of 220 to 223 nm. These values are definitely lower than those calculated from the ORD data (Table 2). This observation will be discussed later.

Figure 14 shows residual ellipticities in aromatic region (250 - 300 nm). The apoprotein does not exhibit any extrema in this region, whereas the riboflavin flavoprotein showed two negative bands at 294 and 272 nm, and a positive band

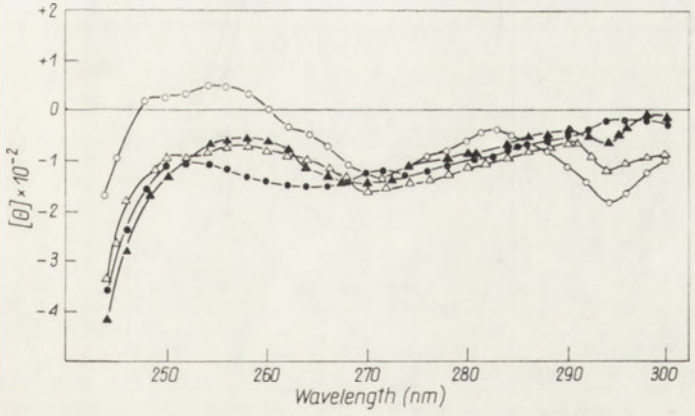

Fig. 14. Near-ultraviolet circular dichroism spectra of ●, apoprotein, and of apoprotein complexes with: ○, riboflavin; △, FMN; and ▲, FAD. Protein concentrations were 4.73 mg/ml in 0.1 M-Na-phosphate buffer, pH 7.0.

at 255 nm. Complexes with FMN and FAD showed negative ellipticities only at 294 nm.

All three studied flavoproteins exhibit right circularly-polarized components centered at 340 and 455 nm, and a negative absorption band at 375 nm but with decreasing intensity for 340 and 455 nm in the order: complex with riboflavin, with FMN and with FAD (Fig. 15). The visible CD spectrum of riboflavin flavoprotein corresponds to the absorption spectrum (Fig. 16A, B) and the positive band at 455 nm has almost the same shape with a shoulder at about 480 nm.

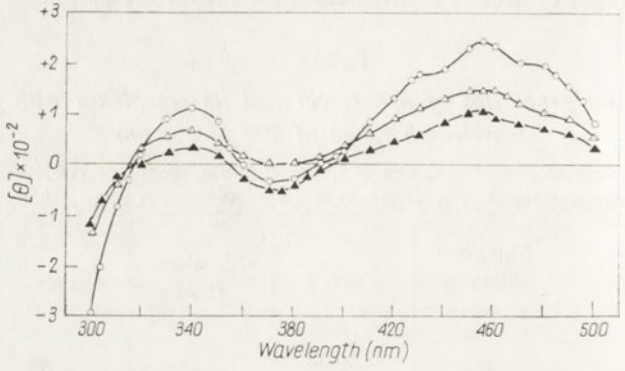

Fig. 15. Visible circular dichroism spectra of apoprotein complexes with ○, riboflavin; △, FMN; and ▲, FAD. Protein concentrations were 2.45 mg/ml in 0.1 M-Na-phosphate buffer, pH 7.0.

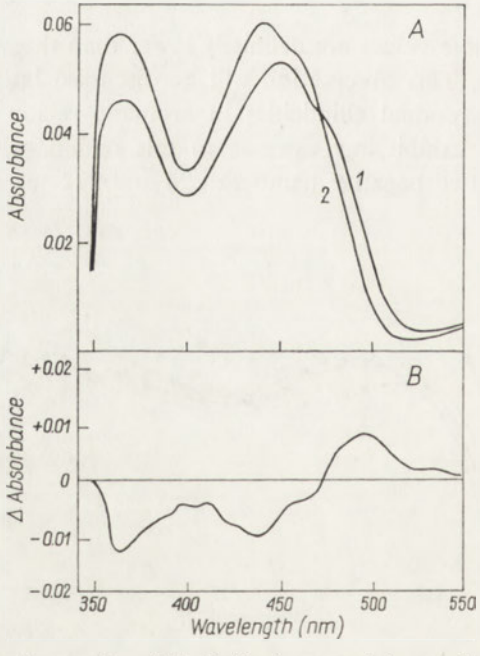

Fig. 16. *A*, Visible absorption spectra of *1*, riboflavin-apoprotein complex and *2*, free riboflavin. *B*, Difference spectrum (absorbance of flavoprotein minus that of riboflavin). Protein and riboflavin concentrations were 1.8×10^{-7} M in 0.1 M-Na-phosphate buffer, pH 7.0.

Immunological reactivity of flavo- and apoprotein. It was previously shown (Żak, Weber & Ostrowski, 1968) that flavoprotein and apoprotein are indistinguishable in the precipitation test. However, conformational changes in the apoprotein after splitting off riboflavin could be expected to become apparent in the complement fixation test.

The data presented in Fig. 17 illustrate the different serological activity of riboflavin flavoprotein and apoprotein with antibodies against native flavoprotein.

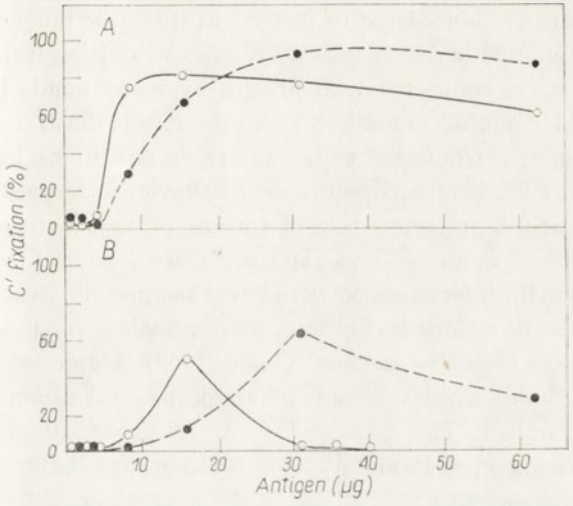

Fig. 17. C′-fixation curves for ●, apoprotein and ○, riboflavin-apoprotein complex. Antiserum against riboflavin flavoprotein was diluted *A*, 1:512, and *B*, 1:2048.

C′-fixation assays were carried out at two different dilutions of antiserum, 1:512 and 1:2048. In both cases apoprotein showed different C′-fixation curves than flavoprotein, with characteristic lateral shift similar to that observed for proteins with unfolded polypeptide chain (Levine, 1967; Heidelberger, 1967). These results are consistent with the change in conformation upon splitting off riboflavin from flavoprotein shown by the ORD and CD measurements.

DISCUSSION

The riboflavin-binding protein is unique in its role as carrier and storage protein for vitamin B_2, probably in all egg-laying species (Clagett, 1971). Absence of this protein in recessive birds leads to riboflavinuria accompanied by production of eggs deficient in riboflavin (Blum, 1967). Synthesis of riboflavin-binding protein can be induced by treating animals with estradiol (Blum, 1967). The present work was aimed at studying in more detail the properties and structure of riboflavin-binding protein.

Our previous experiments (Żak *et al.*, 1969) showed that riboflavin in egg-yolk flavoprotein may occur in three different forms: oxidized, semiquinone, and in the

fully reduced state. In this study, photoreduction of flavoprotein in the presence of EDTA has confirmed this property. Moreover, it was demonstrated that riboflavin may occur in the complex in radical form (Fig. 2), which indicates that riboflavin--binding protein stabilizes the radical form of flavin, similarly as in other flavin complexes (Hemmerich, 1960; Hemmerich, Veeger & Wood, 1965; Müller *et al.*, 1970; Hemmerich, Nagelschneider & Veeger, 1970).

As shown by Hais & Pećaková (1949) and Treadwell *et al.* (1968), photolysis of free riboflavin leads to a great number of photoproducts, most of which are still unidentified. It was considered of interest to study the influence of apoprotein on the course of photolytic degradation of riboflavin, starting from the assumption that the flavin ring is connected with protein by many bonds (Swoboda, 1969), which should hinder photodegradation. From the results illustrated in Fig. 3 it can be seen that riboflavin irradiated under anaerobic conditions in the presence of apoprotein at pH 8.45, compared with free riboflavin, undergoes only limited degradation, indicating a protective role of specific protein in this process.

The fluorescence spectra of flavo- and apoprotein point to a significant role of indole residues in the interaction between flavin and protein. In solutions of neutral pH, the role of tyrosine residues in fluorescence of protein is small, while fluorescence of indole derivatives is nearly maximal (Teale, 1960). Moreover, our experiments showed that the fluorescence spectrum of apoprotein corresponds closely to the spectrum of tryptophan. The average loss of apoprotein fluorescence intensity after binding of riboflavin, is about 65% (Fig. 7), indicating that tryptophan residues in contact with the solvent are involved in the interaction of flavin with protein. This suggestion is supported by the results of Farrell *et al.* (1969) on the role of tryptophan residues in the binding site of riboflavin flavoprotein from hen egg-white.

The fluorescence experiments also show that riboflavin is bound more strongly than FMN, FAD or FMS and that riboflavin is the most specific prosthetic group for egg-yolk apoprotein, as shown by the dissociation constant values (cf. Table 1). This property is similar to that of other flavoproteins, which usually bind strongly only one type of flavin, e.g. the "old yellow enzyme" of Warburg, which binds FMN many times more strongly than FAD or riboflavin (Nygaard & Theorell, 1955).

The differences between ORD and CD spectra of the apoprotein in the short ultraviolet range, and those of the native flavoprotein, indicate a distinct conformation change in the protein molecule on removal of the flavin. This change seems to be fully reversible, because on recombination of these components the ORD and CD spectra become identical with those of the native complex.

The α-helix content calculated from ORD data is for the apoprotein about 30% lower than for the riboflavin flavoprotein. The percentage of α-helix calculated from CD data were for both proteins about 50% lower than those calculated from ORD. A possible explanation of this fact may be a lower optical activity of peptide chromophores in short segments of α-helix (Perutz, Kendrew & Watson, 1965), as well as the participation of other, non-peptide chromophores in shaping the spectra (Perutz *et al.*, 1968). There is also a possibility that the method of calculation

of the α-helix content, based on the data for synthetic polypeptides, is not quite adequate for complex proteins (cf. Rupley & Gates, 1968; Rosenkrantz & Scholtan, 1971). Nevertheless, it can be concluded that the presence of the flavin stabilizes the helical structure in the flavoprotein molecule. ORD data indicate that, on binding of the flavin with the apoprotein, distinct changes in the conformation of the peptide chain take place. The additional Cotton effect at 292 - 298 nm, most pronounced for the riboflavin flavoprotein, suggests the involvement of aromatic amino acid residues — most probably tryptophan — in the binding of isoalloxazine ring with the protein (Glazer & Smith, 1961; Fujioka & Imahori, 1962). The shift of the minimum of the Cotton effect from 235 nm to 228 nm can be explained by the influence of those residues on the region of peptide transitions (Rosenberg, 1966).

The CD spectra in the region of aromatic transitions also indicate a role of tryptophan in the interaction of flavin with apoprotein. Several authors have found (Omenn, Cuatrecases & Anfinsen, 1969; Strickland, Horwitz & Billups, 1969; Teichberg, Kay & Sharon, 1970) that the band of negative ellipticity in the 294 nm region is connected with indole transition. The riboflavin flavoprotein has the highest value of negative ellipticity at 294 nm (Fig. 14) as compared with apoprotein and complexes with FMN and FAD. On the other hand, there are practically no differences in optical activity between apoprotein and flavoprotein in the 270 - 280 nm band, indicating that tyrosine residues play a small part in the direct binding of flavin with apoprotein (Hashizume, Shiraki & Imahori, 1967). The positive ellipticity in 250 - 260 nm band suggests participation of disulphide bridges in stabilization of the flavin-protein complex, analogous to that observed for the lysozyme (Beychok, 1965).

The CD and absorption spectra at longer wavelengths suggest that the isoalloxazine ring is buried within the polypeptide chain, probably in the hydrophobic region of the apoprotein molecule (Edmondson & Tollin, 1971).

Immunological studies provided additional information about the conformational changes in protein upon splitting off flavin. Rabbits immunized with flavoprotein produced antibodies which reacted with the homologous antigen, and also cross-reacted with apoprotein, as shown by the precipitin reaction (Weber *et al.*, 1966) and C′-fixation test in our experiments. The C′-fixing ability of flavoprotein and apoprotein are different, as can be seen by the lateral shift of the fixation curve for apoprotein and the necessity of using higher concentrations of antigen to obtain maximum fixation. This type of changes in immunologic reactions unequivocally indicates essential conformational changes in polypeptides (Levine, 1967) and suggests unfolding of protein structure (Heidelberger, 1967).

This work was partially supported by a grant from the Committee of Science and Technology, Warsaw. The authors wish to thank Miss A. Bulsiewicz for the help in C′-fixation experiments and mgr B. Panczenko for fluorescence measurements.

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RYBOFLAWINOWY FLAWOPROTEID ŻÓŁTKA JAJA. WŁASNOŚCI WIDMOWE I ZMIANY KONFORMACYJNE BIAŁKA PODCZAS WIĄZANIA FLAWIN Z APOPROTEIDEM

Streszczenie

1. Apoproteid ryboflawinowego flawoproteidu żółtka jaja najsilniej wiąże ryboflawinę, a następnie FMN i FAD. Podczas fotoredukcji ryboflawinowego flawoproteidu w obecności EDTA jako donatora elektronów, ryboflawina występuje w postaci semichinonowej i wykazuje charakterystyczny dla formy rodnikowej flawin sygnał rezonansu elektronowego.

2. Ryboflawina w postaci kompleksu ze swoistym apoproteidem staje się bardziej odporna na fotolityczną degradację.

3. Odszczepianie ryboflawiny z flawoproteidu powoduje około dwukrotny wzrost intensywności fluorescencji białka kompleksu. Z modelowych badań wynika, że reszty tryptofanowe są głównie odpowiedzialne za fluorescencję apoproteidu.

4. Za pomocą widma ORD i CD oraz przez badanie wiązania dopełniacza wykazano, że podczas interakcji flawiny z apoproteidem zachodzą istotne zmiany konformacji białka, najsilniej zaznaczone podczas wiązania ryboflawiny. Zawartość α-struktury ryboflawinowego flawoproteidu jest o 30 - 50% wyższa niż wolnego apoproteidu.

Received 2 March, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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MECHANISM OF UREA DECOMPOSITION BY UREASE-LESS *TORULOPSIS UTILIS*

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It has been found that urea is metabolized by the urease-less *Torulopsis utilis via* allophanate: 67% of radioactivity from labelled urea was found in CO_2 and 33% in allophanate. The evidence suggests that the process is mediated by two separate enzymes.

The yeast *Torulopsis utilis* is an urease-less organism which can grow on urea as the sole nitrogen source. One may assume therefore that an enzymic system other than urease is responsible for urea metabolism. Roon & Levenberg (1968) found that in *Candida utilis* urea is decomposed in the presence of ATP to CO_2 and NH_3, and recently they proved (1970) that this transformation is a two-step process involving an acid-labile allophanate (carboxylurea) as an intermediate. Both enzymic functions have been ascribed by these authors to the same enzymic protein — ATP : urea amidolyase (ADP) (UALase). The same type of reaction was also observed by Whittney & Cooper (1970) in the urease-less mutant of *S. cerevisiae*. In our preliminary communication (Kleczkowski, Bralczyk & Tarantowicz-Marek, 1972a) we reported on the formation of an ureidopositive, urease-resistant product formed by the enzymic preparation isolated from *Torulopsis utilis* grown on urea. The product has now been identified as allophanate and the mechanism of this reaction is discussed.

MATERIALS AND METHODS

Reagents. ATP-Na_2 (Reanal, Budapest, Hungary, and Sigma, St. Louis, Mo., U.S.A.); urease, type III, 2530 units/g (Sigma); 2,5-diphenyloxazole (PPO) scintillator (Reanal); 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), (NEN Chemicals, New England Nucleic Corporation, Boston, Mass., U.S.A.); [^{14}C]urea (The Radiochemical Centre, Amersham, England). All other reagents were supplied by Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland). Allophanate was synthesized according to Dains & Wertheim (1920).

Thin-layer chromatography. Incubation mixture and standard synthesized allophanate were chromatographed on Kieselgel-G (Merck, Darmstadt, West Germany) TLC-plates in methanol - chloroform - 17% ammonia (2:2:1, by vol.) as solvent system, at 4°C. The spots were developed using Ehrlich spray reagent and radioactivity was determined after scraping and elution of spots.

Decomposition of [^{14}C]*urea* in vivo

The release of CO_2 *by the growing cultures. Torulopsis utilis* was grown at 30°C in the medium of the following composition: 3% glucose, 0.5% KH_2PO_4, 0.12% $MgSO_4$, and 0.5% urea. Portions of the exponentially growing cultures (2 ml, $E_{650nm} = 0.5$) were pipetted into the outer part of Conway vessel, and 2 ml of 2 N-KOH was added to its inner part. Finally, 100 µl of [^{14}C]urea (1.57 µCi/ml) was added to the outer part of the vessel, stirred, and the vessel was incubated for 30 min at 30°C.

The reaction was terminated by adding 0.25 µl of 80% trichloroacetic acid (TCA) to the outer part of the previously cooled vessel. The vessel was immediately closed and absorption of the released $^{14}CO_2$ was conducted for 12 h at room temperature.

Subsequently, 100 µl of the liquid from the inner part of the vessel was transferred to a disc of Whatman no. 3 paper, dried at room temperature, and the radioactivity was counted in vials containing 5 ml of scintillation fluid (3 g of PPO, 0.3 g of POPOP in 1000 ml of distilled toluene) in Packard tri-carb scintillation counter.

Measurement of the acid-stable radioactivity in the cells. Cells from the exponentially growing cultures ($E_{650} = 0.55$) were collected by centrifugation, transferred to the medium devoid of urea and incubated for 2 h at 30°C. To 39 ml of the suspension of starved culture was added 1 ml of 20% urea solution containing 112 µCi of [^{14}C]urea. After appropriate time of incubation (10 - 60 min), 10 ml portions were withdrawn and transferred to a tube containing 0.5 ml of ice-cold 80% TCA.

The mixture was centrifuged and the pellet washed three times with 5% TCA; the supernatants were combined and the pellet was suspended in 10 ml of 5% TCA, disrupted in the frozen Hughes press block, recentrifuged and washed twice with 5% TCA. The respective supernatants were collected.

The final pellet was transferred to Whatman glass fibre G.F. 83 discs and the radioactivity measured as described above. TCA was removed from supernatants by several-fold extraction with ethyl ether. The TCA-free extracts were evaporated to dryness under vacuum, and dissolved in 2 ml of water. Aliquots of 100 µl were added to 5 ml of absolute ethanol and 8 ml of the scintillation fluid, and radioactivity was measured in Packard tri-carb 3003 scintillation counter.

Decomposition of [^{14}C]*urea* in vitro

The release of $^{14}CO_2$ *by the enzymic preparation.* The enzymic preparation purified about 3 times was obtained as described previously (Kleczkowski *et al.*, 1972a). The incubation mixture contained in 1 ml: 2 µmol of urea, 3 µmol of ATP,

10 μmol of $MgSO_4$, 5 μmol of $NaHCO_3$, 100 μmol of KCl, 50 μmol of tris-HCl buffer, pH 7.6, and 1 - 3 mg of enzymic protein. The mixture was placed in the outer part of the Conway vessel, and 1 ml of 0.5 N-KOH was added to the inner part. The vessels were closed and incubated for 40 min at 30°C. The incubation was terminated by adding 0.5 ml of 70% $HClO_4$, the vessels were closed, the contents carefully stirred and left for distillation for 12 h at room temperature.

Portions of 50 ml of 0.5 M-$BaCl_2$ solution were pippetted on the planchets, followed by 50 μl of KOH from the inner part of Conway vessels. The planchets were carefully dried under an infrared lamp. Radioactivity was measured with EKCO counter (4% efficiency for ^{14}C).

Measurement of the acid-stable radioactivity in the reaction mixtures. Incubation of the enzymic preparation with [^{14}C]urea was performed under conditions previously described (Kleczkowski *et al.*, 1972a). After incubation, urease was added to decompose the non-metabolized urea prior to treatment with 0.1 ml of 70% $HClO_4$. The samples were stirred vigorously for 10 min to remove CO_2, neutralized with 0.3 ml of 5 N-KOH and centrifuged; 100 μl of supernatant and 100 μl of 20% acetic acid were put on planchets and dried under an infra-red lamp. Radioactivity was measured in the EKCO counter.

Parallel samples were incubated as above but without urease, acidified to remove CO_2, and the radioactivity was measured. Difference between radioactivity added and found after incubation was used to calculate the amount of released CO_2.

RESULTS

Release of $^{14}CO_2$ from [^{14}C]urea by intact cells and the isolated enzymic preparation. It appears from data presented in Fig. 1 that the amount of $^{14}CO_2$ released by the cells was proportional to the time of incubation with [^{14}C]urea. The radioactivity was also measured in the extract of the cells fed with [^{14}C]urea. To increase the uptake of labelled urea, the cells were previously starved for 2 h in the medium devoid of urea. The results of these experiments showed that 99.8% of the taken up urea was metabolized to CO_2 during 1.5 h of incubation, and only 0.2% was found in the 5% TCA precipitate. This trace incorporation could be due to carboxylation of $^{14}CO_2$.

These results point to instantaneous decomposition of urea *in vivo*. Roon & Levenberg (1970) observed a similar complete decomposition of urea to CO_2 and NH_3 by the enzymic preparation isolated from *Candida utilis*. Our earlier results (Kleczkowski *et al.*, 1972a) show that at least some part of allophanate, the presumed intermediate, remains in the incubation mixture.

As it may be seen in Table 1, decomposition of urea to CO_2 and NH_3 by the isolated enzymic preparation is ATP and K-ion dependent. Results given in Table 2 show that about 33% of the metabolized urea remained in the form of the urease-resistant, ureido-positive product, and 67% was decomposed to CO_2.

Identification of the urease-resistant product of urea decomposition. As our attempts to isolate the urease-resistant, acid-labile intermediate of urea decomposition were

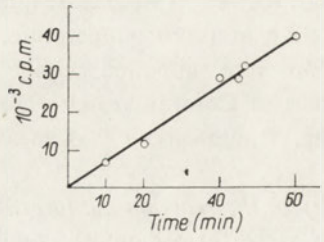


Fig. 1

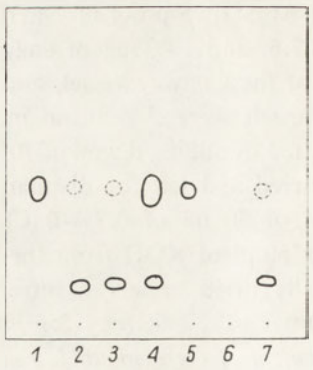


Fig. 2

Fig. 1. Time-course of $^{14}CO_2$ evolution in *Torulopsis utilis* growing on urea. Conditions as described under Methods.

Fig. 2. Chromatographic identification of urease-resistant intermediate with the chemically synthesized allophanate. Conditions as described under Methods. *1*, Urea + allophanate, acidified; *2*, Na-allophanate; *3*, the reaction mixture treated for 15 min with urease, deproteinized in 75% methanol; *4*, as under *3* but without treatment with urease; *5*, as under *3* but acidified; *6*, as under *5*, then neutralized and again treated with urease; *7*, as under *3*, repeatedly treated with urease.

Table 1

The release of $^{14}CO_2$ *from* [^{14}C]*urea by the enzymic preparation from* Torulopsis utilis

Assay conditions as described under Methods, except that to each sample 5000 counts/100 sec of [^{14}C]urea was added.

Component omitted	$^{14}CO_2$ (counts/100 sec/sample)
None	1780
ATP+Mg^{2+}	220
KCl	360

unsuccessful (Kleczkowski *et al.*, 1972a), we based our identification evidence on comparison of the properties of this product with those of the chemically synthesized allophanate.

Both the product and allophanate at pH above 7.0 were urease-resistant and gave a positive reaction in the Archibald (1944) test. At pH below 3 allophanate decomposed rapidly to urea and CO_2, and the same behaviour was observed with the studied urease-resistant intermediate (Table 3). On acidification of the urease--treated incubation mixture, practically no decrease in radioactivity was found. This treatment, however, led to formation of urea, which was then detected by subsequent treatment with urease. Consequently, a complete loss of radioactivity was noted under these conditions.

Table 2

Distribution of label from [^{14}C]*urea between the ureido-positive urease-resistant product and* CO_2 *when incubated with enzyme preparation from* Torulopsis utilis

The reaction mixture was incubated for 45 min at 37°C. The amount of urease-resistant product was calculated from the radioactivity remaining in incubation mixture after 15 min treatment with urease and acidification. The content of CO_2 was calculated from the difference between the radioactivity added and found after acidification of the incubated mixture, not treated with urease.

Assay conditions	Component omitted	Radioactivity in sample (counts/sec)	Radioactivity distribution (counts/sec)	
			urease-positive product	CO_2
Urease-treated	none	343	323	—
	ATP	20		
Untreated	none	674	—	656
	ATP	1330		

Table 3

Lability of the urease-resistant intermediate of urea decomposition

The enzymic preparation was incubated for 45 min at 37°C with [^{14}C]urea. Radioactivity was determined as described under Methods.

Assay conditions	Radioactivity (counts/100 sec/sample)
1. The incubation mixture was treated for 15 min with urease, then deproteinized in 75% methanol at pH 8.0	2845
2. As under 1, followed by acidification	2800
3. As under 2, neutralized, and again treated with urease	32

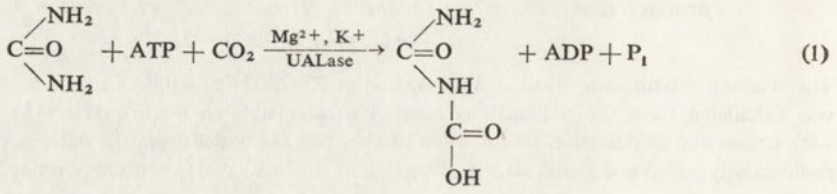
The identity of the studied intermediate with allophanate was confirmed by thin-layer chromatography of allophanate and the reaction products of the successively treated incubation mixture (cf. Table 3). As it may be seen in Fig. 2, R_F of allophanate and the intermediate was the same, i.e. 0.15.

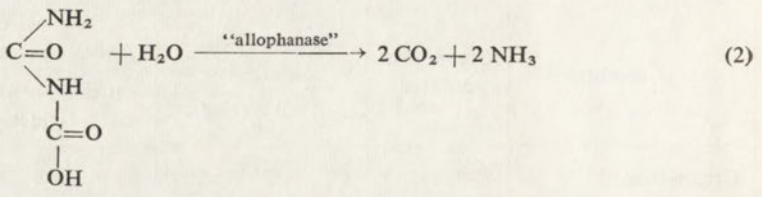
This evidence proves that the intermediate of urea decomposition by the urease-less *Torulopsis utilis* is identical with allophanate.

DISCUSSION

Our results show that urea is decomposed to CO_2 and NH_3 by urease-less *Torulopsis utilis* by an energy-dependent mechanism similar to that proposed by Roon & Levenberg (1970). Whittney & Cooper (1970), Roon & Levenberg (1970) and our group (Kleczkowski *et al.*, 1972a) have demonstrated the absolute requirement of a catalytic amount of HCO_3^- in this process.

The presented data indicate that this decomposition proceeds *via* allophanate as intermediate in a two-step process:





Roon & Levenberg (1970) suggested that UALase shows both enzymic functions, as on a 160-fold purification no significant change in the ratio of both these activities was observed. The estimated molecular weight of about 600 000 is compatible with the enzyme protein being composed of several subunits, one or more of which are likely to possess biotin-free sites for allophanate binding (Roon & Levenberg, 1970).

The obtained results imply that the enzymic preparation from *Torulopsis utilis* catalyses *in vitro* the two steps of urea transformation with different efficiency (Table 2) and that in the steady state of the reaction about 67% of urea is decomposed into CO_2 and 33% remains in the incubation mixture in the form of allophanate.

The possible discrepancy between our results and those of Roon & Levenberg (1970) and Whittney & Cooper (1970) arises from the fact that these authors determined only CO_2 without measuring allophanate in the incubation mixture. For estimation of the non-metabolized urea they used the method of Archibald (1944) directly. As allophanate is decarboxylated to urea with Archibald reagent, it was impossible under these conditions to differentiate between urea and urease-resistant allophanate. In our experiments, the treatment of the incubation mixture with urease before acidification removed selectively the non-metabolized urea.

It seems that transformation of urea in urease-less organisms is catalysed by two separate enzymes of different catalytic efficiency *in vitro*. In the complete incubation mixture, both allophanate and CO_2 were detected.

It is noteworthy that Roon & Levenberg (1968) and Reinbothe (private communication) found that *Candida utilis* grown on ammonium sulphate as the sole nitrogen source does not show the activity of UALase.

The results of our other experiments published elsewhere (Kleczkowski, Bralczyk & Tarantowicz-Marek, 1972b) indicate that the organisms deficient in urease grown on ammonium sulphate show the activity of UALase but not that of "allophanase". It should be therefore concluded that under these conditions the process of urea transformation is stopped at the allophanate step.

After completion of this manuscript, two publications: of Thompson & Muenster (1971) on *Chlorella*, and of Whittney & Cooper (1972) on *Saccharomyces cerevisiae* mutants, came to our notice. Both these papers support our suggestion that urea is metabolized in the urease-less organisms by two separate enzymes.

The authors are thankful to Prof. Dr H. Kating from the Marburg University, Institute of Pharmacognosy for the kind gift of *Torulopsis utilis* strain. The excellent technical assistance of Miss Krystyna Nowosielska is gratefully acknowledged.

This work was supported by the Polish Academy of Sciences within the project 09.3.1.1.1.10.

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MECHANIZM ROZKŁADU MOCZNIKA PRZEZ BEZUREAZOWY SZCZEP *TORULOPSIS UTILIS*

Streszczenie

Badano wykorzystanie mocznika jako jedynego źródła azotu przez bezureazowy szczep *Torulopsis utilis*. Stwierdzono, że produktem pośrednim tej przemiany jest allofanian; 67% radioaktywności pochodzącej z mocznika odzyskiwano w postaci CO_2, a 33% w postaci allofanianu. Na podstawie tych wyników stwierdzono, że proces wykorzystania mocznika jest katalizowany przez dwa enzymy.

Received 15 March, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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PHOSPHATE AND VITAMIN D SENSITIVE MOVEMENT OF CALCIUM IN RAT DISTAL ILEUM

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1. The everted sacs technique was used to study the changes of radioactive and total calcium concentrations in the serosal and mucosal incubation media of different segments of rat small intestine. 2. In duodenum the net increase of both radioactive and total calcium in the serosal medium was observed, in distal ileum the increase of the total calcium only, and no change in the proximal jejunum. 3. The net increase of total calcium in serosal medium of ileal, but not duodenal, sacs was dependent on the presence of phosphate and was arrested by Merphtallil and NEM. Low temperature blocked the serosal increase of total calcium both in duodenum and ileum. 4. Vitamin D was necessary for the serosal increase of calcium concentration both in duodenum and ileum. Actinomycin D abolished the effect of vitamin D in duodenum but not in distal ileum.

The evidence available so far supports the idea that some segments of rat intestine can actively transport calcium across gut wall. The intensity of active translocation of calcium studied *in vitro*, varies with the species of animal and location of the investigated intestinal segment. Transport of calcium is greater in the duodenum than in the distal ileum in the rat but not in chick and hamster (Schachter, Dowdle & Schenker, 1960b; Kimberg, Schachter & Schenker, 1961; Michalska, 1968; Adams & Norman, 1970). In some respects the mechanism of active transport in rat duodenum and ileum is similar, however, differences exist in the response of comparable segments to anaerobic conditions, mode of vitamin D action and role of inorganic phosphate (Schachter, Dowdle & Schenker, 1960a; Schachter, Kimberg & Schenker, 1961; Michalska, 1968). Distal small intestine of rat is relatively impermeable to the diffusion of calcium (Schachter *et al.*, 1961) and a metabolically dependent block to calcium absorption is postulated (Wasserman, 1964). Translocation of calcium across intestinal wall may be looked at as a three-step process: (i) uptake of calcium at microvilli surface, (ii) transfer of calcium inside the cell, and (iii) release of calcium at the serosal surface (Adams & Norman, 1970). The precise site of active process of calcium transfer, i.e. the uptake at the microvilli surface or/and the release at the basal membrane is still a subject of speculation

(Wasserman, 1964; Helbock, Forte & Saltman, 1966; Schachter, Kowarski, Finkelstein & Ma, 1966; Martin & DeLuca, 1969; Adams & Norman, 1970; Adams, Wong & Norman, 1970).

By using the everted sacs technique we have demonstrated that the release of calcium to the medium bathing serosal surface of the distal ileum of rat is a metabolic process, sensitive to the prior vitamin D administration, temperature, some metabolic poisons and the presence of inorganic phosphate in the medium.

MATERIALS AND METHODS

Reagents. Vitamin D_3 (sol. aquosa) and Merphtallil (methoxythephyllinmercuripropylphtalimide) were from Polfa (Tarchomin, Poland). *N*-Ethylmaleimide (NEM) was from BDH Chemicals Ltd (Poole, England). Dicumarol was from Schuchardt GmbH (München, German Federal Republic). Actinomycin D was a generous gift from Merck, Sharp & Dohme Research Lab. (Rathway, N.J., U.S.A.) through the courtesy of Mr. W. B. Gall. $^{45}CaCl_2$ (specific activity 312 mCi/mmol) was supplied by Biuro Dystrybucji Izotopów (Warszawa, Poland). All other reagents were analytical grade products.

Animals. Wistar albino rats of both sexes 4 - 6 weeks old were used. The animals were fed normal laboratory diet or vitamin D-free diet described by Oser (1965). The vitamin D-free diet was introduced starting from the 10th day after delivery. The animals were fasted 16 hours and killed by a blow on the head followed by decapitation.

Preparation of segments of the small intestine. Immediately after killing of the animal, the small intestine (from pylorus to the ileocoecal valve) was removed, washed with 0.9% NaCl solution and immersed in ice-cold 0.9% NaCl - 4 mM-KCl solution. A few minutes later the intestine was placed on wet filter paper and five 6-cm long segments were taken at equal intervals. The first segment corresponded to duodenum. The second, third and fourth segments are referred to in the text as proximal jejunum, middle jejunum and distal ileum, respectively (Fig. 1).

Incubation. The basal incubation medium consisted of 151 mM-NaCl, 20 mM-glucose, 0.8 mM-NaH_2PO_4, 0.4 mM-$CaCl_2$ and 14.7 mM-$NaHCO_3$. Before the experiment the medium was saturated for 5 min with CO_2 to obtain a pH value of 7.4, and in isotope experiments $^{45}CaCl_2$ (37.5 μCi/100 ml) was added.

The intestinal segments were everted using a plastic rod, blotted and weighed. One end was tied with a ligature and the serosal compartment was filled with 0.7 ml of incubation medium using a syringe fitted with a blunt needle. The sacs were placed in 25 ml Erlenmayer flasks containing 5 ml of incubation medium and incubated with shaking for 3 h at 37°C under continuous flow of O_2+CO_2 (95:5), then the sacs were removed and the fluids bathing the mucosal and serosal surfaces were collected for total and radioactive calcium determination.

Determination of radioactivity and calcium. The sample was plated on aluminium planchette and the radioactivity was determined in a proportional scintillation counter provided with solid plastic scintillator. The scaler type PEL-5 and scintillator

counter SSU-3 were products of Biuro Urządzeń Techniki Jądrowej (Warszawa, Poland). The counting surpassed background at least 80 times.

Total calcium was determined by complexometric titration using murexide as indicator according to Schachter *et al.* (1960a).

Presentation of results. Movement of calcium was expressed as the ratio of the final concentrations of ^{45}Ca (count/min/ml) or ^{40}Ca (μmol/ml) in the serosal medium to that in the mucosal medium (S/M ratio) or as an increase or decrease of the total amount of calcium in the serosal or mucosal media expressed in nmol Ca/g wet tissue/h.

RESULTS

The amount of calcium in the incubation fluids on both sides of the everted duodenal sacs after three hours of incubation was changed. The observed decrease in the mucosal medium was equivalent to the increase in the serosal medium (Table 1). In the ileal segment, the increase of calcium in serosal medium exceeded approximately three times the decrease in mucosal medium. Proximal jejunum seemed to be inert.

Table 1

Effect of temperature and some metabolic poisons on the amount of calcium in incubation medium both sides of everted gut-sac

Incubation was carried out for 3 h with basal medium at 37°C, except where otherwise indicated. Figures represent the average of at least two experiments.

Incubation conditions	ΔCa of incubation fluid (nmol/l g wet tissue/l hour)					
	Duodenum		Proximal jejunum		Distal ileum	
	Muc.	Ser.	Muc.	Ser.	Muc.	Ser.
Basal medium (control)	−172	+165	−3	+2	−61	+202
Basal medium at 5°C	−23	±0	±0	±0	±0	±0
Basal medium+5 mM-KCN	−18	+9	−36	−2	−100	+66
Basal medium+0.25 mM-dicumarol	+7	+3	−55	±0	−53	+92
Basal medium+0.2 mM--NEM	−75	+300	—	—	−25	+25
Basal medium+0.2 mM--Merphtallil	−80	+260	—	—	+15	−60

The calculated serosal/mucosal calcium concentration ratio (S/M) exceeded for duodenum 1.0 both for ^{40}Ca and ^{45}Ca. This could be interpreted as an active transport against the concentration gradient. Such interpretation does not take into account the size of calcium pool of intestinal wall cells. There is evidence that the accumulation of ^{45}Ca by intestinal slices (Schachter *et al.*, 1960a) and the release of ^{40}Ca from blood platelets (Mürer & Holme, 1970) are metabolic processes. One could expect that during the incubation time intestinal wall cells may release calcium and thus modify the amount of calcium in both incubation fluids, i.e. serosal

and/or mucosal. Figure 1 demonstrates the calcium concentration ratio S/M for ^{40}Ca and ^{45}Ca in successive segments of rat small intestine. On the basis of ^{40}Ca measurements, duodenum and the segment corresponding to distal ileum showed S/M ratio exceeding 1. On the basis of ^{45}Ca measurements only duodenal S/M ratio exceeded 1. The discrepancy of isotopic and non-isotopic measurements in the ileal segment may be due to the modifying effect of ^{40}Ca pool of intestinal wall cells. To obtain more data concerning the changes in the amount of ^{40}Ca in mucosal

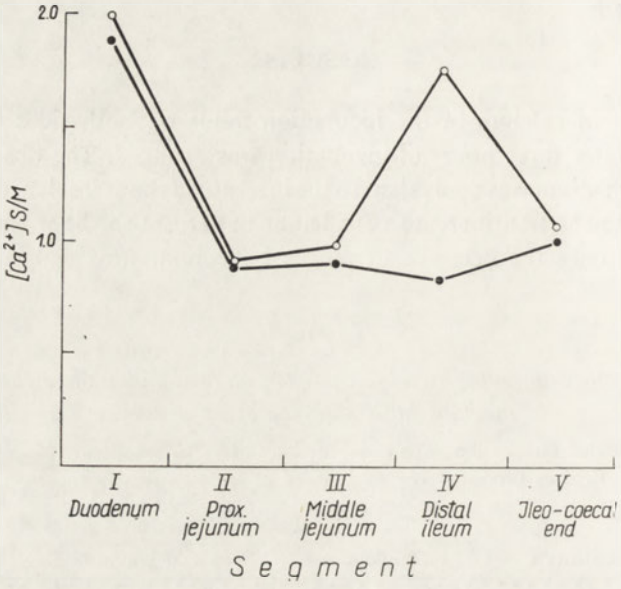

Fig. 1. Ratio of calcium concentration in the incubation medium on the serosal and mucosal side (S/M) of rat small intestine. ●, ^{45}Ca; ○, ^{40}Ca.

and serosal fluids, the everted sacs were incubated under different conditions (Table 1). Low temperature inhibited completely any changes of calcium amount in duodenum and ileum. Respiratory poisons had a greater inhibitory effect on duodenum than on ileum, whereas Merphtallil and NEM affected mainly ileum.

The effect of phosphate on the changes of calcium amount in the incubation fluids is shown in Table 2. In the control experiment, the medium on both sides of the intestinal sac contained 0.8 mM-NaH_2PO_4. In phosphate-free medium in distal ileum no changes in calcium amount were observed. On addition of phosphate to the mucosal fluid only, changes in the amount of calcium were the same as in the control, whereas addition of phosphate to the serosal fluid only, brought about reverse changes. Duodenum and proximal jejunum were not affected by the presence or absence of phosphate.

The results of experiments performed on the everted sacs from the vitamin D deficient rats and after treatment with vitamin D are shown in Table 3. Vitamin D

Table 2

Effect of inorganic phosphate on the amount of calcium in incubation medium both sides of everted gut-sacs

Basal medium containing 0.8 mM-phosphate or/and phosphate-free medium were used. Figures represent the average of at least two experiments.

Incubation media	ΔCa of incubation fluid (nmol/l g wet tissue/l hour)					
	Duodenum		Proximal jejunum		Distal ileum	
	Muc.	Ser.	Muc.	Ser.	Muc.	Ser.
Phosphate both sides (control)	−180	+165	+4	+2	−75	+186
Phosphate-free both sides	−182	+181	+6	−6	−6	−3
Phosphate on mucosal side only	−187	+193	−14	−6	−66	+210
Phosphate on serosal side only	−190	+203	±0	−13	+1100	−95

Table 3

Effect of vitamin D deficiency and vitamin D and actinomycin D administration to rats, on the amount of calcium in incubation medium both sides of everted gut sacs

Vitamin D_3, 750 i.u., was administered by stomach intubation to vitamin D-deficient rats 20 h before killing the animal. Actinomycin D, 1 μg/g of body weight, was injected intraperitoneally 2 h prior to vitamin D administration. Mean values from 4 - 6 animals, ±S.D., are given.

Treatment of vitamin D-deficient rats	Incubation medium	ΔCa of incubation fluid (nmol/l g wet tissue/l hour)					
		Duodenum		Proximal jejunum		Distal ileum	
		Muc.	Ser.	Muc.	Ser.	Muc.	Ser.
None	Basal	−3±14	+7±17	±0± 9	−2±14	−2± 12	+1±16
Vitamin D	Basal	−190±14	+205±90	+4± 9	−3±12	−350±120	+225±76
Actinomycin D + vit. D	Basal	+13±13	+20±17	+13±14	−4±10	−80± 20	+265±68
Vitamin D	Phosphate omitted	−205±27	+245±14	−2±14	+1±10	−10± 12	+5±10
Vitamin D	Basal + NEM	−263±30	+256± 5	−6± 6	±0± 0	+6± 7	+4± 4

seemed to be necessary for the increase of serosal calcium amount both in duodenum and distal ileum. Actinomycin D abolished this effect of vitamin D in duodenum but not in distal ileum.

DISCUSSION

The net change in the amount of ^{40}Ca in media on mucosal and serosal sides of intestinal sacs showed that in duodenum the uptake at the microvilli side was equivalent to the release at the serosal side. In the distal ileum the release of ^{40}Ca at the serosal side exceeded significantly the uptake at the mucosal surface indicating that this release is a process relatively independent from the uptake of calcium at the mucosal side.

Transfer of calcium across the mucosal cell is complex and may involve several processes. Many authors believe (Schachter *et al.*, 1961; Harrison & Harrison,

1965; Martin, Melancon & DeLuca, 1969; Adams & Norman, 1970) that the major consequence of vitamin D administration is the increase in calcium uptake across the microvilli side of cell and hence availability of calcium to the serosal transport. The accumulation of ^{45}Ca by duodenum was shown to be dependent on oxidative phosphorylation and the dietary vitamin D (Schachter *et al.*, 1960a). On the other hand, mucosal cells isolated from the intestine of vitamin D-deficient rats lost their ability to release calcium (Hashim & Clark, 1969).

As demonstrated in the present experiments, the release of ^{40}Ca by ileum at the serosal side could be arrested by low temperature, metabolic poisons and vitamin D deficiency, suggesting an association with metabolic processes. Inorganic phosphate seems to be an obligatory factor for calcium release in ileal segment, but not in duodenum. Merphtallil and NEM were found by Meijer, Groot & Tager (1970) and Aleksandrowicz & Świerczyński (1971) to be inhibitors of phosphate exchange in mitochondria. These agents show an inhibitory effect on ^{40}Ca release in ileum, confirming the role of phosphate in this process. The role of vitamin D in accumulation of calcium in ileum seems to be different from that observed in duodenum. Actinomycin D administered to vitamin D-treated rats completely abolished the effect of vitamin D in duodenum, but had no effect in ileum. Considering that several active metabolites of vitamin D were isolated (Lawson *et al.*, 1971) and that the formation of some of them could be depressed by inhibitors of protein synthesis (Gray & DeLuca, 1971), it is possible that different active forms of vitamin D could be operative in duodenum and ileum.

The authors wish to acknowledge the valuable assistance of Miss Gabriela Nagel.

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WPŁYW FOSFORANU I WITAMINY D NA TRANSPORT WAPNIA W JELICIE KRĘTYM SZCZURA

Streszczenie

1. Metodą odwróconych woreczków jelitowych badano zmiany stężenia wapnia całkowitego i radioaktywnego w płynie otaczającym surowicówkową i śluzówkową stronę różnych odcinków jelita cienkiego szczura.

2. W dwunastnicy po stronie surowicówkowej woreczka stwierdzano wzrost stężenia zarówno wapnia całkowitego, jak i radioaktywnego, a w dystalnej części jelita krętego — jedynie wzrost wapnia całkowitego. W początkowym odcinku jelita czczego nie stwierdzano zmian w stężeniu wapnia.

3. Wzrost stężenia wapnia całkowitego w płynie po stronie surowicówkowej jelita krętego jest związany z obecnością fosforanów w środowisku inkubacyjnym, natomiast w dwunastnicy nie ma tej zależności. Niska temperatura hamuje wzrost stężenia wapnia całkowitego po stronie surowicówkowej zarówno w dwunastnicy, jak i w jelicie krętym, natomiast Merphtallil i NEM tylko w jelicie krętym.

4. Witamina D jest czynnikiem niezbędnym dla wzrostu stężenia wapnia w płynie po stronie surowicówkowej w dwunastnicy oraz w jelicie krętym. Aktynomycyna D znosi działanie witaminy D tylko w dwunastnicy.

Received 26 March, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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EFFECT OF IONIZING RADIATION ON THE REACTIVITY OF ε-AMINO GROUPS IN TROPOCOLLAGEN

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After X-ray irradiation of tropocollagen solution in the presence of nitrogen with 30 kilorad, the content of free ε-amino groups decreased from 36 to 26 per α-chain. This decrease was but apparent and resulted from conformational changes in tropocollagen (formation of new interchain cross-linking bonds) which block the access of fluorodinitrobenzene to some of free ε-amino groups.

ε-Amino groups are involved in the formation of cross-linkages in collagen. *In vivo*, some of collagen ε-amino groups are oxidatively deaminated to aldehydes which, in turn, react with each other or with ε-amino groups to form aldol condensation product or aldimine link derived *via* Schiff base formation (Bailey & Peach, 1968; Bornstein & Piez, 1966; Kang, Piez & Gross, 1969; Rojkind, Gutierrez, Zeichner & Lent, 1969).

Similar cross-links are formed also spontaneously during fibril formation *in vitro* upon heat gelation of collagen solution (Kang, Faris & Franzblau, 1970; Kang & Gross, 1970; Tanzer, Mechanic & Gallop, 1970). This observation prompted us to investigate whether ε-amino groups are involved also in the formation of radiation-induced cross-links.

MATERIALS AND METHODS

Preparation of collagen and irradiation procedure. Neutral-salt-soluble collagen was prepared from skin of young rats (30 - 40 g) according to the procedure described by Bornstein & Piez (1964). For irradiation collagen solution containing 1.5 mg/ml was placed in a test tube, deoxygenated by passing oxygen-free nitrogen for 30 min, then sealed under the stream of nitrogen, immersed in a water-ice bath and irradiated.

The radiation was delivered from Stabilipan-250 X-ray apparatus at 200 kV, 20 mA and filtered through 0.5 mm Cu. The dose rate estimated with the Fricke dosemeter was 410 rad/min.

Analytical methods. Disc electrophoresis was carried out in polyacrylamide gel according to Nagai, Gross & Piez (1964), as described previously (Dancewicz & Majewska, 1971). Free ammonia was determined with Conway's microdiffusion technique using Nessler reagent. The number of ε-amino groups substituted with fluorodinitrobenzene was determined according to Szumiel & Jeleńska (1970), sodium lauryl sulphate being added to the dinitrophenylated collagen solution; this made possible direct spectrophotometric determination of DNP-derivative concentration. Amino acid analysis was performed on Beckman Model 120 B automatic amino acid analyser. The duplicate samples containing about 5 mg of protein were hydrolysed in sealed test tubes with 6 N-HCl in nitrogen atmosphere at 110°C. The hydrolysates were dried in vacuum over KOH, the residue was washed twice with redistilled water and dried again over KOH. The analysis was performed according to the procedure of Spackman, Stein & Moore (1958). Samples of collagen to be analysed for α-aminoadypic-δ-semialdehyde content were oxidized with performic acid according to Moore (1963) prior to hydrolysis. Protein was determined by the Kjeldahl micromethod.

Tryptic digestion. Before digestion collagen samples were dialysed against 0.1 M-calcium acetate, pH 7.3, and then denatured in boiling water for 5 min. The digestion was carried out for 20 h at 20°C with trypsin to substrate weight ratio of 1:100. The resulting tryptic peptide mixture was treated with fluorodinitrobenzene (FDNB). The excess of reagent was extracted with ethyl ether, the water layer was evaporated to dryness and the residue was hydrolysed with 6 N-HCl. The hydrolysate was desalted on a Dowex 50 X8 column and subjected to amino acid analysis. The results were quantified taking arginine content as an index of the amount of collagen analysed.

RESULTS

The content of free ε-amino groups accessible to FDNB in tropocollagen solution irradiated in nitrogen atmosphere with the dose of 30 kilorad was considerably lower than in non-irradiated sample (Table 1). This radiation effect could be due to different mechanisms. The following were considered in the present study: (i)

Table 1

The effect of radiation on the collagen ε-amino groups reactive toward fluorodinitrobenzene

Figures represent the number of ε-NH_2 groups per α-chain (95 000 mol. wt.). Mean values from 10 determinations ±S.D. are given.

Irradiation dose (kilorad)	Number of ε-NH_2/α-chain
0	36.3±0.56
10	33.2±0.64
20	30.9±0.59
30	26.1±0.69

oxidative deamination of ε-amino groups, (ii) involvement of these groups in the formation of new bonds and (iii) inaccessibility of ε-amino groups to the reagent.

The occurrence of oxidative deamination was excluded as the same amount of α-aminoadypic acid deriving from lysine in collagen hydrolysed and oxidized with performic acid was present both in samples irradiated with 30 kilorad and in non-irradiated samples. No difference in the free ammonia content was found by the Conway's microtechnique in irradiated and non-irradiated samples. Moreover, the lysine and hydroxylysine content in collagen hydrolysates remained unchanged upon irradiation of collagen solution.

The involvement of ε-amino groups in Schiff base formation was evaluated from changes in collagen subunit composition and was found to be insignificant. The separation pattern of irradiated collagen prior to and after reduction with sodium borohydride, is presented in Fig. 1. The composition of the subunits estimated from the densitometric tracings (Table 2) was practically the same in reduced and non-reduced samples of irradiated collagen.

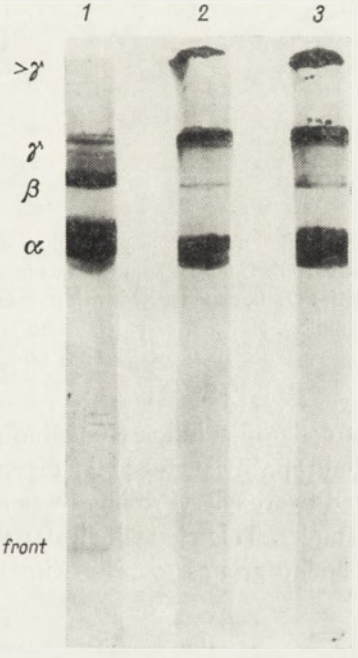

Fig. 1. Electrophoresis in polyacrylamide gels of heat-denatured neutral-salt-soluble collagen. *1*, Control; *2*, irradiated with 30 kilorad; *3*, irradiated and treated with sodium borohydride. The reduction of collagen with $NaBH_4$ was performed according to Deshmukh & Nimmi (1969).

The inaccessibility of ε-amino groups to FDNB was tested by analysing the amount of free, DNP-unsubstituted lysine and hydroxylysine in the heat-denatured dinitrophenylated and then hydrolysed collagen. Figure 2 shows that in control, non-irradiated sample of collagen 0.6 lysyl and 2 hydroxylysyl residues per 1000 aminoacids are unsubstituted by FDNB. This value corresponds to the amount of lysine released on hydrolysis from dinitrophenylated standard lysine.

In irradiated collagen the number of unsubstituted lysyl residues amounted to 2.6 and that of hydroxylysyl to 6 per 1000 amino acids. Hence, on irradiation

Table 2

Subunit composition of irradiated and borohydride-treated collagen

The values presented in the table were obtained by integration of the densitometric tracings of the developed gels (see Fig. 1) and calculated as percentages of the total colour yield. The mean values from 10 determinations ±S.D. are given. Probability (*P*) represents the comparison of the irradiated samples, untreated and treated with $NaBH_4$. N.S. means non-significant.

Subunit	Control (non-irradiated)	Irradiated (30 kilorad)	Irradiated (30 kilorad) and treated with $NaBH_4$	*P*
α	60.6±0.61	46.7±0.70	45.3±0.84	N.S.
β	20.6±0.64	12.5±0.64	14.5±0.70	0.05
γ and $> \gamma$	18.8±0.71	40.8±1.38	40.0±1.30	N.S.

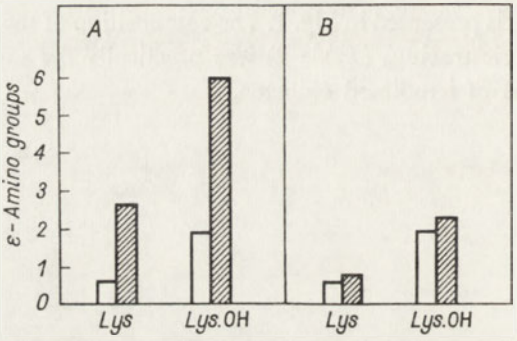

Fig. 2. The effect of collagen irradiation on the content of lysine and hydroxylysine groups inaccessible to FDNB. Collagen solution was irradiated with 30 kilorad, then heat-denatured and: *A*, treated with FDNB, or *B*, treated with trypsin and then with FDNB. Outlined bars, control (non-irradiated samples). Hatched bars, irradiated samples. The results are expressed as Lys or Lys.OH residues per 1000 total amino acid residues present in native tropocollagen molecule.

of collagen solution 6 ε-amino groups became unable to react with FDNB. In the dinitrophenylated tryptic digestion products, the number of unsubstituted lysyl and hydroxylysyl residues was almost the same in control as in irradiated collagen samples. This result indicates that radiation-induced changes which prevent some ε-amino groups to react with FDNB could be abolished by trypsin digestion.

DISCUSSION

The decrease in the number of ε-amino groups due to irradiation of oxygen-free collagen solution, as determined by the dinitrophenylation method (Table 1), is in agreement with the radiation-induced oxidative as well as reductive deamination of nitrogenous compounds such as amino acids, peptides and proteins (Dale, Davies & Gilbert, 1949; Liebster & Kopoldova, 1964, 1966). However, we were unable to confirm that deamination is the reaction which accounts for the decreased number of ε-amino groups in irradiated collagen. We found no increase in the content of free ammonia and of α-aminoadypic-δ-semialdehyde and no decrease in lysyl and

hydroxylysyl residues content in irradiated collagen solution. According to Van Caneghem & Lapiere (1970) the content of lysyl and hydroxylysyl residues remained unchanged even upon irradiation of collagen solution in air with still higher doses of radiation.

These discrepancies may be explained by the changes in the reactivity of some ε-amino groups toward FDNB observed on irradiation of collagen. The existence in collagen of ε-amino groups able and unable to form Schiff bases, was demonstrated by Page & Benditt (1969). This ability seemed to be unaffected by irradiation with 30 kilorad (Table 2). However, the reactivity toward FDNB was affected by radiation. An example of such a radiation effect is given by Alexander (1962) who demonstrated changes in ε-amino groups reactivity in γ-globulin and in albumin irradiated with thousand-fold larger doses of radiation.

The changes in reactivity of ε-amino groups in irradiated proteins are probably due to radiation-induced changes in protein molecule conformation (Alexander, 1962). The exact nature of these conformational changes is unknown. In the case of collagen irradiated in the absence of oxygen there is an increase in the number of cross-links (Bailey, 1967; Dancewicz & Majewska, 1971) leading eventually to higher molecular weight aggregates of collagen.

The nature of these radiation-induced cross-links has not so far been established. They might be the interchain C-C linkages. Liebster & Kopoldova (1966) have shown that such bonds are formed in amino acid solution irradiated under anaerobic conditions. Bailey, Rhodes & Cater (1964) assume formation of these bonds in collagen fibers irradiated with high doses of radiation. Whatever their nature, the radiation-induced cross-links form a steric hindrance preventing the access of FDNB molecule to some ε-amino groups.

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WPŁYW PROMIENIOWANIA JONIZUJĄCEGO NA REAKTYWNOŚĆ GRUP ε-AMINOWYCH W TROPOKOLAGENIE

Streszczenie

Napromienienie roztworów kolagenu w obecności azotu dawką 30 kiloradów promieniowania X powoduje zmniejszenie zawartości wolnych grup ε-aminowych z 36 do 26 w łańcuchu *α*.

Zmniejszenie to jest pozorne i wynika ze zmian konformacyjnych w tropokolagenie (powstanie nowych wiązań poprzecznych międzyłańcuchowych), które zamykają dostęp fluorodwunitrobenzenu do niektórych wolnych grup ε-aminowych.

Received 24 April, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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A NEW PROCEDURE FOR PURIFICATION OF CHROMATIN

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A simple procedure for purification of chromatin based upon direct solubilization of crude chromatin and gel chromatography on Sepharose 4B is described. Spectral properties, chemical composition and electrophoretic patterns of histones from chromatin preparations of hog kidney cortex, spleen and lungs indicate that the extent of purification obtained with the new procedure is similar to that achieved by density-gradient centrifugation.

In 1959 Zubay & Dotty described the procedure for isolation of soluble nucleohistone (chromatin) from calf thymus glands. Subsequently, several modifications of this method, adapted for preparation of chromatin from different tissues, were published (Dingman & Sporn, 1964; Marushige & Bonner, 1966; Bonner *et al.*, 1968). In most of these methods the isolated crude chromatin was further purified by ultracentrifugation through 1.7 M-sucrose solution, followed by dialysis and solubilization; ultracentrifugation was found to be essential for separation of chromatin from the accompanying non-chromosomal protein.

In the present paper an alternative method for purification of chromatin is described, consisting in direct solubilization of crude chromatin followed by gel chromatography on Sepharose 4B. Some properties of chromatin preparations, purified by the new procedure from hog kidney cortex, spleen and lungs, are presented.

MATERIALS AND METHODS

Preparation of chromatin. Hog kidney, spleen and lungs were obtained from slaughterhouse immediately after killing of the animal, frozen in solid CO_2 and stored not longer than for three days. Kidney cortex was isolated immediately before preparation of chromatin. All further operations were performed in the cold-room at 0 - 4°C. Frozen tissue (15 g) was ground in a blendor (Niewiadów, Poland) with 300 ml of 0.075 M-NaCl - 0.024 M-EDTA - 0.05 M-sodium bisulphite solution (saline--EDTA solution), pH 8.0, for 1 min at 150 V and 4 min at 100 V. The homogenate

was filtered through two layers of dense gauze and centrifuged at 2000 *g* for 10 min. The sediment was dispersed (by mixing for 5 - 10 sec at 70 V) in 140 ml of saline--EDTA solution and recentrifuged. The pellet was suspended in 120 ml of 0.05 M-tris-HCl buffer, pH 8.0, using Potter-Elvehjem homogenizer, and sedimented at 2000 *g* for 15 min. The preparation was then washed twice in 100-ml aliquots of the same buffer; each time the pellet was spun down at 10 000 *g* for 15 min. The final sediment was designated crude chromatin.

Crude chromatin was suspended in 50 ml of 0.01 M-tris-HCl buffer, pH 8.0, sheared in the Unipan type 203 homogenizer for 90 sec at 150 V and centrifuged at 12 000 *g* for 30 min. The supernatant, containing solubilized chromatin which corresponded to more than 80% of DNA of crude chromatin, was used for gel chromatography.

Gel chromatography. This was performed on Sepharose 4B column (3.5×30 cm) equilibrated with 0.01 M-tris-HCl buffer, pH 8.0. The solubilized chromatin was diluted with buffer to contain 6 - 8 extinction units at 260 nm, and 30 - 50 ml of this solution was applied to the column. Elution was carried out with the same buffer at a rate of 30 - 40 ml per hour. The eluate was collected in 10-ml fractions and analysed for optical density at 230 and 260 nm. Fractions containing chromatin, i.e. those eluted in the range of V_e from 70 ml to 120 ml, were pooled and considered as purified chromatin.

Determination of DNA and RNA. DNA and RNA were separated as recommended by Munro & Fleck (1966). RNA was determined spectrophotometrically at 260 nm and DNA by the method of Burton (1956) with calf thymus DNA as a standard.

Determination of histones and non-histone protein. Histones were extracted twice with 0.3 N-HCl for 30 min at 0°C and assayed by the microbiuret method of Goa (1953). The non-histone protein was determined in the 0.3 N-HCl-precipitable material by the same method after removal of nucleic acids by heating in 10% trichloroacetic acid at 90°C for 10 min. In both cases bovine serum albumin was used as a standard.

Polyacrylamide-gel electrophoresis. Gel electrophoresis of histones was performed according to Johns (1967) except that the gel containing 15% of acrylamide instead of 20%, was used. Gels were stained overnight with 1% of Amido Black in 7% acetic acid and destained by repeated washing with 7% acetic acid. The protein bands were scanned using ERJ 65 Densitograph (Zeiss, Jena, G.D.R.) adapted for gel scanning.

Reagents. Sepharose 4B was from Pharmacia (Uppsala, Sweden); acrylamide, *N,N*-bisacrylamide, TEMED and sodium persulphate were from Serva (Heidelberg, G.F.R.); calf thymus DNA was from Worthington Biochemical Corporation (Freehold, N.J., U.S.A.), yeast RNA, from Light & Co. Ltd (Colnbrook, England) and bovine serum albumin from Michrome (London, England). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS AND DISCUSSION

Crude chromatin was isolated according to Marushige & Bonner (1966) except that the homogenization medium contained additionally 0.05 M-sodium bisulphite to prevent degradation of histones by cell proteases (Panyim & Chalkley, 1969). The preparations were purified by direct solubilization and gel chromatography on Sepharose 4B. Table 1 presents data on weight ratios of protein and RNA to DNA in chromatin preparations from hog kidney cortex, spleen and lungs, obtained at three steps of purification (crude, solubilized, and gel-purified chromatin). It is evident that the solubilization of crude chromatin by the recommended procedure resulted in almost complete separation of chromatin from non-chromosomal protein, the remaining contaminants being subsequently removed by gel chromatography on Sepharose 4B (Fig. 1).

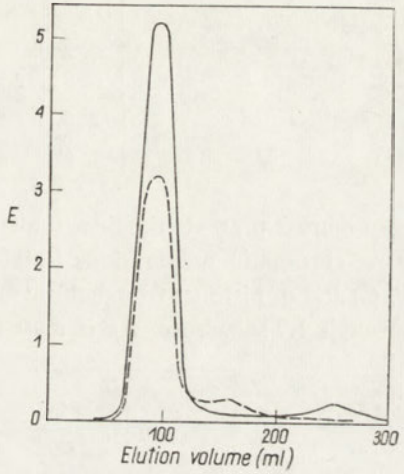

Fig. 1. The elution profile of hog spleen chromatin on Sepharose 4B. Solubilized chromatin, 30 ml, containing 180 extinction units at 260 nm, was applied to a Sepharose 4B column (3.5×30 cm) and chromatographed as described under Materials and Methods. The eluate was collected in 13-ml fractions at a rate of 1 fraction per 20 min. ——, Extinction at 260 nm; - - -, extinction at 230 nm.

Table 1

Weight ratios of DNA, RNA and protein in chromatin preparations from hog tissues at successive steps of purification

Chromatin preparations: 1, crude; 2, solubilized; and 3, purified. For details see Materials and Methods.

Tissue	Preparation	Weight ratios		
		DNA	RNA	Protein
Kidney cortex	1	1.00	0.09	4.80
	2	1.00	0.08	1.67
	3	1.00	0.08	1.60
Spleen	1	1.00	0.07	2.94
	2	1.00	0.05	1.82
	3	1.00	0.04	1.63
Lungs	1	1.00	0.0	4.25
	2	1.00	0.05	1.77
	3	1.00	0.05	1.73

Composition and the $E_{280\ nm}/E_{260\ nm}$ ratio of chromatin preparations purified by the described method, are given in Table 2. Attention should be drawn to a great similarity of the composition of chromatins from hog tissues with those purified by the ultracentrifugation method from calf thymus (Toczko & Jaźwiński, 1971), rat liver and pea bud (Elgin & Bonner, 1970).

Table 2

Composition and spectral properties of the purified chromatin preparations from hog tissues

Chromatins were isolated as described under Materials and Methods. NHP, non-histone protein.

Tissue	Content in % of dry weight				Weight ratios				E_{280}/E_{260}
	DNA	RNA	Histones	NHP	DNA	RNA	Histones	NHP	
Kidney cortex	33.9	2.8	39.0	14.8	1.00	0.08	1.16	0.44	0.63
Spleen	34.0	1.5	40.0	16.1	1.00	0.04	1.16	0.47	0.65
Lungs	32.9	1.6	36.4	20.3	1.00	0.05	1.11	0.62	0.58

Figure 2 presents the densitometric scans of electrophoretic patterns of histones from chromatin preparations isolated by the new procedure from hog tissues (*A*, *B*, *C*) and of histones from calf thymus chromatin (*D*) isolated by ultracentrifugation through 1.7 M-sucrose. As it may be seen, there are some differences in relative

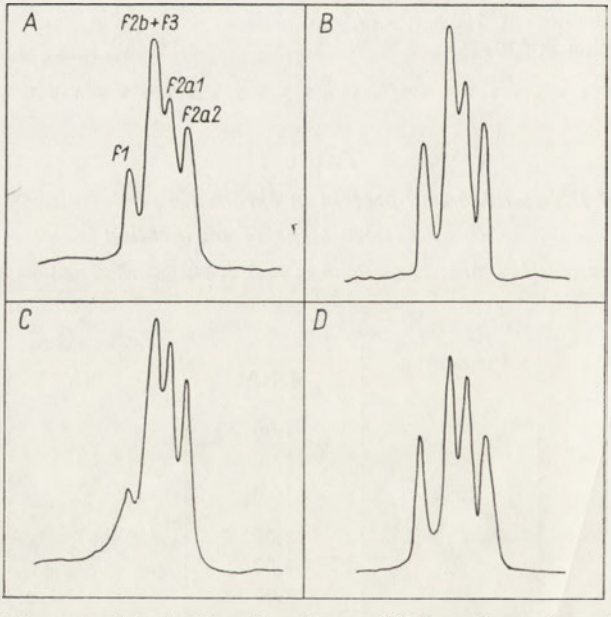

Fig. 2. Densitometric scans of electrophoretic patterns of histones from chromatin of hog tissues: *A*, kidney cortex; *B*, spleen; and *C*, lungs. *D*, Histones from chromatin of calf thymus, isolated by the method of Marushige & Bonner (1966). Histones are designated according to the nomenclature of Phillips & Johns (1965).

content of individual histones; the most variable component is lysine-rich histone (histone *f1*). Similar observations on the difference in contribution of individual histones were reported by Fambrough, Fujimura & Bonner (1968) for different parts of pea plant, and by Hnilica, Edwards & Hey (1966) for animal tissues.

The authors are deeply grateful to Professor Dr. Irena Chmielewska for her comments on the manuscript. This work was supported in part by the Department of Medical Sciences, Polish Academy of Sciences.

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NOWA METODA OCZYSZCZANIA CHROMATYNY

Streszczenie

Opisano nową, prostą metodę oczyszczania chromatyny, polegającą na zastąpieniu ultrawirowania w gradiencie gęstości sacharozy przez przeprowadzenie chromatyny w formę rozpuszczalną i chromatografię molekularną na Sepharozie 4B. Skład chemiczny, własności spektralne oraz obraz elektroforetyczny histonów wskazują, że preparaty chromatyny otrzymane opisaną metodą z kory nerek, śledziony i płuc wieprza wykazują podobny stopień czystości co preparaty otrzymane przy zastosowaniu ultrawirowania w gradiencie gęstości sacharozy.

Received 29 April, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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SECONDARY STRUCTURE AND TRANSCRIPTION OF THE "SUPER--FAST" REASSOCIATING DNA IN RAT LIVER

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1. A component of reiterated rat liver DNA reassociated to $C_0t=2\times10^{-4}$ has ordered secondary structure as revealed by melting profile and hydroxyapatite chromatography. 2. This component after digestion with single-strand specific endonuclease S_1 reveals about 6% of mismatched bases after reassociation. 3. On alkaline CsCl gradient no strand separation of DNA reassociated to $C_0t=2\times10^{-4}$ was observed. However, single-stranded molecules from light and heavy regions of the gradient exhibited ordered secondary structure when mixed together. 4. The component reassociating to $C_0t=2\times10^{-4}$ was poorly transcribed by *E. coli* RNA polymerase as compared to total DNA.

Previously (Szala & Chorąży, 1972) we have shown that the highly reiterated nucleotide sequences (about 6×10^6 copies) in rat genome are composed of three components with different rates of reassociation. The fastest reassociating component which amounts to 2 - 4% of the total DNA has $C_0t=10^{-5}$ (sec × mol × 1^{-1}); a second component amounting to 1 - 2% of the total DNA was half-renatured at $C_0t=5\times10^{-4}$ and the rest (2 - 4%) had $C_0t_{1/2}$ of 5. In this report we describe an attempt at separation of the complementary strands, and *in vitro* transcription of DNA reassociated to $C_0t=2\times10^{-4}$.

MATERIALS AND METHODS

Rat liver DNA was extracted and purified according to methods described previously (Szala, Kilarski & Chorąży, 1970). DNA solution (1 mg/ml) in 0.02 M-sodium phosphate buffer, pH 6.8, was gassed with N_2 and sonicated for 2 min in an ice-bath with a Dawe Sonicator set at position 8 (Melli & Bishop, 1970). After denaturation at 100°C for 10 min and fast cooling in an ice-bath, DNA preparations were reassociated to a $C_0t=2\times10^{-4}$ at 65°C in 0.02 M-sodium phosphate buffer, pH 6.8 (Yasmineh & Yunis, 1971). Separation of reassociated DNA from non-reassociated DNA was achieved by chromatography on hydroxyapatite column at 70°C. Reassociated DNA was digested with nuclease S_1 prepared from *Aspergillus*

oryzae according to Sutton (1971). The reaction mixture contained: 1 ml of DNA having $A_{260}=2.0$ in 0.02 M-NaCl, 1 ml 0.09 M-acetate buffer, pH 4.5, containing 9×10^{-5} M-$ZnCl_2$ and 0.03 M-NaCl, and 1 ml of nuclease S_1 (protein concn. 0.28 mg/ml). The mixture was incubated at 50°C for 30 to 90 min. After deproteinization with chloroform - isoamyl alcohol (10:1, v/v), the nuclease-resistant double-stranded fraction of DNA was separated from the degradation products by chromatography on hydroxyapatite column at 70°C. Degradation products were selectively eluted with 0.125 M-phosphate buffer, whereas the nuclease-resistant fraction was eluted with 0.250 M-phosphate buffer; this last fraction for convenience will be called the "super-fast" component (SF component). Ultracentrifugation of DNA in alkaline caesium chloride density gradients was performed according to Flamm, McCallum & Walker (1967). The reaction of SF component with formaldehyde (CH_2O) was conducted as described by Lazurkin, Frank-Kamenetskii & Trifonow (1970).

RNA polymerase was purified from *E.coli* K 12 by the method of Chamberlin & Berg (1962). The transcription of SF component *in vitro* was carried out at 37°C in the mixture containing: 0.025 ml of 1 M-tris buffer (pH 7.9), 0.025 ml of 6 mM-β-mercaptoethanol, 0.025 ml of 1 mM-solution each of ATP, GTP, CTP (Sigma Chem. Co.) and [^{3}H]UTP (The Radiochemical Centre, Amersham; spec. activity 1000 mCi/mmol), 0.010 ml of 0.2 M-$MgCl_2$ and 50 mM-$MnCl_2$, 0.100 ml DNA ($A_{260}=0.7$) in 0.02 M-NaCl, and 0.04 mg protein of RNA polymerase from *E.coli* in 0.025 ml. After incubation the reaction mixture was chilled in ice and 1 ml of bovine serum albumin (0.4 mg) was added, followed by 1.25 ml of 10% cold trichloroacetic acid. The precipitate was collected on HA 0.45 μ filters (Millipore), and washed three times with 3 ml of cold trichloroacetic acid. The filters were placed in scintillation vials and dissolved in a mixture of dioxane (1 ml) and concn. HCOOH (0.5 ml). After addition of 10 ml of scintillation liquid (3 g PPO+1 g POPOP in 1000 ml of toluene+methylene glycol, 7:10, v/v) the radioactivity was counted with a "Corumatic 200" liquid scintillation spectrometer (Tracerlab).

RESULTS AND DISCUSSION

Melting curves of DNA reassociated to a $C_0t=2\times10^{-4}$ are presented in Fig. 1. It may be seen that DNA in 0.25 M-phosphate buffer (pH 6.8) melted and reassociated almost completely (about 94% recovery of hyperchromicity) in three cycles of melting and cooling. The hyperchromic effect was in the range of 27 - 29%; the base composition determined from hypochromic spectrum according to Felsenfeld (1968) was 41 - 43 mol% G+C.

After digestion with nuclease S_1 which splits preferentially single-stranded DNA (Ando, 1966; Sutton, 1971), SF component dissolved in 0.02 M-NaCl showed a hyperchromic effect of 35%, and a more narrow transition (with T_m of 72°C), as compared to 27% and T_m of 69°C for a non-digested preparation (Fig. 2). The high hyperchromic effect of SF component indicates well matched DNA duplex (Britten & Kohne, 1968; Brahic & Fraser, 1971). However, the differential melting

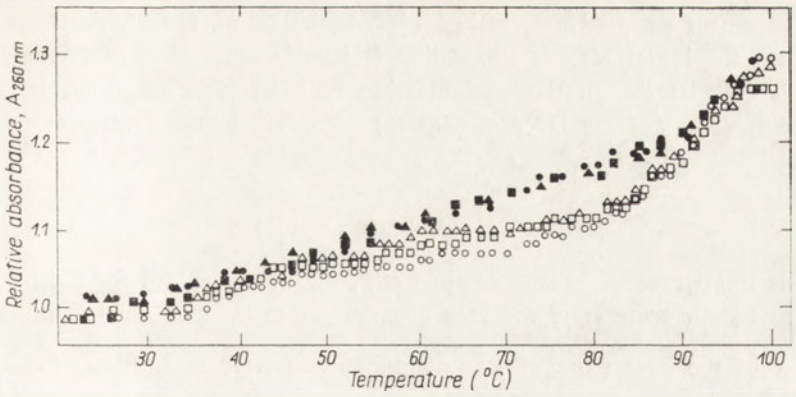

Fig. 1. Thermal denaturation and renaturation profiles of DNA reassociated to $C_0t=2\times10^{-4}$ and non-treated with nuclease S_1. DNA was dissolved in 0.250 M-Na-phosphate buffer, pH 6.8. Cycle of heating: (○) first, (△) second, and (□) third; cycle of cooling: (●) first, (▲) second, and (■) third.

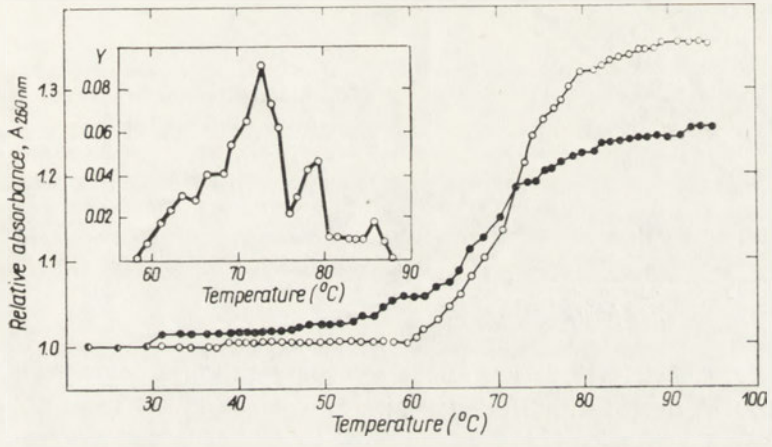

Fig. 2. Melting curve of DNA reassociated to a $C_0t=2\times10^{-4}$ (○), treated and (●), non-treated with nuclease S_1. Inset: Differenciated form of melting curve for DNA treated by nuclease S_1 $Y=\frac{A_{t_1}-A_{t_2}}{A_{100}-A_{25°}}(t_1-t_2)$ (according to Bernardi *et al.*, 1970). All DNA samples had $A_{260}=0.4$ and were dissolved in 0.02 M-NaCl.

profile (Bernardi *et al.*, 1970) of SF component in 0.02 M-NaCl showed (Fig. 2, inset) at least three peaks with T_m of 63.7°C, 72.9°C and 79.4°C, with G+C contents of 21.7 mol%, 44.2 mol% and 60 mol%, respectively, calculated according to Schildkraut & Lifson (1965).

From previous work (Szala & Chorąży, 1972) we know that multipeak patterns of differential elution profile of fast reassociated DNA reflect differences in base pairing rather than differences in base composition. In order to calculate the degree of defectiveness in secondary structure of DNA or percentage of mismatched bases

in SF component we used the method of Lazurkin *et al.* (1970). At a moderate temperature the initial rate of unwinding (I) under the action of formaldehyde (CH_2O) is correlated with the concentration of local violations (defects) in the secondary structure of the DNA duplex (c) according to the formulas:

$$I = pv + 2vc \tag{1}$$

$$\vartheta = \exp\{-(pv+2vc)\,t - pvt^2\} \tag{2}$$

where ϑ is the degree of helicity at time t; p, the rate constant for the initiation of the unwinding centers; v, the rate constant of growth of unwinding centers, v, number of the base pairs of an unwinding center. In coordinates $(-\frac{1}{t} \ln \vartheta)$ and (t) a straight line (Fig. 3) was obtained with the tangent of the slope being pv and the intercept $I = pv + 2vc$. Taking the data given by Lazurkin *et al.* (1970) for $pv \simeq 10^{-5}$ and for $v = 2.5$ min^{-1}, c for DNA non-treated with S_1 is equal to 0.10.

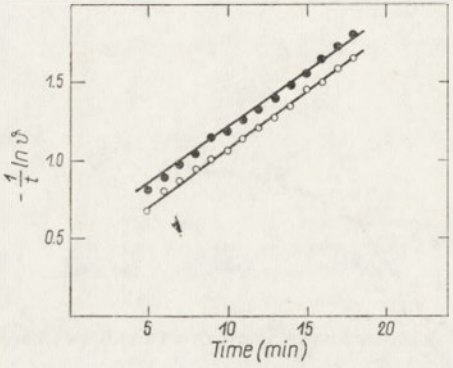

Fig. 3. Linear transformations of unwinding kinetic curves for DNA reassociated to a $C_0t = 2 \times 10^{-4}$, (○), treated and (●), non-treated with nuclease S_1. DNA samples having $A_{260} = 0.5$ were dissolved in 0.25 M-phosphate buffer, at the concentration of CH_2O of 3% (v/v); temperature 74.5°C. See text for other explanations.

That is, approximately 10 defects in the secondary structure occur for 100 base pairs. For DNA treated with S_1, $c = 0.06$, i.e. SF component has about 6% of mismatched bases. This value is low as compared to data of Brahic & Fraser (1971) who suggested that in the rapidly renaturing fraction of mouse DNA about 21% of bases are mismatched.

DNA reassociated to a $C_0t = 2 \times 10^{-4}$ and treated with nuclease S_1 was centrifuged in alkaline CsCl density gradient in a fixed-angle rotor (Flamm *et al.*, 1967). Only one peak was observed with a density of 1.760 g/cm^3 (Fig. 4). In a further search for a bias between complementary polynucleotide chains, the fractions corresponding to shadowed areas (called "H" and "L") were purified by chromatography on hydroxyapatite at 70°C (Fig. 5): about 60% of "H" fraction and about 60% of "L" fraction were eluted with 0.125 M-phosphate buffer, i.e. in a single-strand region. These denatured "H" and "L" fractions when mixed in equimolar proportions and reannealed at $C_0t = 10^{-4}$, reassociated to about 35% (Fig. 5). This simple experiment could be interpreted as showing that the fractions from the leading and trailing edges of the alkaline CsCl gradient contained the separated complementary strands of the SF component.

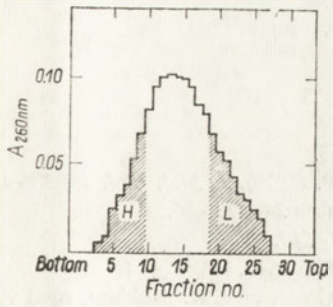

Fig. 4

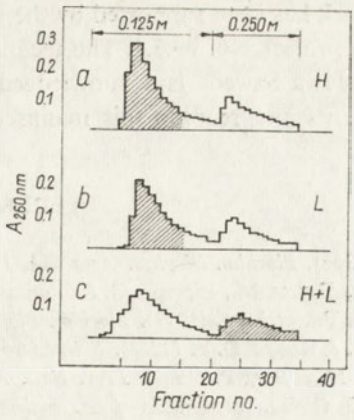

Fig. 5

Fig. 4. Alkaline CsCl sedimentation pattern of the DNA reassociated to $C_0t=2\times10^{-4}$, and digested with nuclease S_1. Centrifugation was conducted at 15° in Spinco no. 50 rotor, operated at 43 000 rev./ /min. for 24 h. Initial density $\rho=1.760$, pH 12.4, DNA concentration was $A_{260}=1.0$.

Fig. 5. Step-wise elution pattern of SF component from hydroxyapatite column after alkaline CsCl density gradient centrifugation; *a*, fractions "H"; *b*, fractions "L"; *c*, mixture of single-stranded fractions "H" and "L" (shadowed areas in diagrams *a* and *b*), incubated to a $C_0t=10^{-4}$. Fractions were dialysed against 0.02 M-NaCl, then loaded on a hydroxyapatite column (1.0×2.0 cm) and eluted at 70°C with indicated concentrations of Na-phosphate buffer, pH 6.8. Fractions of 15 drops were collected.

DNA reassociated at $C_0t=2\times10^{-4}$ was poorly transcribed by *E.coli* RNA polymerase. Figure 6 shows the amounts of [^{3}H]UMP incorporated into the trichloroacetic acid insoluble fraction at 37°C. With DNA reassociated to a $C_0t=2\times10^{-4}$ the synthesis of RNA was much smaller (after 60 min of incubation about 17 times) than that with native, sheared unfractionated rat DNA. The experiments confirm the results from Bishop's laboratory (Melli & Bishop, 1970; Melli *et al.*, 1971) that the rapidly renaturing sequences of rat liver DNA are poorly transcribed *in vitro* with bacterial RNA polymerase. The reason why these highly reiterated DNA sequences cannot be efficiently transcribed and the biological meaning of this phenomenon demand further studies.

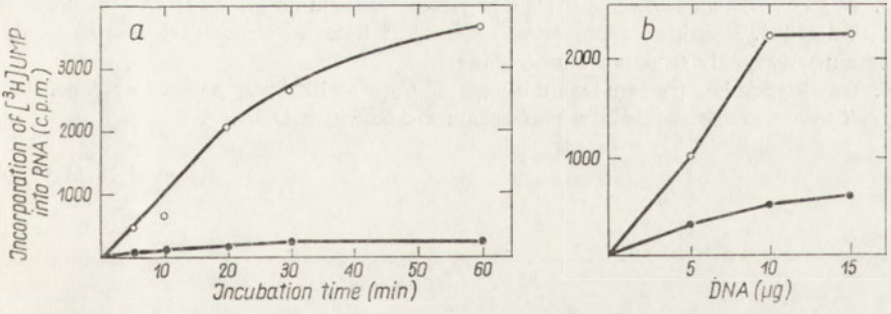

Fig. 6. Transcription *in vitro* with *E. coli* polymerase of (○), native sheared DNA and (●), SF component. *a*, Time-course; *b*, effect of DNA concentration. Incubation time was 60 min.

This work has been supported by the grant from the Polish Academy of Sciences within the project No. 09.3.1. The technical assistance of Miss Elżbieta Gara and Mrs. Zdzisława Szweda is acknowledged. We are grateful to Dr. E. M. Southern for comments and reading this manuscript.

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STRUKTURA DRUGORZĘDOWA I TRANSKRYPCJA SZYBKO REASOCJUJĄCEGO KOMPONENTU DNA WĄTROBY SZCZURA

Streszczenie

1. Komponent powtarzającego się DNA wątroby szczura reasocjujący do wartości $C_0t = 2\times10^{-4}$ posiada zorganizowaną strukturę drugorzędową, co wykazano krzywymi topnienia i chromatografią na hydroksyapatycie.

2. Komponent ten, po trawieniu specyficzną dla jednopasmowych cząsteczek endonukleazą S_1, wykazywał po reasocjacji około 6% niesparowanych zasad.

3. W gradiencie alkalicznego CsCl nie uzyskano rozdziału na pasma DNA renaturowanego do $C_0t = 2\times10^{-4}$. Jednakże jednopasmowe cząsteczki z lekkich i ciężkich rejonów gradientu po zmieszaniu wykazywały strukturę drugorzędową.

4. Transkrypcja komponentu renaturującego do $C_0t = 2\times10^{-4}$ przy pomocy RNA polimerazy z *E. coli* była znacznie obniżona w porównaniu z całkowitym DNA.

Received 18 May, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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INTERMEDIATES IN THE SYNTHESIS OF PURINES AND PTERIDINES: SELECTIVE HYDROLYSIS OF CHLOROPYRIMIDINES

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1. Alkaline hydrolysis of 2,4,6-trichloropyrimidine at room temperature leads to 4,6-dichloro-2-hydroxypyrimidine, which may be isolated on a preparative scale in good yield. At higher temperatures, the final product is 6-chlorouracil. The latter may be more readily obtained by acid hydrolysis at room temperature of 4,6-dichloro-2-hydroxypyrimidine. 2. Alkaline hydrolysis of 2,4-dichloropyrimidine to uracil has been shown to proceed *via* 4-chloro-2-hydroxypyrimidine, which may be isolated on a preparative scale in crude form. 3. The foregoing product may be converted directly to 4-alkoxy-2-hydroxypyrimidines, providing a simpler route to these useful derivatives than alkaline hydrolysis of 2,4-dialkoxypyrimidines. 4. Both 4-chloro-2-hydroxypyrimidine and 4,6-dichloro-2-hydroxypyrimidine are acid labile. Their $t_{1/2}$ values for conversion to uracil and 6-chlorouracil at pH 1 and room temperature are, respectively, 26 and 31 min. 5. Some applications of the foregoing derivatives in various syntheses are described.

Various chloropyrimidines are useful intermediates in the synthesis of purines and pteridines (Nübel & Pfleiderer, 1962), as well as other pyrimidine analogues (Hilbert & Johnson, 1930; Kulikowski & Shugar, 1971).

While there does not appear to be any established order of attack of nucleophiles on the various positions of the chloropyrimidine ring, there is a clear specificity with regard to the nature of the nucleophile. For example, treatment with sodium methoxylate of 2,4-dichloropyrimidine and 2,4,6-trichloropyrimidine gives initially 4-methoxy, then 2,4-dimethoxy- and, finally (in the case of the tri-chloro derivative), 2,4,6-trimethoxypyrimidine (Fisher & Johnson, 1932; Kenner, Reise & Todd, 1955).

Alkaline hydrolysis of halogenopyrimidines has not been widely investigated. Hydrolysis of 4,6-dichloro- or 4,6-dibromopyrimidine in NaOH yielded 4-halogeno--6-hydroxypyrimidines[1] (Brown & Harper, 1961). Two stages have been noted in the alkaline hydrolysis of 2,4,6-trifluoropyrimidine, the first leading to 4,6-difluoro-

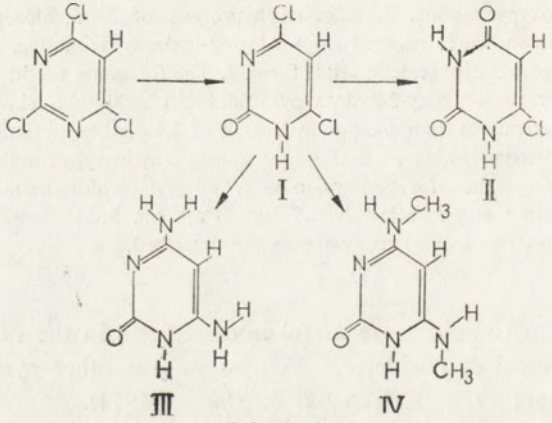
[1] The nomenclature used does not necessarily reflect the tautomeric forms of the compounds.

-2-hydroxypyrimidine, the second to 6-fluorouracil (Nemets, Ivin & Slesarev, 1965).

We herein describe the results of a brief study on the alkaline hydrolysis of 2,4-dichloropyrimidine and 2,4,6-trichloropyrimidine, both of which are readily accessible as starting products (Whittaker, 1951; Wempen & Fox, 1963). It had previously been observed by Cresswell & Wood (1960) that alkaline hydrolysis of 2,4,6-trichloropyrimidine yields 6-chlorouracil. We now show that, as might have been anticipated, this reaction proceeds in two stages, the first of which leads to 4,6-dichloro-2-hydroxypyrimidine, which is readily isolated. It has also been found that the hydrolysis of 2,4-dichloropyrimidine is a two-stage process, leading to the potentially useful 4-chloro-2-hydroxypyrimidine and the even more useful 4-alkoxy-2-hydroxypyrimidines, normally attainable by the rather tedious alkaline hydrolysis of 2,4-dialkoxypyrimidine (Hilbert & Jansen, 1935; Szer & Shugar, 1968).

RESULTS AND DISCUSSION

Treatment of 2,4,6-trichloropyrimidine in aqueous dioxane, brought to alkaline reaction with NaOH, led to hydrolysis at room temperature with formation of 4,6-dichloro-2-hydroxypyrimidine (I, see Scheme 1). The sodium salt of the latter



Scheme 1

precipitated out during the course of the reaction and, at room temperature, no futher hydrolysis (to 6-chlorouracil, II) was detectable when the reaction was followed chromatographically (Table 1). The use of aqueous dioxane or acetone makes it possible to conduct hydrolysis in a single phase, at the same time permitting direct isolation of the product because of the low solubility of the sodium salt.

Further hydrolysis of 4,6-dichloro-2-hydroxypyrimidine may be carried out in the same medium, but under considerably more drastic conditions, viz. heating under reflux for 18 hours (Cresswell & Wood, 1960). However, 4,6-dichloro-2--hydroxypyrimidine is extremely labile in acid medium, undergoing ready conversion to 6-chlorouracil (II). The course of this reaction at pH 1 can readily be followed spectrophotometrically, as shown in Fig. 1. The isosbestic point indicates that

Table 1

R_F *values of pyrimidine derivatives*

Ascending paper chromatography with Whatman paper no. 1 in the solvent systems: (*A*) water-saturated *n*-butanol; (*B*) *n*-butanol - acetic acid - water (4:1:1, by vol.); (*C*) isopropanol - conc. NH_4OH - water (7:2:1, by vol.).

Compound	Solvent systems		
	A	*B*	*C*
4,6-Dichloro-2-hydroxypyrimidine (I)	0.27	0.82	0.67
6-Chlorouracil (II)	0.21	0.58	0.45
4,6-Diamino-2-hydroxypyrimidine (III)	0.08	0.17	0.17
4,6-Dimethylamino-2-hydroxypyrimidine (IV)	0.03	0.28	0.04
4-Chloro-2-hydroxypyrimidine (V)	0.17	0.51	0.41
Uracil (VI)	0.30	0.32	0.30
Cytosine (VII)	0.24	0.18	0.36
4-Ethoxy-2-hydroxypyrimidine (VIII)	0.41	0.45	0.54

hydrolytic conversion is quantitative, in agreement with the known acid stability of 6-chlorouracil (Wempen & Fox, 1964; Kazimierczuk & Shugar, 1970). This acid lability of I was profited from on a preparative scale, as described in Experimental.

As might be anticipated, 4,6-dichloro-2-hydroxypyrimidine exhibits a single ionic equilibrium with a pK, determined by spectral titration (Fig. 2), of 4.05. The strong acid character of this derivative is due to the inductive effect of the chlorine substituents, as in 6-chlorouracil (Pfleiderer & Deiss, 1968) and 6-fluorouracil (Wempen & Fox, 1964) the pK values of which are 5.75 and 4.03, respectively.

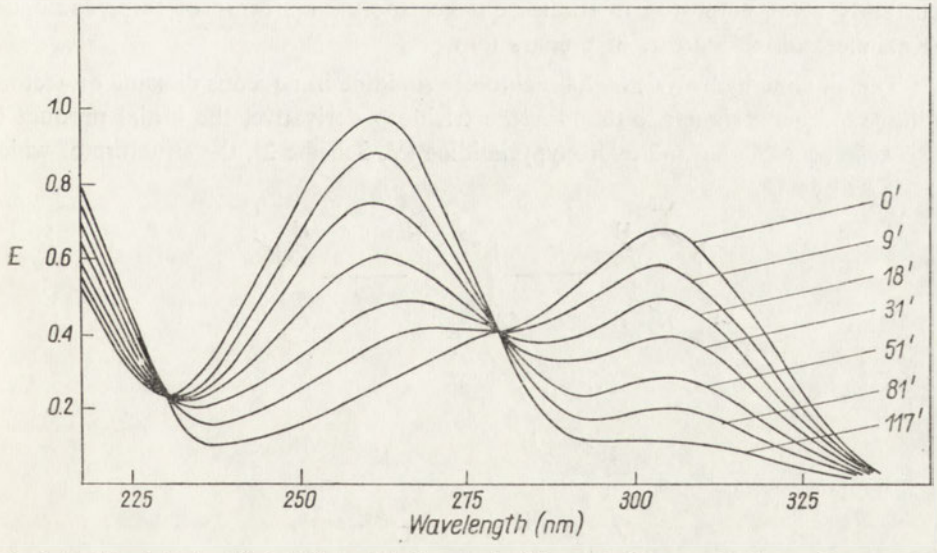


Fig. 1. Acid hydrolysis of 4,6-dichloro-2-hydroxypyrimidine to 6-chlorouracil. Conversion at pH 1.0 and 20°C, followed spectrally in a 10-mm cuvette. Numbers beside each curve refer to the time, in minutes. The $t_{1/2}$ for the reaction is 31 min.

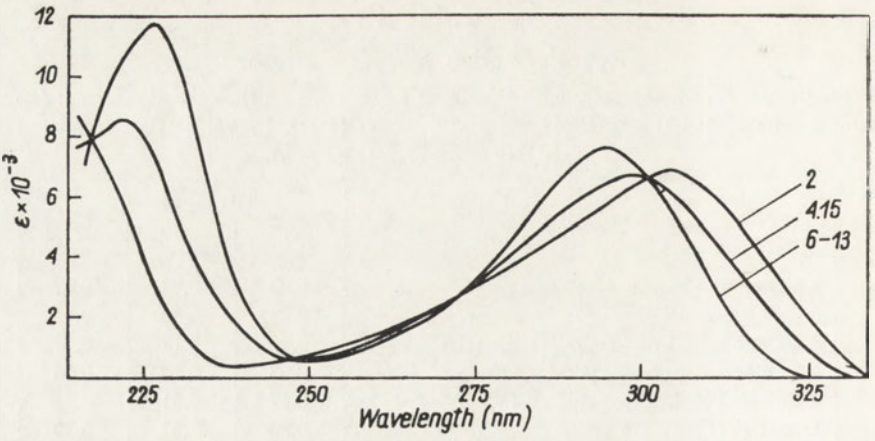
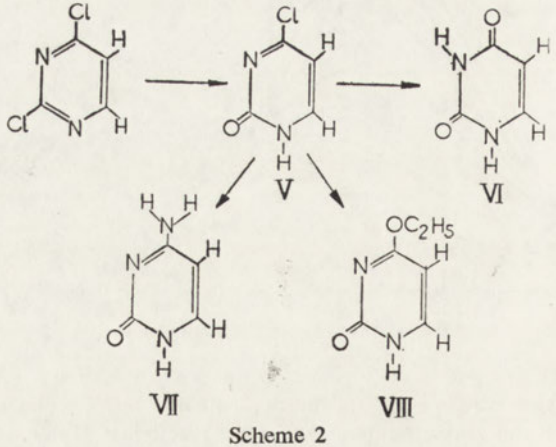


Fig. 2. UV absorption spectrum of 4,6-dichloro-2-hydroxypyrimidine at pH values indicated beside each curve. The 4 isosbestic points indicate the presence of one ionic equilibrium, with a pK of 4.05±0.05.

Like other chloropyrimidines, 4,6-dichloro-2-hydroxypyrimidine readily undergoes nucleophilic substitution, and treatment of this compound with alcoholic ammonia or methylamine, under anhydrous conditions at 140 - 150°C, gave 4,6--diamino-2-hydroxypyrimidine (III, Scheme 1) and 4,6-dimethylamino-2-hydroxypyrimidine (IV). It should be noted that at a lower temperature, 100 - 120°C, amination of I gave, in addition to III, 4-amino-6-chloro-2-hydroxypyrimidine, i.e. 6-chlorocytosine (Pfleiderer & Fink, 1962). The foregoing have been used as intermediates in the preparation of a series of alkylated isoguanines, which are currently being employed in studies on the tautomeric forms of these analogues (Kazimierczuk & Shugar, in preparation).

The alkaline hydrolysis of 2,4-dichloropyrimidine in aqueous dioxane or acetone follows a course similar to that for the trichloro derivative, the initial product in this case being 4-chloro-2-hydroxypyrimidine (V, Scheme 2), the structure of which



Scheme 2

was confirmed by its conversion to cytosine (VII) and 4-ethoxy-2-hydroxypyrimidine (VIII), as shown in Scheme 2. Attempts to obtain V in crystalline form were unsuccessful, largely due to contamination by uracil, which could not be removed without further hydrolysis of the monochloro derivative.

However, treatment of the crude 4-chloro-2-hydroxypyrimidine with sodium ethoxylate in anhydrous ethanol led to the ready isolation in crystalline form of 4-ethoxy-2-hydroxypyrimidine (VIII) in more than 40% yield. This procedure should be quite general for the synthesis of 4-alkoxy-2-hydroxypyrimidines from 2,4-dichloropyrimidine, as shown in Scheme 2, and therefore provides a convenient method of obtaining these compounds for various preparative purposes.

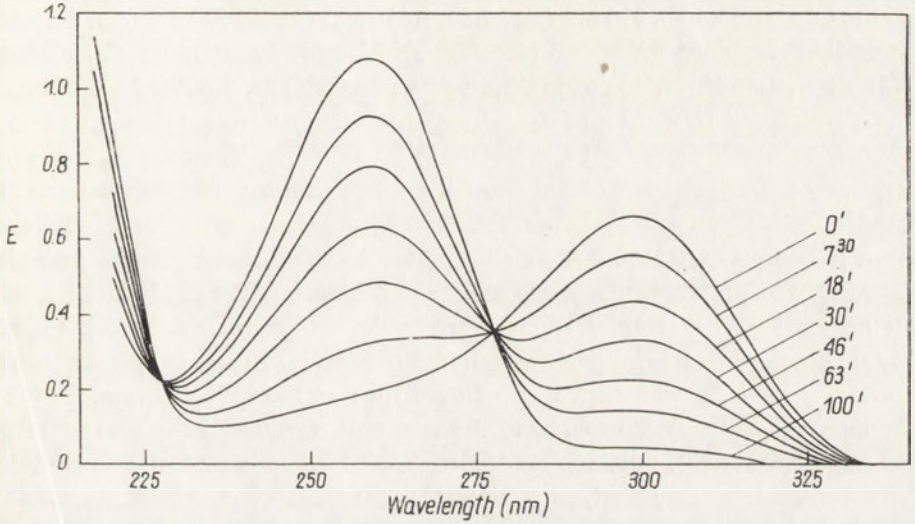


Fig. 3. Acid hydrolysis of 4-chloro-2-hydroxypyrimidine to uracil. Conversion at pH 1 and 20°C, followed spectrally in a 10-mm cuvette, the figures beside each curve indicating the time in minutes. $t_{1/2}$ for reaction is 26 min.

Mention should be made also of the extreme acid lability of 4-chloro-2-hydroxypyrimidine, accompanied by quantitative conversion to uracil, a reaction which is readily followed spectrally (see Fig. 3), and which proceeds at room temperature with a $t_{1/2}$ of only 26 min.

EXPERIMENTAL

Materials and Methods. Preparations of the starting compounds, 2,4,6-trichloropyrimidine (Wempen & Fox, 1963) and 2,4-dichloropyrimidine (Whittaker, 1951), were according to standard procedures. Both of these are also readily available commercially.

Melting points (uncorrected) were measured on a Boetius microscope hot plate. Ultraviolet absorption spectra were run on a Zeiss VSU-2 instrument and on a Unicam SP-800 recording spectrophotometer. Measurements of pH made use

of a Radiometer PHM-22 meter fitted with a glass electrode. Chromatographic data for the various compounds and intermediates are provided in Table 1.

4,6-Dichloro-2-hydroxypyrimidine (*I*): To a solution of 7 g (38 mmol) of 2,4,6-trichloropyrimidine in 100 ml dioxane was added 3.1 g (78 mmol) of NaOH in 30 ml water, and the whole stirred vigorously for 3 h, during the course of which a white precipitate of the sodium salt of 4,6-dichloropyrimidine settled out. The reaction mixture was then carefully brought to neutrality with dilute HCl, and concentrated almost to dryness. The residue was filtered off on a sintered glass filter, washed with a small volume of water, and dried to give 5.5 g (75%) of a white product, m.p. 273 - 275°C (decomp.), (reported 262°C, Koppel, Springer, Robins & Cheng, 1961) suitable as such for further preparations. UV spectrum, λ_{max} (pH 12) 225 nm (ε_{max} 11.6×10^3), 296 nm (ε_{max} 7.5×10^3); λ_{max} (pH 2) 304 nm (ε_{max} 6.2×10^3); spectrally determined $pK = 4.05$ (see Fig. 3). Elementary analysis: Calculated C, 23.41%; H, 1.46%; N, 13.66%; Obtained C, 22.95%; H, 1.68%; N, 13.91%.

6-Chlorouracil (*II*): 12 g of 2,4,6-trichloropyrimidine was taken up in 75 ml dioxane, to which was added 6 g NaOH in 50 ml water. The mixture was stirred vigorously for 3 h and left overnight at room temperature. The solution was then acidified by addition of 30 ml conc. HCl and heated under reflux for 2 h, following which it was concentrated to a small volume, and the resulting white precipitate filtered off and washed with a small volume of cold water. The product (8.5 g, 90%) was identical with that obtained by other methods (Cresswell & Wood, 1960).

4,6-Diamino-2-hydroxypyrimidine (*III*): 1 g of I in 10 ml anhydrous ethanol saturated with NH_3 was heated in a sealed tube at 140 - 150°C overnight (12 h). The mixture was cooled, brought to dryness, and crystallized from water to give 0.54 g of white needles, m.p. 300°C (decomp.), of 4,6-diamino-2-hydroxypyrimidine. Yield 88%. Elementary analysis: Calculated: C, 38.09%; H, 4.76%; N, 44.44%; Found: C, 37.78%; H, 4.91%; N, 44.63%. UV spectrum: pH 2: λ_{max} 272 nm; λ_{min} 242 nm. pH 12: λ_{max} 269 nm; λ_{min} 245 nm. The product was identical with that reported by Bendich, Tinker & Brown (1948), obtained by another route.

4,6-Dimethylamino-2-hydroxypyrimidine (*IV*): 4 g of I and 30 ml dimethylamine in 50 ml anhydrous ethanol was heated in a steel bomb at 140 - 150°C for 18 h. The mixture was cooled, brought to dryness and the residue crystallized from 2 N-H_2SO_4 to yield 2.2 g of 4,6-dimethylamino-2-hydroxypyrimidine sulphate ($C_6H_{10}N_4O\cdot H_2O\cdot{}^1/_2H_2SO_4$); yield 52%. Elementary analysis: Calculated: C, 32.46%; H, 5.86%; N, 25.44%; Found: C, 31.93%; H, 5.46%; N, 24.78%. UV data: pH 2: λ_{max} 278 nm; λ_{min} 242 nm. pH 12: λ_{max} 273 nm; λ_{min} 248 nm.

4-Chloro-2-hydroxypyrimidine (*V*): 3 g (0.02 mol) of 2,4-dichloropyrimidine in 20 ml dioxane was added to 40 ml 1 N-NaOH (0.4 mol) slowly, with stirring. Following overnight storage in the cold, a precipitate was removed, and the filtrate concentrated at the lowest possible temperature to yield 1 g of the sodium salt of V, and an additional 0.5 g from the filtrate, contaminated with 15% uracil. Total yield 45%.

Cytosine (*VII*): 1.5 g of the crude sodium salt of V in 20 ml of ammoniacal anhydrous ethanol was heated at 120°C for 12 h. The resulting crude, white, product

following crystallization from methanol - water, yielded 0.45 g (41% yield) of cytosine, identical chromatographically and spectrally with a standard sample.

4-Ethoxy-2-hydroxypyrimidine (*VIII*): 252 mg (11 mmol) sodium was dissolved in 20 ml anhydrous ethanol, to which was added 850 mg (7 mmol) of the sodium salt of 4-chloro-2-hydroxypyrimidine. The mixture, in a sealed tube, was heated on a water bath for 4 h. The mixture was then brought to dryness under reduced pressure, the residue dissolved in 50 ml water and the pH brought to 3.5 with acetic acid. The solution was extracted three times with 50-ml portions of chloroform, and the combined chloroform extracts brought to dryness. The resulting residue was crystallized from water to give 310 mg of VIII (42% yield), m.p. 165 - 168°C (reported 167 - 167.5°C, Szer & Shugar, 1968), chromatographically and spectrally identical with the sample prepared by alkaline hydrolysis of 2,4-diethoxypyrimidine (Hilbert & Jansen, 1935; Szer & Shugar, 1968).

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SUBSTRATY W SYNTEZIE PURYN I PTERYDYN. SELEKTYWNA HYDROLIZA CHLOROPIRYMIDYN

Streszczenie

1. W wyniku hydrolizy alkalicznej 2,4,6-trójchloropirymidyny w temperaturze pokojowej otrzymuje się 4,6-dwuchloro-2-hydroksypirymidynę, którą można z dobrą wydajnością wyodrębniać w skali preparatywnej. W wyższej temperaturze produktem końcowym hydrolizy alkalicznej jest 6-chlorouracyl. Jednakże związek ten można łatwiej uzyskać przez hydrolizę kwasową 4,6-dwuchloro-2-hydroksypirymidyny w temperaturze pokojowej.

2. Wykazano, że 4-chloro-2-hydroksypirymidyna jest związkiem pośrednim w hydrolizie alkalicznej 2,4-dwuchloropirymidyny do uracylu. Ten związek pośredni można wyodrębniać w surowej postaci w skali preparatywnej.

3. 4-Chloro-2-hydroksypirymidynę można przekształcić bezpośrednio w 4-alkoksy-2-hydroksypirymidyny, co pozwala na otrzymanie tych związków w prostszy sposób niż przez alkaliczną hydrolizę 2,4-dwualkoksypirymidyn.

4. 4-Chloro-2-hydroksypirymidyna jak i 4,6-dwuchloro-2-hydroksypirymidyna rozkładają się w kwaśnym środowisku. Wartości $t_{1/2}$ w przemianie prowadzącej do uracylu i 6-chlorouracylu w pH 1 w temperaturze pokojowej wynoszą odpowiednio 26 i 31 minut.

5. Omówiono niektóre zastosowania otrzymanych pochodnych w różnego rodzaju syntezach.

Received 18 May, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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RESISTANCE TO SULPHATE ANALOGUES IN *SALMONELLA TYPHIMURIUM*

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Mutants of *Salmonella typhimurium* — named *cysL* — resistant to chromate and selenate were isolated; the mutation mapped in a locus closely linked with the gene *cysA*. *CysL* mutants showed elevated activity of sulphate permease and sulphite reductase. The previously isolated mutants *cysB* with constitutive control of cysteine biosynthesis were found to be resistant to selenate but not to chromate. Selenate-resistance and the enzymic activities were repressed in *cysL*, but not in *cysB*, mutants by growing the bacteria in the presence of methionine. Chromate and selenate had a smaller inhibitory effect on sulphate transport in *cysL* mutants as compared with *cysL*$^+$ strain.

Inhibition of bacterial growth by selenate was shown to be reversible by cysteine; this suggested that the toxic effect was due to interference with sulphate metabolism (Pasternak, 1962). Subsequently it was proved by Thompson (1967) that selenate is a substrate of APS[1] synthetase, and that the APS selenium analogue formed can be phosphorylated by APS kinase. The same author has also established that the selenium analogues of APS and PAPS are less stable than the natural intermediates of sulphate assimilation pathway. In addition, therefore, to competition for active sites of the two sulphate activating enzymes, selenate leads to energy drain.

The inhibitory effect of chromate is not reversible by cysteine; one can assume, therefore, that its toxic action is not concerned with cysteine biosynthesis or has multiple targets (Roberts & Marzluf, 1971). The latter alternative is supported by the finding that chromate-resistant mutants are cysteine auxotrophs, defective in sulphate transport (Pardee, Prestidge, Whipie & Dreyfuss, 1966).

In this study prototrophic mutants of *Salmonella typhimurium* resistant to selenate and chromate were isolated. They map in locus named *cysL*, which is located close to *cysA* gene coding sulphate permease. In the isolated mutants grown in the presence

[1] Abbreviations: APS, adenosine 5′-phosphosulphate; ICR 191, code name for a quinacrine derivative; NG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PAPS, 3′-phosphoadenosine 5′-phosphosulphate.

of sulphate as a sole sulphur source, the activity of sulphate permease was increased as well as that of the other enzymes of sulphate assimilation. Mutants *cysB* with constitutive cysteine biosynthesis have been found to be resistant to selenate, but not to chromate.

MATERIALS AND METHODS

Strains. All bacterial strains listed in Table 1 were derivatives of *Salmonella typhimurium* LT2. For transduction mutant L4 of phage P22 was used (Smith & Levine, 1967).

Table 1

Strains of S. typhimurium *used*

Strain designation	Genotype	Origin
LT2	wild type	B. N. Ames
cysA20	*cysA20*	D. A. Smith
purC7	*purC7*	J. S. Gots
purF145	*purF145*	J. S. Gots
TK292	*hisD3512 serA13* HfrK5	T. Kłopotowski
TK302	*hisD3512* HfrK5	by transduction of TK292
TK344	*cysL1366, hisD3512* HfrK5	isolated in TK302, on selenate plate
TK345	*cysL1367 hisD3512* HfrK5	isolated in TK302, on chromate plate
TK346	*cysL1373 purF145*	isolated in *purF145*
TK358	*cysL1368 purC7*	isolated in *purC7*, on selenate plate
TK359	*cysL1369 purC7*	isolated in *purC7*, on chromate plate
TK360	*cysL1373 purF145*/F′ *purG*$^+$ *metG*$^+$	by episome transfer from TR340 to TK346
DW25	*cysB1352*	N. M. Kredich
TR340	*hisE35 metG319 purG302*/F′ *purG*$^+$ *metG*$^+$	J. R. Roth

Media, culture, conditions, growth measurement and techniques of transduction and conjugation were the same as previously described (Hulanicka, Kłopotowski & Smith, 1972).

Selenate was added to the medium to 2 mM final concentration before autoclaving. Chromate containing medium was prepared by addition of the inhibitor to 0.25 mM final concentration after autoclaving.

Enzyme assays. The activity of sulphate permease was determined in the cells harvested in logarithmic phase, washed twice and resuspended in sulphate-free medium BS containing 30 μg of chloramphenicol and 2 mg of glucose per ml. To 0.45 ml of cell suspension (about 25 μg of dry wt.) preincubated for 10 min at room temperature, 0.05 ml of substrate was added. The substrate solutions contained alternatively in 0.05 ml: 5 nmol of Na-[^{35}S]sulphate (about 1 μCi), 0.5 nmol of

Na-[^{51}Cr]chromate (about 1 μCi) or 1.5 nmol of Na-[^{75}Se]selenate (about 1 μCi). After incubation, cells were separated from incubation medium by filtration on membrane filters (Coli 5, Biomed, Warszawa or Synpor 6, pore size 0.4 μm, Chemapol, Prague, Czechoslovakia), and extensively washed with about 30 ml of sulphate-free medium. Filters were dried under infrared lamp and counted in the standard scintillation fluid using Packard Tri-Carb counter, Model 3320. In the alternative procedure, the reaction mixture contained 2 - 5 mg of dry weight of bacteria and the incubation was terminated by pouring the mixture into columns of 1 cm diameter containing 7 ml (bed volume) of Sephadex G-50, coarse, soaked in BS medium. Bacteria usually passed through the column in less than 30 sec. Unabsorbed [^{35}S] sulphate did not appear earlier than in the sixth drop. The first three drops of turbid bacterial suspension were collected, diluted and their optical density measured at 420 nm for calculating dry weight. Aliquots of the cell suspension were dried on aluminium planchets and counted in Nuclear Chicago Biospan gas-flow counter. The uptake of [^{35}S]sulphate and [^{75}Se]selenate could be measured by either procedure; for determination of [^{51}Cr]chromate uptake only the column procedure was used.

Sulphite reductase was assayed in bacteria grown and harvested as described above, and washed with 0.1 M-tris buffer, pH 7.8. Cell suspensions were shaken at 37°C for 15 min with 10 μl of toluene per ml. In the toluenized cells the enzyme was determined by the procedure of de Vito & Dreyfuss (1964).

Chemicals: Glucose 6-phosphate and disodium ATP (Sigma, St. Louis, Mo., U.S.A.); 1,2,4-triazole and L-aminoacids (Calbiochem., Los Angeles, Calif., U.S.A.); chloramphenicol (Polfa, Warszawa, Poland); sodium [^{51}Cr]chromate and carrier-free sodium [^{35}S]sulphate (Instytut Badań Jądrowych, Świerk, Poland); sodium [^{75}Se]selenate (The Radiochemical Centre, Amersham, England).

RESULTS

Isolation of cysL *mutants and their mapping*. Inoculation of minimal agar plates containing 2 mM-Na-selenate or 0.25 mM-Na-chromate with about 2×10^8 cells of *Salmonella typhimurium*, gave rise to several colonies of resistant mutants after three to four days of incubation at 37°C. Number of the mutants could be increased by putting NG, diethylsulphate or ICR 191 on plates seeded with bacteria. Mutagen-induced resistant mutants appeared as rings of colonies growing around the inhibition zones produced by the mutagens.

For mapping, the isolated mutants of Hfr strains were used as donors in conjugation with a series of auxotrophic recipients. For finer mapping, phages grown on *cysL* mutants were used as donors. Table 2 shows that *cysL* mutations are 84 to 96% linked to *cysA* gene. As all the sulphate permease negative mutants map within the region deleted in mutant *cysA20* (Ohta, Galsworthy & Pardee, 1971) and the linkage of *cysL* with *cysA20* was less than 100%, it can be concluded that *cysL* locus maps outside *cysA20* deletion and thereby, quite likely, outside the structural gene for sulphate permease.

Table 2

Mapping of cysL *mutants by transduction*

Strain *cysA20* was recipient for all the crosses. In each case 200 colonies were tested.

Donor strain	Pertinent genotype	Recombinant colonies		Linkage (%)
		Sel-R	Chr-R	
TK344	*cysL1366*	192	192	96
TK345	*cysL1367*	174	174	87
TK358	*cysL1368*	180	180	90
TK359	*cysL1369*	176	176	88

The gene order in this region was previously established to be *cysA cysL trzA* (Hulanicka & Kłopotowski, 1972). This order was confirmed with several *cysL* and *cysA* mutants used.

Properties of cysL *mutants.* As shown in Fig. 1, growth of sensitive strain TK302 was inhibited more than 90% by 0.2 mM-selenate and completely by 0.1 mM-chromate. Mutant TK344 (*cysL1366*) isolated on selenate plate, grew in the presence of either inhibitor at about the same rate as in their absence. However, selenate — depending on concentration — delayed normal growth by 30 - 60 min. Growth of mutant TK345 (*cysL1367*) resistant to chromate was precisely the same as that of mutant TK344.

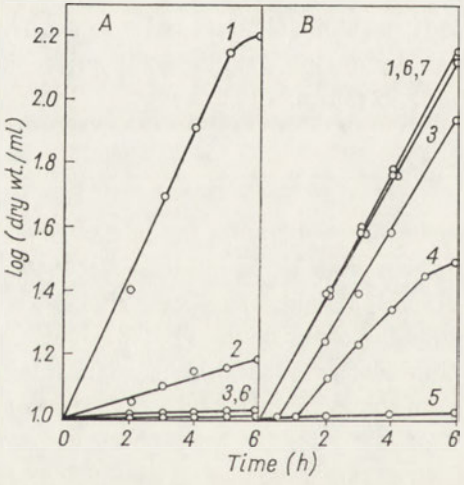

Fig. 1

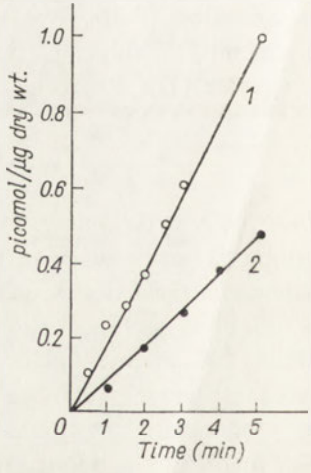

Fig. 2

Fig. 1. Resistance of *cysL* mutants to selenate and chromate. Mutants: *A*, TK302 (*cysL*$^+$) and *B*, TK344 (*cysL1366*). *1*, Control; media supplemented with selenate: 0.2 mM (*2*), 2 mM (*3*), 4 mM (*4*), 10 mM (*5*), or chromate: 0.025 mM (*6*), 0.25 mM (*7*).

Fig. 2. Transport of $^{35}SO_4$ by *cysL* mutants: *1*, TK344 (*cysL1366*) and *2*, TK302 (*cysL*$^+$). Cells were incubated at 22°C in the medium supplemented with glucose (0.2%), chloramphenicol (0.003%) and 0.01 mM-$Na_2{}^{35}SO_4$ (1 µCi/ml); at proper intervals the samples were withdrawn, cells collected on membrane filters and immediately washed with saline.

Comparative data presented in Table 3 illustrate growth behaviour of *cysL*, *cysA* and constitutive *cysB* mutants in the presence of chromate or selenate on the minimal agar plate with sulphate or L-djenkolate as a sole sulphur source.

Table 3

Growth of wild type and cys *mutants in the presence of selenate and chromate*

Sulphate or L-djenkolate in concn. of 10^{-4} M were used as alternative sulphur sources. Concentration of added selenate and chromate was 2 mM and 0.25 mM, respectively. Scoring was performed by replica-plating and 24 h of incubation at 37°C. Growth was recorded as (+) or (−).

Strain	Pertinent genotype	Sulphate			L-Djenkolate		
		without inhibitor	selenate	chromate	without inhibitor	selenate	chromate
TK302	*cysL*$^+$	+	−	−	+	+	−
TK344	*cysL1366*	+	+	+	+	+	+
TK345	*cysL1367*	+	+	+	+	+	+
DW25	*cysB1352*	+	+	−	+	+	−
cysA20	*cysA20*	−	−	−	+	+	+

These results demonstrate three different types of resistance to sulphate analogues. Strain TK302, sensitive to both sulphate analogues, could grow on selenate plate when L-djenkolate was a sulphur source, while chromate inhibited growth of this strain both on sulphate and L-djenkolate. *CysL* mutants grew well under all conditions studied. Cysteine constitutive mutant *cysB1352* was resistant to selenate, but not to chromate. Mutant *cysA20* having a deletion in sulphate permease gene (Ohta *et al.*, 1971) did not grow on sulphate but grew well on the djenkolate plates; it was resistant to chromate and apparently to selenate.

More pronounced differences in toxic effects of chromate and selenate have been found when methionine-supplemented plates were used (Table 4). Methionine abolished the resistance to selenate of *cysL* mutants, but did not affect that of *cysB* constitutive mutant. It had no effect on chromate sensitivity or resistance in any of the strains examined.

Table 4

Effect of L-*methionine upon growth of mutants in the presence of selenate or chromate*

Methionine (0.1 mM) was added to the plates containing alternatively selenate (2 mM) or chromate (0.25 mM). Scoring was performed by replica-plating and 24 h of incubation at 37°C. Growth was recorded as (+) or (−).

Strain	Pertinent genotype	Selenate		Chromate	
		without methionine	with L-methionine	without methionine	with L-methionine
TK344	*cysL1366*	+	−	+	+
TK345	*cysL1367*	+	−	+	+
DW25	*cysB1352*	+	+	−	−

Interference by methionine was observed not only with *cysL* mutants but also with *cysL*$^+$ and *cysB* strains. This has been proved in the following experiment. Few drops of nutrient broth cultures of the strain LT2 were diluted with warm 0.6% agar and poured over minimal and methionine-supplemented agar plates. After solidification of agar, 5 µmol of Na-selenate was pipetted onto filter paper disc put on the agar surface. Inhibition zones were observed after overnight incubation at 37°C. On methionine plate these zones were of a diameter three times greater than that on the unsupplemented plates, i.e. 60 and 20 mm, respectively.

Transport of sulphate, chromate and selenate. Transport of sulphate proceeded in *cysL* mutants at rates about three times higher than in the control *cysL*$^+$ strain (Table 5). Transport of chromate was reduced in these mutants and that of selenate was unchanged in the only *cysL* mutant studied in this respect. Sulphate permease negative mutant *cysA20* was completely unable to transport any of the studied anions.

Table 5

Transport of sulphate and its analogues

The investigated strains were grown on sulphate, except for *cysA20* which was grown on djenkolate as sulphur source.

Strain	Pertinent genotype	(pmol/min/µg of dry wt.)		
		[^{35}S]sulphate	[^{51}Cr]chromate	[^{75}Se]selenate
TK302	*cysL*$^+$	0.12	0.017	0.016
TK344	*cysL1366*	0.33	0.007	0.018
TK345	*cysL1367*	0.38	0.005	—
cysA20	*cysA20*	0.00	0.000	0.000

Kinetics of sulphate uptake by *cysL* mutants is presented in Fig. 2. It can be seen that sulphate was taken up linearly with time by cells of both TK344 (*cysL1366*) mutant and TK302 (*cysL*$^+$) strain, but at different rates. This proves that *cysL* mutants are able to transport sulphate at increased rates.

An attempt was made to compare K_m values for sulphate permease in *cysL*$^+$ strain and *cysL* mutants. The calculated K_m values fall in the range 0.015 - 0.05 mM (Table 6).

Table 6

K_m *values of sulphate permease*

Strain	Pertinent genotype	K_m (mM)
LT2	*cysL*$^+$	0.030
TK302	*cysL*$^+$	0.030
purF145	*cysL*$^+$	0.025
TK344	*cysL1366*	0.050
TK346	*cysL1373*	0.015

Data given in Table 7 show that chromate and selenate had less effect on sulphate uptake by *cysL* mutants than by their parent strain; 0.1 mM-selenate decreased sulphate transport in *cysL*$^+$ strain by 43%, but had no effect on *cysL1366* mutant. Chromate present in the assay medium at the same concentration completely abolished sulphate uptake by parental strain, whereas the uptake by the mutant was reduced by a half. These results show that *cysL* mutations provide an advantage for transport of sulphate in competition with chromate or selenate.

Table 7

Inhibition of sulphate uptake by sulphate analogues

Inhibitors were added in concentration of 0.1 mM to the medium containing 0.01 mM-$Na_2{}^{35}SO_4$.

Strain	Pertinent genotype	Inhibition (%)	
		Selenate	Chromate
TK302	*cysL*$^+$	43	100
TK344	*cysL1366*	0	50

Sulphite reductase activity in cysL *mutants.* It was of interest to see whether sulphate permease activity in *cysL* mutants was derepressed alone or together with sulphite reductase, an enzyme of cysteine biosynthesis. In addition, in search for an explanation of the effect of methionine, sulphite reductase was assayed along with sulphate permease in cells grown in the presence of methionine. Data presented in Table 8 demonstrate that *cysL* mutants show increased activities of both these enzymes. The derepression of sulphite reductase was almost of the same magnitude as in *cysB* constitutive mutant. Presence of methionine in the growth medium reduced the activity of both enzymes in *cysL* and its parent strain, whereas it had practically no effect on sulphite reductase in *cysB* mutant.

Table 8

Effect of L-*methionine on the activity of sulphate permease and sulphite reductase*

L-Methionine was added to concn. of 10^{-3} M.

Strain	Pertinent genotype	Sulphate permease (pmol/μg dry wt/min)		Sulphite reductase (mol/mg dry wt. /min)	
		without methionine	with L-methionine	without methionine	with L-methionine
TK302	*cysL*$^+$	0.12	0.045	1.87	0.88
TK344	*cysL1366*	0.28	0.18	5.40	1.16
DW25	*cysB1352*	—	—	6.10	5.80

Dominance test. The TK346 (*cysL1373*) strain was used on minimal plates as a recipient in a cross with donor strain TK340 (*hisE35 metG319 purG302*/F′ *purG*$^+$ *metG*$^+$). Under these conditions only F′ heterogenates carrying episome F′ *purG*$^+$ *metG*$^+$ could grow. None out of the 100 clones checked was resistant to selenate.

One of these clones (TK360) was grown in nutrient broth liquid medium and spread on nutrient broth plates for individual colonies. It has been found that four out of 100 colonies examined lost episome, required adenine for growth and were resistant to selenate. This result indicates that the strain TK360 was carrying autonomous F' *purG*$^+$ *metG*$^+$ episome and its ability to grow on minimal plates and selenate--sensitivity resulted from complementation. It has been therefore concluded that wild type *cysL* allele is dominant over a mutant gene.

DISCUSSION

The two sulphate analogues used in this study, chromate and selenate, affect metabolism of *S. typhimurium* in different ways. This is best illustrated by the fact that cysteine added to the growth medium completely nullifies toxic effect of selenate, but not that of chromate. The same was observed when djenkolate was used as source of cysteine. Furthermore, derepression of cysteine biosynthesis by mutation in cysteine regulatory gene *cysB* rendered the mutant resistant to selenate, but again not to chromate. This can be simply explained, assuming that selenate is an inhibitor of cysteine biosynthesis: when present in growth medium it produces a shortage of cysteine in the cells. Chromate acts probably also on some other process or affects cellular metabolism by its strong oxidizing action. It is also possible that reduction of chromate is responsible for its toxicity.

Mutants of *S. typhimurium* defective in sulphate transport have been shown to be resistant to chromate (Ohta *et al.*, 1971). The obtained results confirm this finding. Mutant *cysA* was unable to take up chromate. However, it was not possible to design an experiment to prove whether *cysA* mutants are resistant to selenate, since they require cysteine or other sulphur sources (sulphite, sulphide, thiosulphate or djenkolate), which reverse toxic action of selenate. Chromate-resistance of *cysA* and inability for uptake of sulphate analogues suggested that chromate and selenate are transported to *S. typhimurium* cells only by sulphate permease system.

This paper describes *cysL*, a novel class of chromate-resistant mutants, which, unlike those isolated by the Princeton group (Ohta *et al.*, 1971), are prototrophs and show increased activity of sulphate permease. They can be isolated on minimal plates containing chromate or selenate and are resistant to either of these sulphate analogues. The mutations *cysL* were found to map very close to *cysA*, but outside *cysA20* deletion which covers all known sulphate permease mutations (Ohta *et al.*, 1971). It should be noted that none of the chromate-resistant mutants of these authors mapped outside but close to *cysA20* deletion.

CysB constitutive mutant was previously shown to be selenate-resistant (Hulanicka & Kłopotowski, 1972). Neither the cysteine enzymes in this mutant nor its selenate-resistance were repressible by methionine. On the contrary, selenate-resistance of *cysL* mutants was reversible by methionine. Since methionine represses cysteine enzymes in *cysL* the repression could be responsible for reappearence of selenate--sensitivity.

It seems a paradox that chromate-resistance can be obtained by mutations either completely abolishing or increasing sulphate permease activity. A similar situation was encountered in arsenate-resistant mutants by Medveczky & Rosenberg (1971). They found that phosphate uptake by the phosphate-starved *Escherichia coli* had a biphasic kinetics. Some of these arsenate-resistant mutants missed the fast component, whereas in others its action lasted longer which resulted in the increased uptake.

In the studied sulphate-starved *S. typhimurium* strains with wild-type and mutant alleles of *cysL* gene, the kinetics of sulphate transport was linear for at least 5 min. In *cysL* mutants the ability to transport sulphate was increased, selenate uptake was unchanged, and that of chromate was reduced by more than 50%. Mutation in *cysA* — gene for sulphate permease — completely abolished transport of these anions.

On the basis of these data one can assume that *cysA* product is obligatory for permeation of sulphate and its analogues, but inactivation of *cysL* product affects transport of these anions in a different way. Evidence for inactivation of *cysL* product was provided by dominance test which showed that *cysL* mutations were recessive to the wild-type allele.

Differentation of *cysL* effect could result from derepression of other enzymes of sulphate assimilation, e.g. sulphite reductase, the activity of which was increased to the level observed with constitutive *cysB* mutant.

It was observed that in *cysL* mutants competition of chromate and selenate in sulphate transport was less efficient. Apparently, derepression of sulphate permease and other enzymes of sulphate metabolism increased the activity at a step limiting sulphate uptake, but not at those which limit chromate and selenate transport.

The author wishes to express her gratitude to Prof. Dr. G. Bagdasarian and Prof. Dr. T. Kłopotowski for their constant interest and helpful suggestions, and to Dr. N. M. Kredich for his interest and supply of *cysB* constitutive mutant. Skilful technical assistance of Mrs. Wiesława Chabros is gratefully acknowledged.

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OPORNOŚĆ NA ANALOGI SIARCZANU U *SALMONELLA TYPHIMURIUM*

Streszczenie

Wyizolowano mutanty *Salmonella typhimurium*, nazwane *cysL*, oporne na chromian i selenian; mutacja ta zlokalizowana jest w miejscu sprzężonym blisko z genem *cysA*. Mutanty *cysL* wykazują zwiększoną aktywność permeazy siarczanowej i reduktazy siarczynowej. Uprzednio otrzymane mutanty *cysB* o konstytutywnej regulacji biosyntezy cysteiny, są oporne na selenian, lecz nie na chromian. Oporność na selenian i aktywność tych enzymów są reprymowane u mutantów *cysL*, lecz nie u *cysB*, przez metioninę. Chromian i selenian w mniejszym stopniu hamują transport siarczanu u mutantów *cysL* niż u szczepu $cysL^+$.

Received 2 June, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 19 1972 No. 4

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DNase-RESISTANT FRAGMENTS OF DNA IN CHROMATIN

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1. The effect of histones on DNA hydrolysis by DNase I was studied in calf thymus chromatin. In the native chromatin about 50% of DNA was released as protein-free oligonucleotides, whereas in completely dehistonized chromatin, more than 90%. 2. DNA fragments resistant to DNase action in native chromatin were of similar, relatively high molecular weight (2.9 S), and their nucleotide composition resembled that of native calf thymus DNA. In chromatin depleted only of histone *f1*, DNase-resistant fragments consisted of rather small stretches of DNA (mol.wt. 2000 - 4000). 3. The obtained results seem to indicate that in chromatin all histones except *f1* are bound point-wise to DNA.

Data on titration of chromatin with polylysine (Itzhaki, 1970; Clark & Felsenfeld, 1971) and the results on binding of toluidine blue (Itzhaki, 1971a) indicate that a large part of DNA phosphate residues of interphase chromatin are "free". Clark & Felsenfeld have also shown that about 50% of the DNA in chromatin is digestible by nucleases. They concluded that half of this DNA is present in zones completely free of protein. However, Itzhaki (1971b) believes that DNA in chromatin is extensively covered by histones, and interpreted the high value of its degradation as a result of release of some protein from chromatin during DNase action.

In the present work, the effect of histones on the extent of degradation of DNA in chromatin by pancreatic DNase was studied with chromatin preparations treated and untreated with formaldehyde. The size of DNA fragments protected by histones was estimated in the DNase digests of native chromatin and chromatin depleted of histone *f1*.

MATERIALS AND METHODS

Preparation of native and partially dehistonized chromatin. Chromatin was isolated from calf thymus as described by Marushige & Bonner (1966) except that the ultracentrifugation through 1.7 M-sucrose was omitted. Final sediment was suspended in 0.01 M-tris-HCl buffer, pH 8.0, in a concentration equivalent to 20 E units at

260 nm, sheared in the Unipan type 203 homogenizer for 90 sec at 120 V and centrifuged at 12 000 *g* for 30 min.

Chromatin preparations depleted of: (1) histone *f1*; (2) histones *f1*, *f2a2*, *f2b* and (3) completely dehistonized, were obtained by extraction with sulphuric acid as described by Toczko, Dobrzańska & Chmielewska (1972). The obtained chromatin preparations were suspended in 0.01 M-tris-HCl buffer, pH 8.0, dialysed for 48 h at 0 - 4°C against 100 vol. (two changes) of the same buffer, and solubilized by homogenization as above.

Preparation of formaldehyde-treated chromatin. Chromatin, native or partially depleted of histones, was treated with formaldehyde (1%) in 0.01 M-triethanolamine--HCl buffer, pH 7.8, as described by Brutlag, Schlehuber & Bonner (1969). The preparations were dialysed in cold-room against 100 vol. (two changes) of 0.01 M--tris-HCl buffer, pH 8.0, for 24 h.

Digestion of chromatin by DNase I. Solution of chromatin (1.0 - 1.2 E units at 260 nm) in 0.01 M-tris-HCl - 3 mM-$MgCl_2$ buffer, pH 8.0, was digested with DNase (20 μg/ml) at 37°C for 30 min, then cooled at 0°C, and centrifuged at 12 000 *g* either directly or after the addition of $HClO_4$ to 2% final concentration. In the supernatants, the content of nucleotides was determined spectrophotometrically at 260 nm. The rate of DNA degradation was calculated in relation to DNA content in chromatin solution, taking into account the hyperchromic effect.

Isolation of DNase-resistant fragments of DNA. Chromatin preparations (20 E units at 260 nm) were digested by DNase (60 μg/ml) for 40 min as described above, cooled at 0°C and centrifuged at 3500 *g* for 10 min. The sediment was suspended in the initial volume of 0.01 M-tris-HCl buffer, pH 8.0, and recentrifuged. The DNA fragments were isolated from the precipitate by the sodium dodecyl sulphate method of Zamenhof (1958) and further purified by digestion with pronase and RNase, followed by chloroform treatment as described by Stern (1968). The purified fragments were characterized by gel filtration, ultracentrifugation and assay of nucleotide composition.

Sephadex-gel filtration. Gel filtration of DNA fragments was performed on Sephadex G-50 column (34×2 cm, V_t 100 ml), equilibrated with 1 M-NaCl in 0.01 M--sodium citrate. Two millilitres of DNA solution (5 E units at 260 nm) were introduced into the column and eluted with 1 M-NaCl - 0.01 M-sodium citrate at a rate of 25 ml/h. Fractions of 3 ml were collected and monitored spectrophotometrically at 260 nm.

Determination of approximate size of DNA fragments. The S_{20} value of DNA fragments from native chromatin was determined by sedimentation velocity method in 0.1 M-NaCl - 0.01 M-sodium citrate. The approximate molecular weight of DNA fragments from chromatin depleted of histone *f1* was estimated by gel filtration on columns of Sephadex G-25 (39×1.1 cm, V_t 37.5 ml) and G-15 (35×1.1 cm, V_t 34 ml). For equilibration and elution 1 M-NaCl in 0.01 M-sodium citrate was used; the rate of elution was about 15 ml/h.

Determination of nucleotide composition of DNA fragments from native chromatin. This was assayed according to Wyatt (1951).

RESULTS AND DISCUSSION

The effect of histones on the extent of hydrolysis of DNA in chromatin. Susceptibility of DNA of native and modified chromatins to DNase was estimated by determining the amount of liberated oligonucleotides. Direct centrifugation of the hydrolysate of chromatin, or centrifugation following $HClO_4$ addition resulted in separation of DNA fragments released from chromatin by DNase. All the protein of chromatin and protein-bound DNA fragments were sedimented.

The time-course of digestion of DNA in native chromatin and chromatin treated with formaldehyde is shown in Fig. 1. As it may be seen, the release of oligonucleotides was in both cases about the same. The extent of hydrolysis after 30 min of incubation as estimated after direct centrifugation, was for native chromatin $51.3 \pm 1.5\%$, and for the formaldehyde-treated chromatin $49 \pm 1.2\%$; the respective values obtained with $HClO_4$ were: $51.1 \pm 2.5\%$ and $47.7 \pm 0.7\%$.

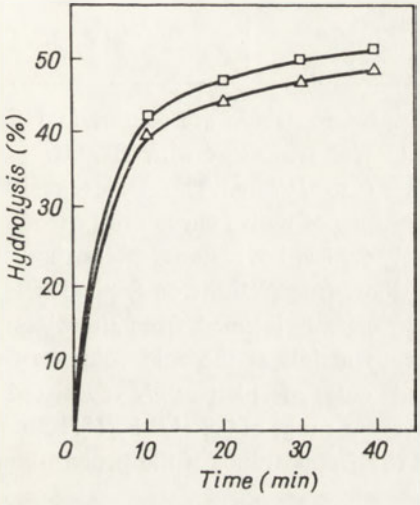

Fig. 1

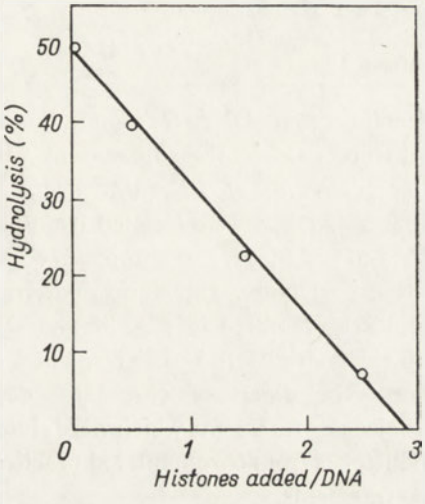

Fig. 2

Fig. 1. Hydrolysis of DNA of native (□) and formaldehyde-treated (△) chromatin. Degradation of DNA was determined in the reaction mixture following the addition of $HClO_4$ to 2% final concentration.

Fig. 2. Digestion of chromatin DNA as a function of added histones.

Addition of histone to chromatin lowered the accessibility of its DNA to the enzyme action (Fig. 2), and, at 3:1 ratio of histones to DNA, prevented completely hydrolysis of DNA. Correspondingly, successive removal of histones from chromatin caused an increase in susceptibility of these preparations to DNase action (Table 1).

The results obtained after $HClO_4$ treatment were somewhat lower for histone--depleted, formaldehyde-fixed preparations as compared with the corresponding preparations not treated with formaldehyde. The observed difference may indicate that digestion of chromatin partially depleted of histones leads to formation of low-

Table 1

The rate of hydrolysis of DNA in native and dehistonized chromatin, treated and non-treated with formaldehyde

Preparation	Hydrolysis of DNA (%) determined	
	directly	after treatment with $HClO_4$
Native chromatin		
formaldehyde-treated	49.0	47.7
untreated	51.3	51.1
Chromatin depleted of histone *f1*		
formaldehyde-treated	55.5	57.5
untreated	60.8	76.2
Chromatin depleted of histones *f1*, *f2b* and *f2a2*		
formaldehyde-treated	83.0	83.5
untreated	89.0	91.5
Dehistonized chromatin		
formaldehyde-treated	90.0	90.0
untreated	89.5	94.0

-molecular-weight DNA fragments bound to protein, which are soluble in $HClO_4$ and do not pass to the supernatant without prior treatment with $HClO_4$.

Characteristics of DNA fragments resistant to DNase. The DNase-resistant fragments of DNA were isolated from preparations of native chromatin, chromatin depleted of histone *f1*, and chromatin completely depleted of histones after exhaustive digestion by DNase. The elution patterns of these preparations on Sephadex G-50 column are presented in Fig. 3. The DNA fragments isolated from the digest of native chromatin were rather large and emerged mainly at the void volume of the column. The digest of chromatin depleted only of histone *f1* consisted of low-molecular fragments which were eluted over the range of 0.8 - 1.0 V_e/V_t. Removal of all histones from chromatin led practically to disappearance of the protein-bound DNA fragments.

On sedimentation the DNase-resistant DNA fragments of native chromatin formed a single fraction, appearing as a distinct, sharp peak (Fig. 5). The mean value of $S_{w,20}$ was 2.9, which corresponds to a molecular weight of about 20 000. The nucleotide composition of these fragments was found to be similar to that of native DNA of calf thymus. The content of adenine, thymine, cytosine and guanine, expressed in mol%, was 28.6, 27.9, 22.1 and 21.4, respectively.

The DNA fragments isolated from DNase digest of the histone-*f1*-depleted chromatin emerged from Sephadex G-15 at the void volume of the column, and from Sephadex G-25 at 0.51 - 0.67 V_e/V_t, with a maximum at 0.59 V_e/V_t (Fig. 4). Calculated from these values $K_{av}=\frac{V_e-V_0}{V_t-V_0}$ was within the range 0.16 - 0.42, which corresponds to the molecular weight of 2000 - 4000.

The presented results imply that the DNase action on formaldehyde-treated chromatin results in the release of 50% of DNA in the form of protein-free oligo-

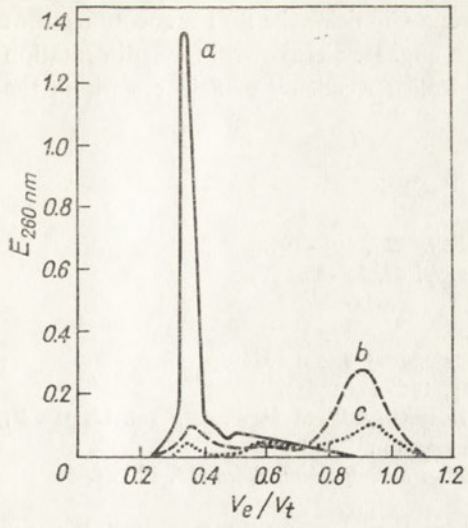

Fig. 3

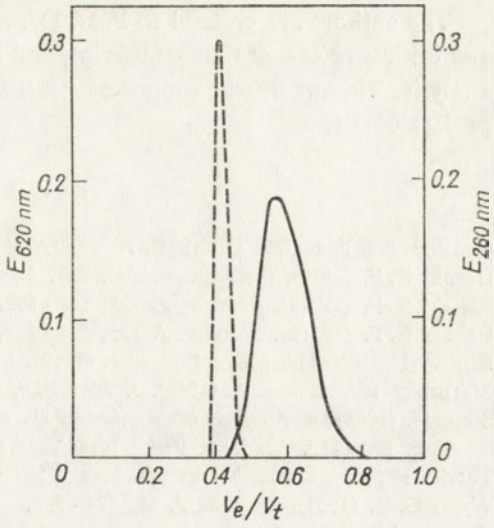

Fig. 4

Fig. 3. Sephadex G-50 gel filtration of DNase-resistant fragments from chromatin: *a*, native; *b*, depleted of histone *f1*; *c*, dehistonized.

Fig. 4. Sephadex G-25 gel filtration; ——, DNA fragments from chromatin depleted of histone *f1* ($E_{260\,nm}$); - - -, Blue Dextran ($E_{620\,nm}$).

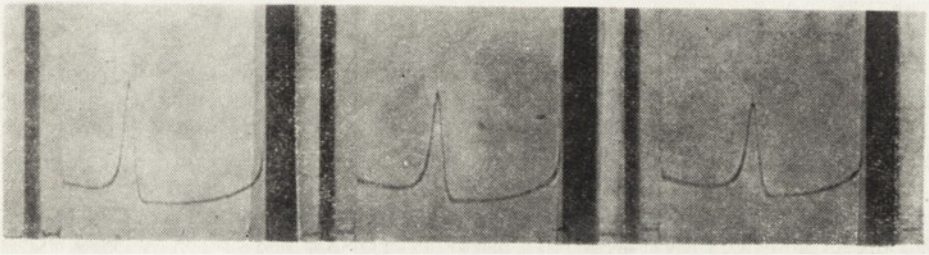

Fig. 5. Ultracentrifuge patterns of DNase-resistant fragments from native chromatin, after 60, 76 and 92 min. Concentration of DNA 0.8%.

nucleotides. These results are in agreement with those reported by Clark & Felsenfeld (1971). As no differences in the digestibility were found between the native and formaldehyde-treated chromatin, it seems that DNA fragments susceptible to hydrolysis are not directly bound to protein. A marked increase in the extent of hydrolysis of DNA observed on removal of histones suggests that DNA fragments resistant to the enzyme are protected mainly by these particular proteins. The removal of histone *f1* was accompanied by a slight increase in the total amount of the released oligonucleotides (from 50% to about 58%). However, the size of the DNase-resistant fragments was altered; they were found to be about eight or ten times smaller than in intact chromatin. The presented results may be interpreted as evidence that in chromatin histones *f2a1*, *f2a2*, *f2b* and *f3* are bound to only short fragments of DNA, whereas histone *f1* covers about ten times larger stretches of DNA.

The authors are grateful to Prof. Dr. Irena Chmielewska for her encouragement and critical review of the manuscript, and to eng. H. Sierakowski for sedimentation analysis. This work was supported by the Polish Academy of Sciences within the project 09.3.1.

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FRAGMENTY DNA CHROMATYNY OPORNE NA DZIAŁANIE DNazy

Streszczenie

1. Zbadano wrażliwość DNA chromatyny grasicy cielęcej na działanie DNazy w zależności od ilości i rodzaju histonów dodanych lub usuniętych z chromatyny. Ustalono, że z chromatyny natywnej w wyniku działania tego enzymu uwalnia się około 50%, a z chromatyny pozbawionej histonów ponad 90% DNA w formie wolnych od białka oligonukleotydów.

2. Stwierdzono, że w chromatynie natywnej fragmenty DNA połączone z białkami, to jest oporne na działanie enzymu, mają jednakową i stosunkowo dużą masę cząsteczkową (2,9 S), a ich skład nukleotydowy nie odbiega od składu nienaruszonego DNA grasicy cielęcej, natomiast w chromatynie, z której usunięto tylko histon *f1*, są to niewielkie odcinki DNA o masie 2000 - 4000.

3. Wyniki pracy wskazują, że w chromatynie wszystkie histony, z wyjątkiem *f1*, są punktowc połączone z DNA.

Received 14 June, 1972.