KOMITET BIOCHEMICZNY I BIOFIZYCZNY
POLSKIEJ AKADEMII NAUK

COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS
POLISH ACADEMY OF SCIENCES

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. 18
No. 1

WARSZAWA 1971
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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CHOLESTEROL ESTERASE ACTIVITY OF RAT PANCREATIC JUICE
EFFECT OF pH AND BILE SALT ON THE ENZYME-CATALYSED EQUILIBRIUM STATE

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1. The cholesteryl ester synthesizing and hydrolysing activities of rat pancreatic juice could not be separated either by gel filtration or by ion exchange chromatography. 2. The same equilibrium position was reached starting from the ester or the component fatty acid and cholesterol. The composition of the mixture at equilibrium is dependent on pH; low pH values favour formation of cholesteryl ester, high pH values the free sterol. 3. Bile salts are necessary for the activity of the enzyme. This did not appear to be specific only for the trihydroxy bile salts. The dihydroxy compounds had a similar effect although the fatty acid exchange was less complete. This incomplete exchange might, however, well be explained by differences in the stability of the enzyme in the presence of various bile salts. 4. The reaction mechanism probably is similar to that described for pancreatic lipase and is explained by the low water concentration at the site of the enzyme action.

Pancreatic juice of rat has been shown to contain enzymic activities catalysing both the formation and hydrolysis of cholesteryl esters. The literature contains conflicting evidence as to the question whether these two activities are due to the action of the same enzyme or two different enzymes. Hernandez & Chaikoff (1957) and Hyun *et al.* (1969) were unable to separate these activities on purification while Murthy & Ganguly (1962) reported their partial separation.

It occurred to us that if these enzymic activities were due to the same enzyme, this enzyme would catalyse an equilibrium that could be reached starting either from the ester or the fatty acid and cholesterol. The reaction could then be analogous to that previously described for pancreatic lipase (Borgström, 1964) and the relatively large proportion of ester present in the equilibrium mixture, explained

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by the low concentration of water at the site of enzymic action. Furthermore, it could then be expected that a cholesteryl ester should exchange its fatty acid with the free fatty acids present in the system. In order to evaluate this postulated exchange reaction as well as the effects of different bile salts and pH, experiments were undertaken aiming at establishing the existence of an equilibrium state between synthesis and hydrolysis of cholesteryl oleate and to prove the fatty acid exchange between the cholesteryl ester and free fatty acid pool.

MATERIAL AND METHODS

Chemicals. Cholesteryl oleate (B.D.H., Poole, Dorset, England) was purified on alumina columns and its purity checked by t.l.c.[1] Cholesterol (Eastman Kodak Co., Rochester, N.Y., U.S.A.) was recrystallized until a chromatographically pure product was obtained. Oleic acid (Applied Science Laboratories, State College, Pennsylvania, U.S.A.) was used without purification. [U-^{3}H]Oleic acid (50 mCi/mmole) was obtained from the Radiochemical Center (Amersham, England); as it contained polar components, it was purified by t.l.c. until its radiopurity was found to be better than 97%. Octadecane (Fluka A.G., Buchs SG, Switzerland) was 99% pure. All these chemicals were stored as stock solutions in hexane.

The conjugated bile salts used were synthesized and purified according to Norman as modified by Hofmann (1963) and were better than 97% pure.

Sephadex G-100 and G-200, and DEAE-Sephadex were from Pharmacia (Uppsala, Sweden). Silica gel G was from Merck (Darmstadt, West Germany).

Analytical procedure. Protein was measured according to the method of Lowry, Rosebrough, Farr & Randall (1951).

Cholesterol and its esters were determined according to Vahouny, Borja & Weersing (1963).

Free fatty acids were titrated according to Dole as modified by Trout, Estes & Friedberg (1960).

Gel filtration was carried out on Sephadex G-100 and G-200. The Sephadex gel was packed in columns of diameter 15 mm, total volume 120 ml. Lyophilized rat pancreatic juice, 10 - 20 mg, in 1 ml of 0.15 M-NaCl was applied to the column and eluted with 0.15 M-NaCl at 4°C. Blue dextran was used to determine the void volume of the gel. Fractions of approx. 2 ml were collected at flow rates of 6 - 10 ml per hour.

Ion exchange chromatography was carried out on DEAE-Sephadex. Cationic proteins were eluted with 0.05 M-tris-HCl buffer, pH 7.4, and anionic proteins by elution with a gradient of 0.05 to 1.0 M-NaCl in this buffer. Lyophilized rat pancreatic juice, 10 - 20 mg, dissolved in 1 ml of the starting buffer, was applied to the column of 2.5 cm × 25.0 cm, and fractions of approx. 2.5 ml were collected.

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[1] Abbreviations used: t.l.c., thin-layer chromatography; NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate; NaGC, sodium glycocholate; NaDC, sodium deoxycholate; NaGDC, sodium glycodeoxycholate; FA, fatty acid.

Substrates. The emulsified substrate for hydrolytic action of the steryl ester hydrolase (EC 3.1.1.13) contained in a volume of 1 ml: 16 µmols of cholesteryl oleate, 8 µmols of octadecane (Borgström, 1967; Arnesjö & Filipek-Wender, 1968), 16 µmols of sodium taurocholate or other bile salt in 0.05 M-buffer at chosen pH.

For esterification studies, the following substrate was used: 4 µmols of cholesterol, 4 µmols of oleic acid, 2 µmols of octadecane and 8 µmols of bile salt, all in 1 ml of 0.05 M-buffer at appropriate pH.

Solutions of cholesteryl oleate or of oleic acid and cholesterol together with the octadecane solution were evaporated under nitrogen at 40°C. To the dry residue 0.05 M-buffer and the bile salt were added. The mixture was then sonicated for 3 min in a Branson sonicator with the power level control switch on 7. In experiments aiming at assessing the equilibrium state of the reaction under study, a trace amount of ^{3}H-labelled oleic acid of high specific activity was included in the substrate.

Buffers. In the pH range from 5 to 7, 0.05 M-sodium phosphate buffer and for pH 8 and 9 a glycyl-glycine - NaOH buffer, were used.

Source of enzyme. Lyophilized rat pancreatic juice, free of bile was used as the source of pancreatic steryl ester hydrolase. The experimental animals were male Sprague-Dawley rats (AB Anticimex, Stockholm, Sweden) reared on a standard pellet diet and weighing 200 - 250 g. The juice was obtained by cannulating the pancreatic duct after draining the bile with a separate cannula. The juice was collected over solid CO_2 and then lyophilized. About 20 mg of the pancreatic juice powder containing 80% of protein was equivalent to 1 ml of fresh juice. When needed for incubations, the lyophilized juice was dissolved in the respective buffer, or for the gel filtration in 0.15 M-saline to yield a concentration equivalent to native juice.

The hydrolytic cholesterol esterase activity was measured by following the release of free fatty acids occurring during enzymic hydrolysis of cholesteryl oleate, and the synthesizing activity, by following the disappearance of oleic acid from the incubation medium.

Incubations. To 0.9 ml of substrate, 0.1 ml of pancreatic juice powder solution was added and incubations continued at 30°C for the times stated in the tables. The enzyme reaction was terminated by adding 4 ml of Dole's extraction mixture (Dole, 1956) and the extracted fatty acid titrated. In experiments with labelled substrates the incubations were run in a total volume of 3 ml (2.7 ml of substrate and 0.3 ml of enzyme). Two 1 ml portions were extracted by Dole's procedure and two 0.4 ml portions were extracted with chloroform-methanol according to Folch, Lees & Sloane-Stanley (1957). The final washed and dried extracts were evaporated to dryness under a stream of nitrogen and then dissolved in 0.4 ml of chloroform. Samples of 100 µl were taken for determination of total radioactivity, and the same amounts were spotted in duplicate on silica-gel G thin layer plates. The plates were developed with hexane - diethyl ether - glacial acetic acid (166 : 32 : 2, by vol.) and the spots visualized with iodine vapour. The cholesteryl ester and fatty acid spots were scraped off and transferred into counting vials. The radioactivity was measured

in an automatic Tri-Carb-Scintillation Spectrometer, using dioxane with PPO and POPOP as scintillators according to Brown & Johnston (1962).

The mean recovery of radioactivity in the FA and cholesteryl ester spots from plates was 87% of the activity applied to the plate; 5 - 12% of the activity remained at the origin.

RESULTS

Gel filtration and ion exchange chromatography. The attempts to separate the synthesizing and hydrolysing activities of cholesterol esterase by means of gel filtration on Sephadex G-100 and G-200 and with the aid of ion exchange chromatography were unsuccessful. In four separations with Sephadex G-100 and in two with Sephadex G-200 the two activities followed each other exactly (Table 1 and Fig. 1).

Table 1

Individual K_{av} values of the hydrolytic and synthetic cholesterol esterase activities of rat pancreatic juice separated on Sephadex G-100 and G-200

Four samples of lyophilized rat pancreatic juice were separated on Sephadex G-100 and two on Sephadex G-200, activities against cholesteryl oleate and cholesterol plus oleic acid determined in collected fractions, and the respective K_{av} values calculated after Laurent & Killander (1964).

Type of gel used	Enzyme activity	
	Hydrolysis K_{av}	Esterification K_{av}
Sephadex G-100	0.190	0.190
	0.185	0.190
	0.200	0.210
	0.180	0.180
Sephadex G-200	0.380	0.380
	0.380	0.380

Three separations were performed using DEAE-Sephadex at pH 7.4. Maximum hydrolytic activity against cholesteryl oleate was eluted with the anionic proteins in exactly the same tube as the maximum esterifying activity, with an identical distribution around the symmetrical peak.

Our results thus confirm in this respect the results of a thorough purification procedure of Hyun *et al.* (1969), suggesting that cholesteryl ester synthesizing and hydrolysing activities in pancreatic juice are a function of the same enzyme.

If this is the case, the enzyme should catalyse an equilibrium state, and the same equilibrium mixture should be obtained starting either from the ester form or from the fatty acid and cholesterol.

The effect of time and pH on the enzyme-catalysed equilibrium state. In preliminary experiments, incubations were carried out at pH values ranging from 5 to 9 for different time intervals from 1 h up to 24 h. It appeared that 6 to 8 h incubation was needed for the reaction to reach a steady state. There was no further release of FA observed in the 24 h samples. No spontaneous hydrolysis of the substrate took place under these conditions.

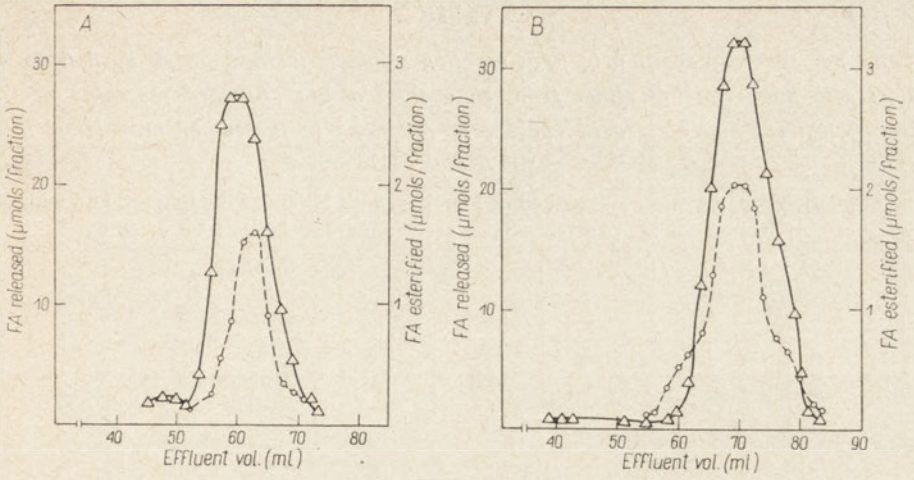

Fig. 1. Chromatography of lyophilized rat pancreatic juice (10 mg) on Sephadex G-100 (*A*) and G-200 (*B*). Temp. 4°C, elution with 0.15 M-NaCl, flow rate approx. 8 ml/h. Void volume for G-100: 46 ml, for G-200: 39 ml. Total volume 120 ml. Enzyme activity was assayed as described in Methods with NaTC as the bile salt; temp. 30°C. △, Hydrolytic activity against cholesteryl oleate (pH 9.0, 5 min); ○, Esterifying activity with cholesterol and oleic acid as substrate (pH 6.0, 30 min).

Table 2 shows the results of a study of the final composition of the products obtained during incubation of both cholesteryl oleate and cholesterol plus oleic acid for 8 h with lyophilized rat pancreatic juice at high enzyme concentration, .e. o ne for which the addition of a new portion of enzyme does not greatly affect the relative composition of end products.

It is evident that the percent molar composition of cholesteryl ester and free fatty acid (free cholesterol) at equilibrium starting either from cholesteryl oleate or from cholesterol and oleic acid at the two chosen pH values are strikingly similar. Incubation of cholesterol and free fatty acid with pancreatic juice resulted in a net formation of steryl ester bonds, as proved by t.l.c., the equilibria being dependent on the pH of the incubation mixture. At low pH the equilibrium was rather far in favour of synthesis, despite the presence in the system of a large excess of water, which would be expected to drive the reaction towards hydrolysis, and at high pH — hydrolysis prevails.

The enzyme-catalysed FA exchange between cholesteryl esters and free fatty acid pool. A more detailed study over a range of pH from 5 do 9 and using different bile salts in the incubation medium was performed with a different experimental approach. This approach was based on the assumption that if there exists an equilibrium state between hydrolysis of cholesteryl oleate and esterification of free cholesterol, the esterified fatty acid should equilibrate with the free fatty acid of the incubation medium, as was found for the hydrolysis of triglycerides by pancreatic lipase (Borgström, 1964).

The exchange of fatty acids was judged from the distribution of radioactivity between the free and esterified fatty acids after incubation with pancreatic juice.

Table 2

Molar per cent composition of final reaction products formed during incubation with pancreatic juice starting either from cholesteryl oleate (forward reaction) or from free cholesterol and oleic acid (backward reaction) in the molar proportion 1:1 at pH *6 and* pH *9*

The incubation mixture was as described in Methods, with NaTC as the bile salt and with 2 mg of lyophilized rat pancreatic juice per sample. Incubation: 8 h at 30°C.

	pH 6		pH 9	
	Esterified FA (%)	Free FA (%)	Esterified FA (%)	Free FA (%)
Forward reaction (hydrolysis)	78	22	39	61
Backward reaction (esterification)	71	29	32	68

The incubations were carried out using the usual cholesteryl oleate substrate with the addition of an untitratable trace of tritium-labelled oleic acid of high specific activity. The released fatty acid was titrated and the final composition of the reaction products were calculated therefrom. The radioactivity of the total extract, corrected for the recovery from t.l.c. plates, per μmol of FA was assumed to account for the specific activity of 100% equilibrium. The radioactivities of the FA and cholesteryl ester spots were calculated to obtain the respective specific activities. The specific activity of the cholesteryl ester spot when compared with that of the whole extract, indicated the actual equilibrium reached.

The effect of pH on the final composition of reaction products at equilibrium when cholesteryl oleate was incubated with rat pancreatic juice cholesterol esterase in the presence of NaTC, is shown in Table 3 and Fig. 2. It can be seen that at low

Table 3

The effect of pH *on the products formed during prolonged incubation of cholesteryl oleate with rat pancreatic juice at high enzyme concentration*

The incubation mixture was as described in Methods, with NaTC as the bile salt, and with 2 mg of lyophilized rat pancreatic juice per 1 ml of the mixture; the substrate contained trace amounts of ^{3}H-labelled oleic acid (spec. act. 1×10^6 d.p.m./mg) Incubation: 8 h at 30°C.

	pH				
	5	6	7	8	9
FA released (μmols)	3.14	3.60	4.36	8.48	9.73
Hydrolysis (%)	19.6	22.5	27.2	53.0	60.8
Specific activity of FA	2746	2719	2404	2640	2655
Specific activity of cholesteryl ester	2472	2481	2551	2162	1958
Specific activity for 100% equilibrium	2520	2535	2510	2416	2382
Per cent of equilibrium reached	98	98	102	89	80

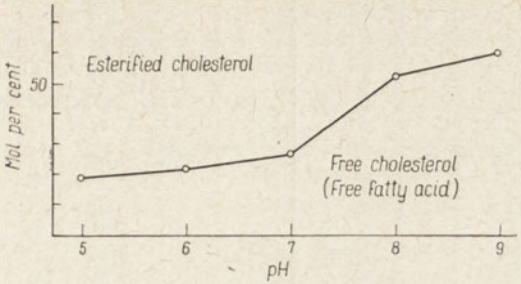

Fig. 2. Effect of pH on the composition of reactants after prolonged incubation of cholesteryl oleate with rat pancreatic juice. Incubation conditions as stated for Table 3. Amount of esterified and free cholesterol or fatty acid plotted on a molar basis in a cumulative fashion.

pH values (5 to 7) the esterified form of cholesterol, though gradually decreasing, predominated in the equilibrium mixture. At pH 8 a significant decrease of the cholesteryl ester moiety was observed, the free sterol form (or free fatty acid) constituting 53% at pH 8.0 and 61% at pH 9.0. At the same time, however, it appeared that while at pH 5, 6 and 7 almost a 100% exchange was observed, at pH 8 and 9 only 90% and 80% respectively, of the FA exchange was attained. This could indicate that at higher pH values the enzyme was gradually inactivated and a true equilibrium has not been achieved.

The effect of different bile salts on the fatty acid exchange reaction catalysed by pancreatic juice cholesterol esterase is presented in Table 4. At pH 6 the position of the equilibrium in the presence of different bile salts was very similar, around 20% (17 to 24%) of the cholesteryl moiety being present in the free form and about 80% in the esterified one. Only with NaTC in the incubation medium was an almost 100% FA exchange observed. With the other bile salts the exchange was not complete after 8 h. The dihydroxy bile salts, however, were almost as effective at that pH in promoting the FA exchange as were the trihydroxy compounds. At pH 9 the exchange was much less complete; with NaTC an 80% level was reached, with the other bile salts it varied between 58 and 26%, the lowest values being found with the dihydroxy compounds.

DISCUSSION

When interpreting these findings, two effects of pH on the enzymic reaction should be considered, a direct effect on the enzyme itself, and an effect which could be related to the physico-chemical interactions between the substrate and its split products. Based on our experiments, no arguments can be provided for a direct effect of pH on the enzyme molecule. It is obvious, however, that an increase in the pH of the medium will necessarily influence the ionization of fatty acids and this in turn may shift the fatty acids from the oil phase to the water phase (Borgström, 1967), thus affecting the availability of the fatty acid for reesterification. This would be in keeping with the evidence provided by Vahouny, Weersing & Treadwell (1964)

Table 4

Effect of different bile salts on the equilibrium state of the reaction catalysed by pancreatic cholesterol esterase at pH *6 and* pH *9*

The incubation mixture was as described in Methods and contained 2 mg of lyophilized rat pancreatic juice per 1 ml; the cholesteryl oleate substrate was prepared with the bile salt indicated in the Table and contained trace amounts of ^{3}H-labelled oleic acid (spec. act. 1×10^6 d.p.m./mg). Incubation: 8 h at 30°C.

	NaTC	NaGC	NaTDC	NaGDC	NaDC	NaTC	NaGC	NaTDC	NaGDC	NaDC
	pH 6					pH 9				
FA released (μmols)	3.60	2.76	3.07	3.94	3.33	9.73	8.98	6.46	8.31	6.13
Hydrolysis (%)	22.5	17.2	19.2	24.6	21.0	60.8	56.0	40.4	52.0	38.0
Specific radioactivity of FA	2719	3760	3820	3640	4220	2655	2910	4730	4360	5410
Specific radioactivity of cholesteryl ester	2481	2080	2060	2330	2260	1958	1320	680	680	1020
Specific radioactivity for 100% equilibrium	2535	2300	2420	2640	2630	2382	2260	2420	2610	2580
Per cent of equilibrium reached	98	90	88	88	85	80	58	28	26	39

that for esterification of cholesterol by pancreatic cholesterol esterase, an emulsified substrate with cholesterol and the fatty acid equally available to the enzyme, was preferred. The ionized fatty acids, due to their disymmetric polar and non-polar areas, may participate in micelle formation in the presence of another effective micellar substance, such as the bile salts, thus increasing the micellar solubility of the cholesteryl ester (Borgström, 1967), rendering the polar ester bond arranged near the polar periphery of the micelle susceptible to splitting by the water-soluble enzyme (Vahouny *et al.*, 1964; Morgan, Barrowman, Filipek-Wender & Borgström, 1968). In fact, it has been postulated by Vahouny *et al.* (1964) that a micellar form of cholesteryl oleate is the preferred substrate for hydrolytic action of cholesterol esterase, and we have gained additional evidence that even in a bile salt emulsified substrate, it is the micellar form of cholesteryl oleate which is attacked by the enzyme (Morgan *et al.*, 1968).

Two facts can be responsible for the low exchange at pH 9. First, as shown by Mattson & Volpenhein (1966), the enzyme most probably identical with cholesterol esterase, is rapidly inactivated when incubated at pH 9 at 40°C; secondly, the trihydroxy bile salts, especially NaTC, protect cholesterol esterase against proteolytic inactivation (Vahouny, Weersing & Treadwell, 1965). It is not clear from the literature whether the inactivation at pH 9 is a proteolytic inactivation, but if so, bile salts should offer protection. The results of Table 4 seem to indicate that the trihydroxy bile salts have a partial protective effect against the inactivation at pH 9. The results, however, clearly indicate that all the bile salts tested, tri- as well as dihydroxy compounds, are active as cofactors for the cholesterol esterase catalysed exchange reaction as well as the establishment of an equilibrium state as regards the composition of the incubation mixture. Whether the differences obtained for different bile salts in these respects are caused by specific effects on the enzyme-catalysed reaction or a protection of the enzyme from inactivation, is not clear. The results are at variance with those of Vahouny *et al.* (1965) who ascribe a specific effect to the trihydroxy bile salts.

REFERENCES

Arnesjö B. & Filipek-Wender H. (1968). *Acta physiol. scand.* **74**, 616.
Borgström B. (1964). *J. Lipid Res.* **5**, 522.
Borgström B. (1967). *J. Lipid Res.* **8**, 598.
Brown J. L. & Johnston J. M. (1962). *J. Lipid Res.* **3**, 480.
Dole V. P. (1956). *J. clin. Inv.* **35**, 150.
Folch I., Lees M. & Sloane-Stanley G. H. (1957). *J. biol. Chem.* **226**, 497.
Hernandez H. H. & Chaikoff I. L. (1957). *J. biol. Chem.* **228**, 447.
Hofmann A. F. (1963). *Acta chem. Scand.* **17**, 173.
Hyun J., Kothari H., Herm E., Mortenson J., Treadwell C. R. & Vahouny G. V. (1969). *J. biol. Chem.* **244**, 1937.
Laurent T. C. & Killander J. (1964). *J. Chromat.* **14**, 317.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.
Mattson F. H. & Volpenhein R. A. (1966). *J. Lipid Res.* **7**, 536.

Morgan R. G. H., Barrowman J., Filipek-Wender H. & Borgström B. (1968). *Biochim. biophys. Acta* **167**, 355.
Murthy S. K. & Ganguly J. (1962). *Biochem. J.* **83**, 460.
Trout D. L., Estes E. H. & Friedberg S. J. (1960). *J. Lipid Res.* **1**, 199.
Vahouny G. V., Borja C. R. & Weersing S. (1963). *Analyt. Biochem.* **6**, 555.
Vahouny G. V., Weersing S. & Treadwell C. R. (1964). *Archs Biochem. Biophys.* **107**, 7.
Vahouny G. V., Weersing S. & Treadwell C. R. (1965). *Biochim. biophys. Acta* **98**, 607.

AKTYWNOŚĆ ESTERAZY CHOLESTEROLOWEJ SOKU TRZUSTKOWEGO SZCZURA
WPŁYW pH I SOLI KWASÓW ŻÓŁCIOWYCH NA STAN RÓWNOWAGI KATALIZOWANEJ REAKCJI

Streszczenie

1. Stosując sączenie molekularne oraz chromatografię jonowymienną nie udało się rozdzielić aktywności syntetyzującej od hydrolizującej esterazy cholesterolowej soku trzustkowego szczura.

2. Skład mieszaniny reakcyjnej w stanie równowagi jest identyczny, niezależnie czy enzym inkubowano z esterem cholesterolu czy z wolnym cholesterolem i kwasem tłuszczowym. Skład mieszaniny reakcyjnej w stanie równowagi jest zależny od pH; niskie wartości pH sprzyjają tworzeniu estrów, a wysokie — hydrolizie.

3. Sole kwasów żółciowych są niezbędne dla aktywności enzymatycznej. Nie stwierdzono specyficznego efektu soli kwasów trójhydroksycholanowych. Pochodne dwuhydroksycholanowe wykazywały podobne działanie, choć reakcja wymiany kwasu tłuszczowego była w ich obecności niekompletna. Różnice te tłumaczyć można odmienną trwałością enzymu w obecności soli kwasów dwuhydroksycholanowych.

4. Mechanizm reakcji jest prawdopodobnie podobny do mechanizmu opisanego dla lipazy trzustkowej i tłumaczy się niskim stężeniem wody w miejscu działania enzymu.

Received 24 March, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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EXTRACTION PROCEDURES AND THEIR INFLUENCE ON THE VALUES OF NICOTINAMIDE-ADENINE DINUCLEOTIDES IN FROZEN BRAIN TISSUE

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1. The procedure of Burch *et al.* (1967) for extraction of nicotinamide-adenine dinucleotides has been modified in order to fit the direct fluorimetric method of their determination in frozen brain tissue. 2. Due to rapid post-mortem changes in the content and oxido-reduction state of nicotinamide-adenine dinucleotides observed in rat brain, it was necessary to apply quick freezing of the brain. 3. The proposed modification permits to determine the sums of $NAD^+ + NADP^+$ and $NADH + NADPH$ in the brain by a rapid and simple non-enzymic procedure.

It has been pointed out in the literature that erroneous values for the nicotinamide-adenine dinucleotides in tissues may be obtained when inappropriate extraction procedures are used. This problem is of special importance in procedures in which a frozen tissue is subjected to analysis.

The procedure of Gordon (1963) for extraction of the reduced and oxidized forms of nicotinamide-adenine dinucleotides in hot 0.1 M-sodium carbonate and in cold 5% trichloroacetic acid, respectively, as applied in our earlier study (Sokal, Tarkowski & Wrońska-Nofer, 1969) is satisfactory for fresh liver tissue. However, it appeared impossible to apply the same procedure for extraction of these coenzymes from fresh brain tissue due to changes occurring in their content and oxido-reduction state in the course of preparation. When applied to the frozen tissue, the procedure also gave erroneous results.

In this report we present the modification which made possible an adaptation of the extraction procedure of Burch, Bradley & Lowry (1967) to the fluorimetric method for determination of nicotinamide-adenine dinucleotides (Sokal *et al.*, 1969) in brain tissue.

EXPERIMENTAL

Preliminary experiments on determination of nicotinamide-adenine dinucleotides in brain

The influence of freezing of brain tissue in liquid nitrogen. The extraction procedure described previously (Sokal *et al.*, 1969; see Fig. 1A) has been applied only to fresh liver tissue. As found now (Table 1), when the brain was subjected to freezing much lower amounts of the reduced forms of the nucleotides were obtained as compared with those in fresh tissue. As there was no difference in the amount of the oxidized forms, it may be assumed that the reduced nucleotides underwent decomposition during the extraction of the frozen tissue. This was confirmed by the simultaneous partial loss of the exogenous NADH in the course of alkaline extraction of the frozen tissue, a fact not observed with the fresh tissue (Table 1). It seems that the loss of the reduced forms of the nucleotides in the frozen brain extract was due to their oxidation and subsequent decomposition in the hot alkaline medium. Several authors attempted to prevent these changes by addition of cysteine (Lowry, Passonneau, Schulz & Rock, 1961a; Neuhoff, 1965; Neuhoff & Desselberger, 1965; Burch *et al.*, 1967), also EDTA appeared partially protective (Lowry, Passonneau & Rock, 1961b). However, when applied to our former procedure, these compounds were ineffective.

Effect of the extraction procedure on fluorimetric determinations. In the procedure of Burch *et al.* (1967), the nucleotides are extracted with a cold alkaline medium in the presence of cysteine. The separation of the reduced and oxidized forms of

Table 1

The influence of freezing and thawing of brain tissue on the content of nicotinamide-adenine dinucleotides and on the recovery of exogenous NADH

Two minutes after decapitation, a part of the brain was frozen in liquid nitrogen; a parallel sample of fresh tissue was taken directly to extraction. Details of the extraction procedure and of determinations were in both cases those of Sokal *et al.* (1969), cf. Fig. 1A. Exogenous NADH, cysteine and EDTA were added to the extraction medium simultaneously with the tissue; their final concentrations in the medium were: 6 μM, 1 mM and 5 mM, respectively. Mean values from 4 - 9 determinations ±S.D. are given

Brain	$NAD^+ + NADP^+$	NADH+NADPH		Recovery of exogenous NADH (%)	
	nmols/g				
	Extraction with TCA	Extraction with		Extraction with	
		Na_2CO_3	Na_2CO_3 +cysteine +EDTA	Na_2CO_3	Na_2CO_3 +cysteine +EDTA
Fresh	399±40	109±9	102±8	96±6	—
Frozen	401±53	45±8	40±8	78±5	75±6

nucleotides is achieved by heating one part of the extract to decompose NAD^+ and $NADP^+$, and by treating the second part with 0.02 N-H_2SO_4 - 0.1 M-Na_2SO_4 in the presence of ascorbic acid, to decompose NADH and NADPH. Then the individual nucleotides are determined by the enzymic cycling method described by Lowry *et al.* (1961a).

To adapt the above extraction procedure for use with the fluorimetric determinations used in our laboratory (Sokal *et al.*, 1969), the following modifications of both procedures appeared useful: I. The extraction of the oxidized nucleotides (Fig. 1B, stage 3) was performed with trichloroacetic acid and without the addition of ascorbic acid, which in the procedure of Burch is used to prevent oxidation of the reduced nucleotides. Ascorbic acid inhibits the fluorescence of the nucleotides in the alkaline medium and also increases the blank values. Actually, it was not necessary to apply an antioxidant as it was found that in the presence of trichloroacetic acid the oxidation of reduced forms of the nucleotides was negligible (Table 2). II. Oxidation of the reduced nucleotides in the alkaline extract (pH about 12.5) was performed in the presence of 91 μM-phenazine methosulphate for 10 min at 0 - 2°C (Fig. 1B, stage 4). As shown in Table 3, the oxidation of nucleotides was complete. This confirms our previous observations (Sokal *et al.*, 1969) that this

Table 2

Oxidation of exogenous NADH in the course of acid extraction

To the alkaline homogenate of brain tissue (Fig. 1B, stage *1*), NADH was added to a concentration exceeding the endogenous amounts by a factor of 10 and 20, and the oxidized nucleotides extracted with acid were determined as described in the text. The endogenous oxidized nucleotides were determined in parallel samples of the alkaline homogenate. Mean values from 2 determinations are given.

NADH added (μM)	Oxidized nucleotides in homogenate (μM)		Recovery of NADH in oxidized form	
	control without added NADH	with the addition of NADH	μM	%
30	22	22.6	0.6	2
60	22	25.9	3.9	6.5

Table 3

The oxidation of NADH by phenazine methosulphate

To 4 ml of NaOH-cysteine solution, containing different amounts of NADH, 0.4 ml of phenazine methosulphate was added and the solution was kept in 0 - 2°C for 10 min. Thereafter 0.4 ml of 100% trichloroacetic acid was added and NAD^+ was determined. Results of two determinations are given.

NADH added (nmols)	NADH oxidized (%)
10	101; 105
20	92; 109
30	99; 100

process occurs readily and is to a great extent independent of the conditions applied. III. Excitation of the fluorescence of the products formed from the nucleotides in strong alkaline medium (Fig. 1, stage 6) was performed in ethanolic medium after Green & Israelstam (1968). This gave a much higher sensitivity of the determinations than in the aqueous medium (Fig 2).

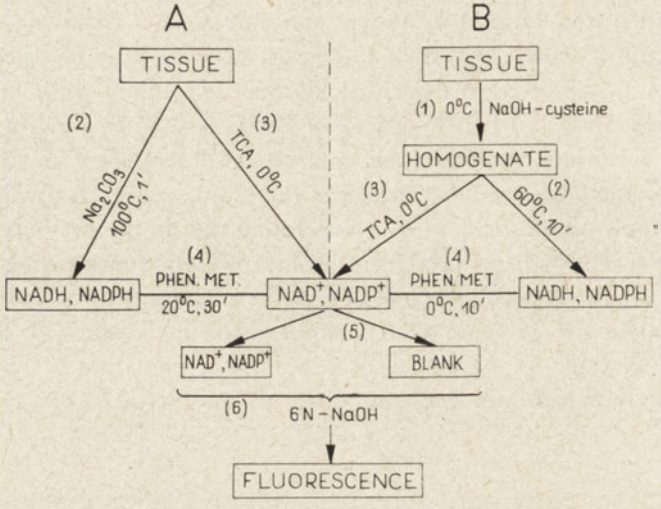

Fig. 1. The scheme of the procedures for determination of the nicotinamide-adenine dinucleotides. A, Procedure for fresh liver tissue (Sokal *et al.*, 1969). B, Procedure for frozen brain tissue. Abbreviations: TCA, trichloroacetic acid; Phen. met., phenazine methosulphate.

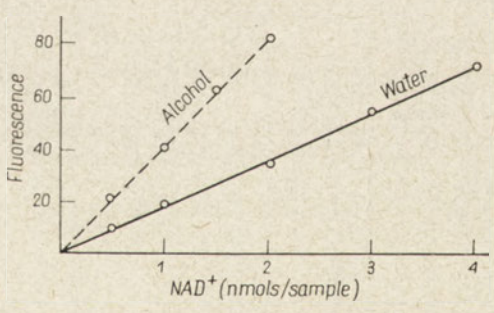

Fig. 2

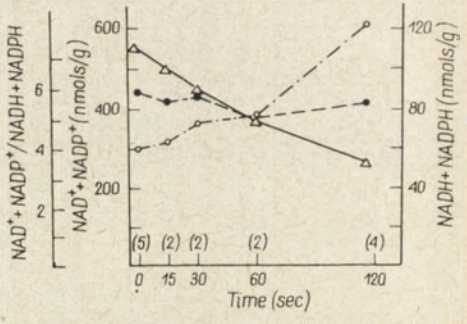

Fig. 3

Fig. 2. Calibration curves for NAD^+ in ethanol and aqueous media. Samples of 0.4 ml containing indicated amounts of NAD^+ were mixed with 0.8 ml of 9 N-NaOH. After incubation at 38° for 60 min, 2.8 ml of water or 50% ethanol was added and the fluorescence was measured.

Fig. 3. The influence of the duration of ischaemia (after decapitation) on the content and oxido-reduction state of nicotinamide-adenine nucleotides in rat brain. Rats were decapitated either directly into liquid nitrogen (zero time) or the head was kept after decapitation for the indicated time at room temperature before being immersed in liquid nitrogen. Determinations were performed using procedure B (Fig. 1). Number of rats in brackets. ●, $NAD^+ + NADP^+$; ○, NADH + NADPH; △, $[NAD^+ + NADP^+] : [NADH + NADPH]$ ratio.

In this way the extraction procedure of Burch has been modified to permit its application together with fluorimetric determinations of the nucleotides in brain tissue. The procedure, described in detail below, appeared to give correct results as the same amount of both oxidized and reduced forms of the nucleotides were found in fresh and frozen brain tissue, and the recovery of exogenous NAD^+ and NADH was satisfactory (Table 4). The results were independent of the amount of tissue (in the range 200 - 400 mg) subjected to extraction (Table 5).

Effect of duration of ischaemia. The effect of time interval between the decapitation of the rat and freezing of the brain on the content and oxido-reduction state of nucleotides, was studied. Figure 3 shows that the amount of reduced nucleotides increased rapidly after decapitation, being about doubled within two minutes. At the same time, there was a decrease of the oxidized forms, and thus the sum of nucleotides remained constant. The ratio $[NAD^+ + NADP^+] : [NADH + NADPH]$ decreased from 7.4 ± 0.4 at zero time to 3.4 ± 0.5 two minutes later. This points to the necessity of rapid freezing of brain tissue for correct determination of the amount and oxido-reduction state of the nucleotides.

Table 4

The content of nicotinamide-adenine nucleotides in frozen and fresh brain tissue and the recovery of exogenous NAD^+ and NADH

Two minutes after decapitation a part of the brain was frozen in liquid nitrogen; a parallel sample of the fresh tissue was taken directly for extraction. The procedure B (Fig. 1) was applied. Exogenous NAD^+ and NADH were added to the extraction medium simultaneously with the tissue to obtain their final concentrations 30 μM and 10 μM, respectively. Mean values from 4 determinations ±S.D. are given.

Brain	Endogenous nucleotides (nmols/g)		Recovery of exogenous NAD^+ and NADH (%)	
	$NAD^+ + NADP^+$	NADH + NADPH	NAD^+	NADH
Fresh	398 ± 70	118 ± 4	—	—
Frozen	408 ± 35	121 ± 17	94 ± 5	90 ± 8

Table 5

The influence of the amount of brain tissue taken to extraction on the content of nicotinamide nucleotides

The frozen brain was subjected to extraction at "zero" time and the procedure B (Fig. 1) was applied. Mean values from 5 - 16 determinations ±S.D. are given.

Amount of tissue (mg)	$NAD^+ + NADP^+$	NADH + NADPH
	nmols/g	
200 - 250	429 ± 18	60 ± 6
250 - 300	402 ± 28	58 ± 8
300 - 350	420 ± 34	57 ± 7
350 - 400	403 ± 28	57 ± 7

Analytical procedures

Reagents. (*a*), L-Cysteine (Fluka AG, Buchs S.G., Switzerland), 25 mM solution in water, prepared immediately before use. (*b*), NaOH-cysteine solution for extraction: 4 ml of 0.05 N-NaOH, 0.1 ml of 25 mM-cysteine, and water up to 5 ml; prepared just before use. (*c*), Phenazine methosulphate (Koch-Light, Colnbrook, Bucks., England), 1 mM-solution in water. (*d*), Trichloroacetic acid, 100% w/v. (*e*), Standards of NAD^+ and NADH (Boehringer, Mannheim, West Germany): NAD^+, 0.75 mM aqueous solution, stored at 0 - 4°C; NADH, 0.375 mM in 0.04 N-NaOH, prepared before use; to remove the NAD^+ present in commercial preparations of NADH, the solution was heated for 10 min at 60°C; the concentration of NAD^+ and NADH in the above solutions was determined spectrophotometrically as described previously (Sokal *et al.*, 1969). (*f*), Fluorescence standard: quinine sulphate, 0.5 µg/ml in 0.1 N-H_2SO_4.

Material. Rats were decapitated directly into liquid nitrogen. Samples of the brain tissue (with the exception of hindbrain) were taken out from the frozen head by means of chisels. To avoid superficial thawing during the preparation, the head was immersed several times in the liquid nitrogen. Samples of the brain were kept in liquid nitrogen till extraction.

Procedure. The scheme of the procedure is presented in Fig. 1B. A sample of frozen brain tissue, 250 - 350 mg, was homogenized rapidly with 5 ml of cold (0 - 2°C) NaOH-cysteine solution (*b*) in a Potter glass homogenizer placed in ice (stage *1*). Two ml of the homogenate were added to 2 ml of the same NaOH-cysteine solution heated previously to 60°C, and incubated at 60°C for 10 min (stage *2*). After cooling, 0.4 ml of phenazine methosulphate (*c*) was added and the sample was kept in an ice-bath for 10 min. Then 0.4 ml of 100% trichloroacetic acid was added, mixed, centrifuged at 8000 *g* at 0 - 2°C for 15 min, and the supernatant was adjusted to 5 ml with water (stage *4*).

A parallel sample of the homogenate (1 ml) was added to 3 ml of NaOH-cysteine solution, placed in an ice-bath, 0.4 ml of trichloroacetic acid was added, and the sample was mixed quickly. Then 0.4 ml of phenazine methosulphate was added, the sample was centrifuged as above and the supernatant adjusted to 5 ml with water (stage *3*).

The solution obtained after stage *3* contained NAD^+ and $NADP^+$ originally present in the tissue; also after stage *4* the oxidized forms of the nucleotides were present in the solution, but their amounts were equivalent to the original content of NADH and NADPH.

From each solution, 1 ml sample was taken; to the sample 0.2 ml of water was added, and the alkaline fluorescence was excited in 0.4 ml of solution to which 0.8 ml of 9 N-NaOH was added. The samples were mixed promptly and heated at 38°C for 60 min. Then 2.8 ml of 50% ethanol was added, the fluorescence measured, and the content of the nucleotides calculated as described in the previous paper (Sokal *et al.*, 1969). Blank samples were prepared as follows: to parallel 1 ml samples of NAD^++$NADP^+$ solutions, 0.2 ml of 3.5 N-NaOH was added and

the samples were heated at 60°C for 10 min (stage 5). Then 0.4 ml samples were treated in the same way as the proper samples.

Content and oxido-reduction state of nicotinamide-adenine nucleotides in rat brain

The determinations were performed on 35 female rats of the Wistar strain; 29 of them were 4 - 6 months old and 6 were aged 15 months. The results are presented in Table 6, in which the data obtained by other authors are also included.

Table 6

The content and oxido-reduction state of nicotinamide nucleotides in the brain of rat according to different authors and methods used

nmols/g								
NAD^+	$NADP^+$	$NAD^+ + NADP^+$	NADH	NADPH	NADH+NADPH	Ratio: $\frac{NAD^+ + NADP^+}{NADH + NADPH}$	Authors	Procedure
161	—	—	137	—	—	—	Jedeikin & Weinhouse (1955)	1 *ds*
200 ±9	3	200	133 ±54	11 ±4	144	1.4	Glock & Mc Lean (1955)	1 *es*
241	10	251	107	12	119	2.1	Lowry *et al.* (1957)	4 *df*
322	7	329	95	23	118	2.8	Lowry *et al.* (1961a)	1 *ef*
289	20	309	46	12	58	5.3	Garcia-Bunuel *et al.* (1962)	2 *ef*
290 ±30	14 ±6	304	—	—	—	—	Coper (1963)	2 *ds*
283 ±23	21 ±3	304	—	—	—	—	Willing *et al.* (1964)	1 *df*
—	—	240	—	—	110	2.2	Neuhoff (1965)	2 *df*
—	—	—	—	—	153	—	Pande *et al.* (1964)	1 *df*
415 ±56	—	—	144 ±83	—	—	—	Brown (1964)	1 *df*
198 ±11	—	—	20 ±3	—	—	—	Lewis & Pollock (1965)	4 *df*
—	5.3 ±0.3	—	—	26.4 ±1	—	—	Burch *et al.* (1967)	3 *ef*
—	—	406±31 [a] 389±28 [b]	—	—	61±6 [a] 57±8 [b]	6.7±0.7 [a] 6.9±0.6 [b]	Present paper	3 *df*

Method of determination: *e*, enzymic cycling; *d*, direct; *f*, fluorimetric; *s*, spectrophotometric. Procedures: 1, without freezing; 2, freezing after isolation of brain; 3, decapitation into liquid nitrogen or freone; 4, no data. [a] Results for 29 rats 4 - 6 months old. [b] Results for 6 rats 15 months old.

DISCUSSION

A fluorimetric method for determination of nicotinamide-adenine dinucleotides in liver tissue was described in the previous paper (Sokal *et al.*, 1969). When attempting to apply this method for determination of the nucleotides in brain tissue, two possible sources of errors had to be taken into account: (a), the post-mortem changes in the content and in the oxido-reduction state of the nucleotides; (b), the formation of artifacts during the extraction of frozen brain by the procedure of Gordon (1963).

It has been demonstrated by Lowry *et al.* (1964) that, after decapitation of the mouse, an increase in NADH and decline of NAD^+ occurs in the brain. However, their data do not include the time-course of post-mortem changes during the very short periods after decapitation. The results obtained in the present work (Fig. 3) show that, in the rat, changes in the oxido-reduction state of nicotinamide nucleotides are almost linear with respect to the time elapsed since decapitation, within 2 min. Thus, when no freezing has been applied, the magnitude of the error increases with time from the moment of decapitation till the extraction of tissue. These errors are the greatest for the reduced nucleotides, and consequently the oxido-reduction state of nucleotides found, is incorrect. It seems probable that the lower ratios of the oxidized-to-reduced nucleotides obtained by the majority of authors (Table 6) may have resulted from the post-mortem changes which had not been satisfactorily prevented. On the other hand, extraction of the frozen brain may bring about additional errors: when applying the extraction with hot sodium carbonate according to Gordon (1963) to the frozen brain tissue, we encountered a loss of the reduced forms of nucleotides (Table 1), due probably to their partial oxidation with subsequent decomposition of the formed oxidized nucleotides. The oxidation of reduced nucleotides was observed by other authors during extraction both of whole tissue (Bassham, Birt, Hems & Loening, 1959; Neuhoff & Desselberger, 1965; Burch *et al.*, 1967) and of mitochondria (Neubert *et al.*, 1964); however, the mechanism of these changes has not yet been satisfactorily explained.

Recently Burch *et al.* (1967) have demonstrated that the disappearance of reduced nucleotides during tissue extraction is due to the presence of haemoglobin which is able to oxidize rapidly the reduced forms of nucleotides both in alkaline and acidic medium. Thus the erroneous low values of NADH and NADPH obtained by procedures requiring freezing and thawing of the tissue, may be explained as an effect of disruption of red cells and tissue cells, resulting in longer contact of the reduced nucleotides with haemoglobin, before the latter was destroyed.

In the procedure presented in this report, the oxidation of the reduced form of nucleotides is prevented by the addition of cysteine as an antioxidant to the first alkaline extraction medium, and by extraction of the oxidized forms with trichloroacetic acid which simultaneously acts as a deproteinizing agent.

The procedures proposed by other authors, such as extraction with a phenol-saturated solution (Neuhoff, 1965; Neuhoff & Desselberger, 1965), or single acidic

extraction with subsequent determination of the degradation products of the reduced nucleotides (Heldt, Klingenberg & Papenberg, 1965) are more complicated and their usefulness is also controversial (Burch *et al.*, 1967).

The author feels greatly indebted to the Head of the Biochemical Department of the Institute, Doc. Dr. J. Piotrowski and to the Chief of the Laboratory of General Biochemistry, Dr. Teresa Wrońska-Nofer, for kind assistance in the preparation of the manuscript and helpful comments. The technical assistance of Mrs. Wiesława Adamiak is highly appreciated.

These investigations have been done under the Polish-American Agreement 05-003-3 with the Occupational Health Program, P.H.S., U.S.A.

REFERENCES

Bassham J. A., Birt L. M., Hems R. & Loening U. E. (1959). *Biochem. J.* **73**, 491.
Brown F. Ch. (1964). *Can. J. Biochem.* **42**, 489.
Burch H. B., Bradley M. E. & Lowry O. H. (1967). *J. biol. Chem.* **242**, 4546.
Coper H. (1963). *Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak.* **244**, 420.
Garcia-Bunuel L., Mc Dougal D. B., Burch H. B., Jones E. M. & Touhill E. (1962). *J. Neurochem.* **9**, 589.
Glock G. E. & Mc Lean P. (1955). *Biochem. J.* **61**, 388.
Gordon E. E. (1963). *J. biol. Chem.* **238**, 2135.
Green W. G. & Israelstam G. F. (1968). *Can. J. Biochem.* **46**, 390.
Heldt H. W., Klingenberg M. & Papenberg K. (1965). *Biochem. Z.* **342**, 508.
Jedeikin L. A. & Weinhouse S. (1955). *J. biol. Chem.* **213**, 271.
Lewis J. J. & Pollock D. (1965). *Biochem. Pharmacol.* **14**, 636.
Lowry O. H., Passonneau J. V., Schulz D. W. & Rock M. K. (1961a). *J. biol. Chem.* **236**, 2746.
Lowry O. H., Passonneau J. V. & Rock M. K. (1961b). *J. biol. Chem.* **236**, 2756.
Lowry O. H., Passonneau J. V., Hasselberger F. X. & Schulz D. W. (1964). *J. biol. Chem.* **239**, 18.
Lowry O. H., Roberts N. R. & Kapphahm J. I. (1957). *J. biol. Chem.* **224**, 1047.
Neubert D., Schulz H. U. & Hoehne R. (1964). *Biochim. biophys. Acta* **92**, 610.
Neuhoff V. (1965). *Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak.* **250**, 302.
Neuhoff V. & Desselberger E. (1965). *Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak.* **252**, 43.
Pande S. V., Bhan A. K. & Venkitasubramanian T. A. (1964). *Analyt. Biochem.* **8**, 446.
Sokal J. A., Tarkowski S. & Wrońska-Nofer T. (1969). *Acta biochim. Polon.* **16**, 1.
Willing F., Neuhoff V. & Herken H. (1964). *Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak.* **247**, 256.

SPOSOBY EKSTRAKCJI I ICH ZNACZENIE DLA OZNACZANIA DWUNUKLEOTYDÓW NIKOTYNAMIDO-ADENINOWYCH W ZAMROŻONEJ TKANCE MÓZGOWEJ

Streszczenie

1. Zmodyfikowano procedurę ekstrakcji dwunukleotydów nikotynamido-adeninowych wg. Burch i wsp. (1967) w celu dostosowania do bezpośredniej fluorymetrycznej metody oznaczania w zamrożonej tkance mózgowej.

2. Stwierdzono szybkie zmiany pośmiertne zawartości i stanu oksydo-redukcyjnego dwunukleotydów nikotynamido-adeninowych w mózgu szczura, które wskazują na konieczność szybkiego zamrożenia mózgu w badaniach tych nukleotydów.

3. Proponowana procedura pozwala na oznaczenie w szybki, prosty, nieenzymatyczny sposób sumy $NAD^+ + NADP^+$ i NADH+NADPH.

Received 27 June, 1970.

A C T A B I O C H I M I C A P O L O N I C A
Vol. 18 1971 No. 1

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INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO PHOSPHOLIPIDS OF THE INTESTINE OF NORMAL RATS

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1. The incorporation of $^{32}P_i$ into phospholipids of rat intestinal mucosa and Ca^{2+} absorption along the entire length of the small intestine were investigated. 2. *In vitro*, $^{32}P_i$ incorporation was age-dependent; it was two to three times higher in the intestine of one-month-old rats than in that of two-month-old and adult rats. 3. In one-month-old rats the segments corresponding to jejunum showed the highest incorporation. 4. The incorporation of $^{32}P_i$ into the main phospholipid fractions decreased in the following order: lecithin, sphingomyelin, lysolecithin, cephalin. 5. The differences in $^{32}P_i$ incorporation into mucosal phospholipids seem to be dependent on the differences of the intracellular pool of P_i and phospholipid precursors. 6. The mechanism of Ca^{2+} absorption does not seem to be dependent on metabolic turnover of phospholipid.

Several authors have suggested that phospholipids might play a role in the movement of cations across cellular membranes (Hokin & Hokin, 1959; Solomon, Lionetti & Curran, 1959; Woolley & Campbell, 1962). The occurrence of active transport of calcium against concentration gradient in rat small intestine is well documented (Schachter, Dowdle & Schenker, 1960a,b; Michalska, 1968), and its intensity is known to vary along the small intestine. It is the highest in two regions: in the duodenum and the segment corresponding to 3/4 of the length of the small intestine (Michalska, 1968).

Schachter & Rosen (1959) by using the everted gut-sack technique have demonstrated that vitamin D increases the transport of calcium against a concentration gradient in the proximal part of small intestine. Harrison & Harrison (1960) have reported that vitamin D increases calcium diffusion across the intestinal wall over the entire length of the intestine. Vitamin D was also shown to influence the incorporation of $^{32}P_i$[1] into phospholipids of intestinal mucosa of rat (Thompson

[1] Abbreviations used: P_i, orthophosphate; t.l.c., thin-layer chromatography.

& De Luca, 1964) and chicken (Neville & Holdsworth, 1968). It could be therefore expected that some connection exists between active transport of Ca^{2+} and phospholipid turnover in the small intestine.

The aim of the present work was to obtain information on $^{32}P_i$ incorporation into phospholipids of intestinal mucosa of young and adult rats. The incorporation of $^{32}P_i$ over the entire length of small intestine was compared with the location of active transport of Ca^{2+}.

MATERIALS AND METHODS

Animals. Wistar rats of both sexes were used. The animals were given a standard diet and water *ad libitum*. The weight of the animals was: at 1 month 50 - 70 g, at 6 weeks 70 - 90 g; at 2 months 100 - 150 g; adult rats weighed 200 - 300 g. The animals were fasted for 18 - 24 h before the experiment; they were killed by a blow on the head followed by decapitation.

Preparation of segments of the small intestine. After killing of the animal, the small intestine was removed rapidly, 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 six 5-cm segments were taken at equal intervals from pylorus to the ileocaecal valve (Fig. 1).

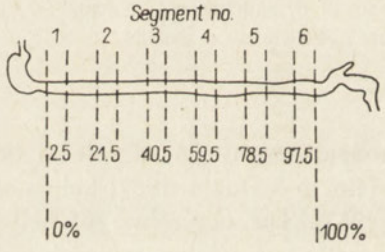

Fig. 1. Diagram of small intestine dissections.

In vitro *incorporation of* $^{32}P_i$. The intestinal segments from one animal were everted, labelled by attaching a thread, and placed together in an incubation vial containing 15 ml of medium consisting of 151 mM-NaCl, 20 mM-glucose, 0.8 mM-NaH_2PO_4, 0.4 mM-$CaCl_2$ and 1.47 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 NaH_2PO_4 (500 μCi/100 ml) was added. The segments were incubated with shaking for 3 h at 37°C, either O_2 - CO_2 (95:5, v/v) or N_2 - CO_2 (95:5, v/v) being passed through the medium. Following incubation, the segments were washed in two successive water baths, then mucosa was removed by scraping with a metal spatula and subjected to analysis.

Everted gut-sack experiments. In a typical experiment, everted sacks from five or six 5-cm segments (see above) were prepared as described by Schachter *et al.*

(1960a,b). The serosal compartment was filled with about 1 ml of incubation medium composed of 151 mM-NaCl - 4 mM-$CaCl_2$ - 20 mM-glucose - 4 mM-phosphate buffer, pH 7.4. The sack was placed in the Warburg vessel containing 3 ml of the above medium, and incubated with shaking at 37°C for 3 h in an atmosphere of O_2. Following incubation, the sack was removed, and the mucosal and serosal fluids were collected for calcium determination.

Experiments in vivo. The rats were injected intraperitoneally with $NaH_2{}^{32}PO_4$ (500 µCi per 100 g of body weight) and three hours later killed by decapitation. A sample of blood was collected, and whole blood or serum were used for determination of radioactivity and total phosphorus. The small intestine was removed, rinsed with 0.9% NaCl solution, cut into segments as above, and mucosa was taken for analysis.

Extraction and fractionation of phospholipids. The mucosa obtained from intestinal segments was homogenized with 10 ml of chloroform - methanol (2:1, v/v). The homogenate was filtered and the extract washed with water (Folch, Lees & Sloane-Stanley, 1957). A sample of the chloroform phospholipid extract was taken for radioactivity determination, and the remaining extract was submitted to thin-layer chromatography on silicic acid in a system of chloroform - methanol - water (65:25:4, by vol.). The spots of the fractionated phospholipids were visualized with Rhodamine 6-G (0.002% aqueous solution) and by exposing t.l.c. plates to X-ray sensitive film for two weeks. The particular spots were scraped off and placed in a small glass column plugged at the bottom with glasswool. Then the lipids were eluted with a mixture of chloroform - methanol (1:1, v/v). The radioactivity of each lipid fraction was estimated and phosphate was determined after digestion with $HClO_4$.

Determination of radioactivity. The sample was plated on an 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 countings surpassed background at least 20 times.

$NaH_2{}^{32}PO_4$, specific activity 160 µCi/µmol P, was supplied by Biuro Dystrybucji Izotopów (Warszawa, Poland).

Presentation of results. The data of *in vitro* experiments are expressed in terms of relative specific radioactivity, i.e. the ratio of phospholipid specific activity to the specific activity of phosphate in the incubation medium. The data of *in vivo* experiments are expressed in terms of the ratio of mucosal phospholipid specific activity to the specific activity of total phosphorus of blood serum.

Analytical procedures. Lipid phosphorus was determined by the method of Ernster, Zetterstrom & Lindberg (1950) as modified by Strickland, Thompson & Webster (1956). Calcium was determined by complexometric titration using murexide as indicator (Schachter *et al.*, 1960a,b). Total nitrogen was determined by the method of Kjeldahl with the Markham all-glass micro-apparatus. Tissue dry weight was determined by dessicating the sample of mucosa at 105°C to constant weight.

RESULTS

Experiments in vitro. Figure 2 shows the amount of mucosal phospholipids of the intestinal segments. The data are expressed either as μmols of lipid phosphorus per 1 mg of total nitrogen, or per 10 mg of dry weight. In young rats there were some differences in the amount of mucosal phospholipid in particular intestinal segments. In one-month-old rats the highest amount was found in segments no. 2 and 3. In rats aged two months, the differences between the segments were not significant. In all animals the amount of phospholipid was the smallest in segments no. 5 and 6.

Preliminary experiments showed that the incorporation of $^{32}P_i$ into phospholipids was almost linear with time over a period of 3 h (Fig. 3) and was strongly dependent on oxygen as the gas phase during the incubation period (Fig. 4).

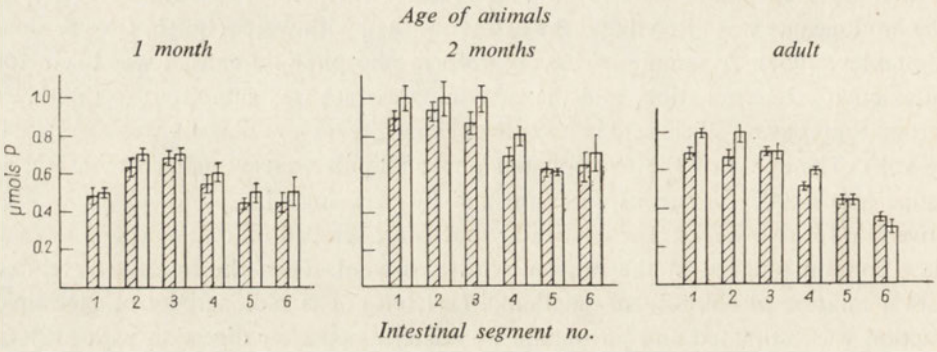

Fig. 2. Phospholipid pool in the small intestine of rats of different age. Dashed columns, μmols P/mg N; outlined columns, μmols P/10 mg of dry tissue. The bars show the S.E.M. from at least 6 determinations.

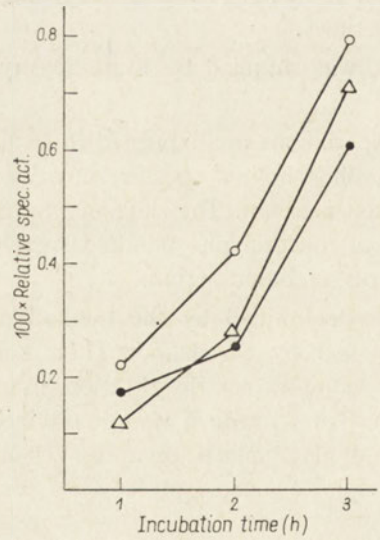

Fig. 3. Effect of incubation time on the incorporation of $^{32}P_i$ into phospholipids of intestinal mucosa of one-month-old rats. Segments no.: ●, *1*; ○, *3*; △, *5*.

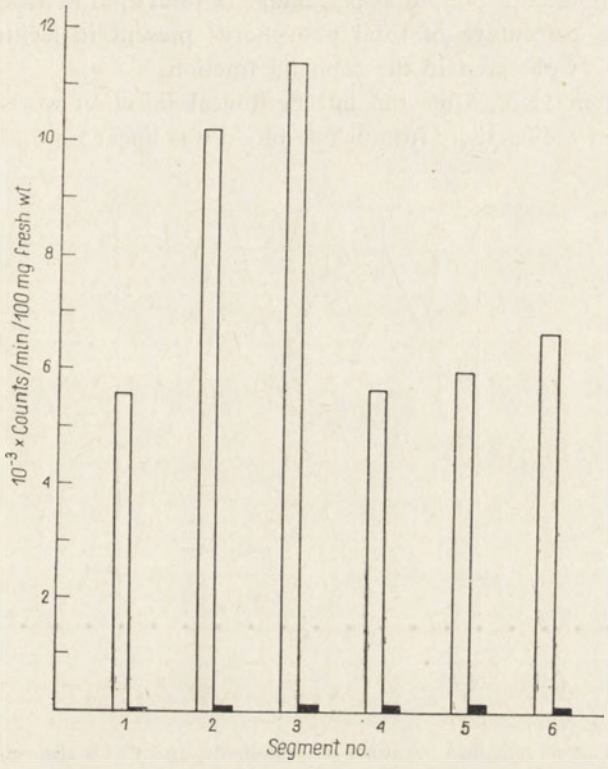

Fig. 4. Effect of aerobic and anaerobic conditions on the incorporation of $^{32}P_i$ into mucosal phospholipids of the intestine of one-month-old rats. The gas atmosphere, both aerobic (outlined columns) and anaerobic (black columns) contained 5% (v/v) of CO_2. Time of incubation 3 h.

The incorporation of $^{32}P_i$ into mucosal phospholipid of one- and two-month-old and adult rats is illustrated in Fig. 5. The results of two typical experiments on the absorption of calcium by six-week-old animals are plotted on the same diagram. The relative specific activity was considerably higher in one-month-old than in older animals. The segments no. 2 and 3 from the younger animals showed the highest incorporation. The incorporation in two-month-old and adult animals was similar, but in the two-month-old rats the differences between separate segments, although smaller than in one-month-old animals, were still apparent. Changes in calcium absorption appear to have an opposite trend than the incorporation of $^{32}P_i$ by the younger animals.

It should be noted that when the incorporation of $^{32}P_i$ into phospholipids by the intestine of one-month-old rats was expressed in terms of counts/min/100 mg of fresh mucosa, the same pattern was obtained.

Fractionation of the whole phospholipid extract on t.l.c. gave in all segments an essentially similar pattern of $^{32}P_i$ incorporation (Fig. 6). The results obtained after 1, 2 or 3 h of incubation were similar. Lecithin was the main phospholipid fraction of intestinal mucosa and showed the highest $^{32}P_i$ incorporation. The radio-

activity of this fraction expressed as percentage of total lipid radioactivity, exceeded significantly the percentage of total phosphorus present in lecithin. The lowest incorporation was observed in the cephalin fraction.

Experiments in vivo. After the intraperitoneal injection of $NaH_2{}^{32}PO_4$, the disappearance of radioactivity from whole blood was linear up to 3 h (Fig. 7), and

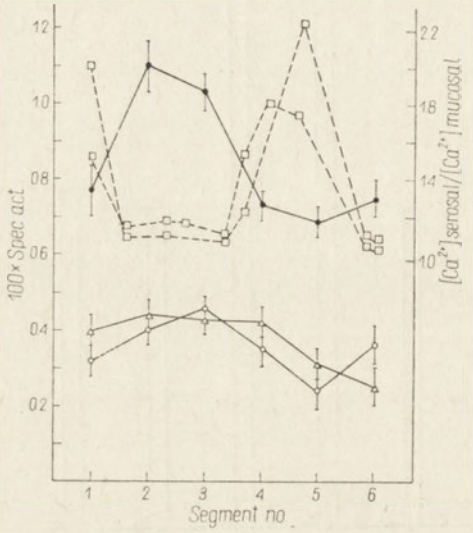

Fig. 5. Incorporation of $^{32}P_i$ into intestinal phospholipids, and Ca^{2+} absorption. Incorporation of $^{32}P_i$ by rats aged: ●, one month; ○, two months; △, adult. The bars indicate the S.E.M. from 12 to 18 experiments. — — —, Ca^{2+} absorption by six-week-old rats (results of two separate experiments).

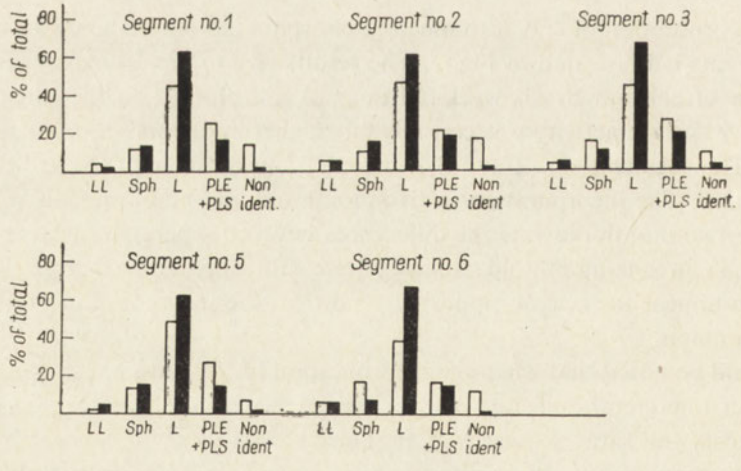

Fig. 6. Incorporation of $^{32}P_i$ into the main phospholipid fractions of intestinal mucosa. Black columns, percentage of total radioactivity; outlined columns, percentage of total phosphorus. *LL*, lysolecithin; *Sph*, sphingomyelin; *L*, lecithin; *PLE*, phosphatidylethanolamine; *PLS*, phosphatidylserine. Each column represents the mean from two to four experiments.

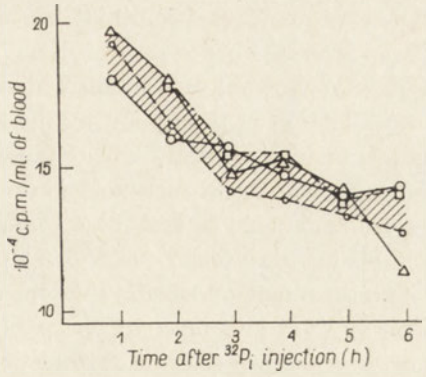

Fig. 7. Time-course of total radioactivity disappearance from whole blood after a single intraperitoneal injection of $NaH_2{}^{32}PO_4$ (6×10^7 counts/min/100 g of body wt.). Each curve represents a single experiment.

a plateau was observed during the fourth and fifth hour. The period of 3 h was therefore chosen for further experiments. The experiments on incorporation of phosphorus into intestinal phospholipids *in vivo* were performed under conditions comparable to those *in vitro*, i.e. analogous segments of the intestine were taken, and amounts of radioactivity given intraperitoneally corresponded to those added to the incubation medium. In contrast to the results obtained *in vitro*, all rats *in vivo* showed a similar pattern of incorporation, the values decreasing from the duodenum towards the ileocaecal valve (Fig. 8).

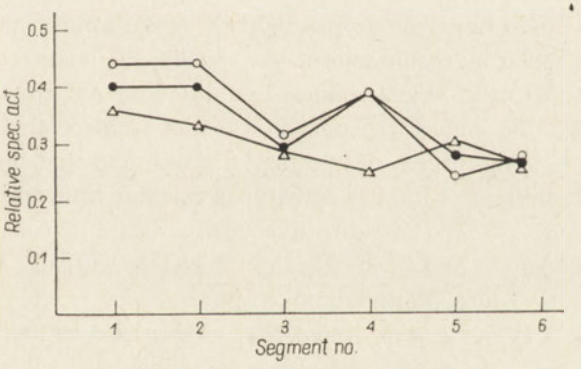

Fig. 8. Incorporation *in vivo* of $^{32}P_i$ into phospholipids of intestinal mucosa. Three hours after a single intraperitoneal injection of $NaH_2{}^{32}PO_4$, radioactivity of blood serum and of mucosal phospholipids was determined. The results are expressed as the ratio of specific activity of mucosal phospholipid to specific activity of total serum phosphorus, in rats aged: ●, one month; ○, two months; △, adult. Each point represents the mean from at least eight experiments.

DISCUSSION

The presented results show that the incorporation of $^{32}P_i$ into phospholipids of intestinal mucosa is age-dependent, and that the highest incorporation occurs in one-month-old rats (Fig. 5). The activity of incorporation varies along the small

intestine. In the intestine of one-month-old rats the activity was found to be the highest in the second and third segment (which correspond to jejunum), both when it was expressed per tissue weight and per μmols of lipid phosphorus. The phospholipid pool does not vary so much along the small intestine to account for such differences in $^{32}P_i$ incorporation by the segments (Fig. 2). The slightly larger amount in the middle segments would cause even lower specific activity if one assumes the same activity of incorporation in all segments. The higher specific activity in the jejunum could be due either to the higher specific activity of intracellular orthophosphate or to faster turnover of phospholipids.

Pretreatment of rachitic chickens with vitamin D resulted in an increased $^{32}P_i$ incorporation into mucosal phospholipids only when Ca^{2+} was present in the incubation mixture (Neville & Holdsworth, 1968); the authors concluded that vitamin D had no effect on phospholipid metabolism but increased phosphate translocation in the presence of calcium. A similar effect of Ca^{2+} on stimulation by vitamin D of $^{32}P_i$ incorporation by suspended intestinal mucosa cells of vitamin D deficient rats, was observed by Thompson & DeLuca (1964).

The observation of Kowarski & Schachter (1969) implies the existence of compartmentalized and channellized transport of phosphate through the mucosal cell, which is relatively independent of the presence of calcium in medium. The phosphate channels could be lined with phospholipid-containing membrane, e.g. the endoplasmic reticulum. Phosphate transport is known to be the greatest in the jejunum (Harrison & Harrison, 1961), hence the greater incorporation of $^{32}P_i$ into phospholipid in jejunum could be secondary to higher mucosal uptake and greater specific activity of ^{32}P-labelled inorganic phosphate. Finally, the studies of Schachter, Kowarski & Reid (1967) and Wasserman & Taylor (1968) on rat and chicken have demonstrated that the active transport of calcium is associated with vitamin D induced calcium-binding protein in intestinal mucosa, and that the calcium-binding protein activity correlates with the activity of calcium transport along the small intestine. The results of our experiments seem to indicate that the activity of Ca^{2+} absorption in duodenum and ileum does not correlate with the maximum activity of $^{32}P_i$ incorporation into phospholipids (Fig. 5).

Hokin & Hokin (1963) demonstrated that an increase in the secretion of proteins and other hydrophilic material was accompanied by an increase in the incorporation of $^{32}P_i$ into phosphatidic acid and phosphoinositide, but not into other phospholipids. Similarly, Johnston & Bearden (1960) demonstrated that in the hamster, during absorption of fatty acids, there was a high incorporation of phosphate but only into phosphatidic acid. In the present studies we could not find any appreciable radioactivity at the position of phosphatidic acid on t.l.c. plate. Nearly all activity was associated, in the decreasing order, with lecithin, sphingomyelin, lysolecithin, and cephalin.

The intestine of one-month-old rats showed *in vitro* two to three times higher incorporation of $^{32}P_i$ into phospholipids than that of two-month-old and adult rats. On the other hand, in the experiments *in vivo* no significant differences were found either between the animals of different age, or between different segments

of intestine (Fig. 8). It seems that *in vitro* at least a part of the $^{32}P_i$ incorporated into phospholipid was due to unidirectional translocation of phosphate from the mucosal to the serosal surface of the intestine. In the experiments *in vivo* this mechanism probably was not operative, as $^{32}P_i$ was injected intraperitoneally and was not present in the lumen of the intestine. Both active transport of phosphate and diffusion have been shown to occur in the intestine (Harrison & Harrison, 1961). The intracellular content of phosphate is dependent on simple diffusion and thus the amount of phosphate and phospholipid precursors is approximately the same along the intestinal wall.

The present experiments indicate that the differences in $^{32}P_i$ incorporation *in vitro* into mucosal phospholipid along the small intestine might depend on the differences in the intracellular pool of phosphate and of phospholipid precursors. These differences are more evident in one-month-old than in older rats. Also, it seems that the mechanism of Ca^{2+} absorption is not coupled with metabolic turnover of phospholipid.

REFERENCES

Ernster L., Zetterstrom R. & Lindberg O. (1950). *Acta chem. scand.* **4**, 942.
Folch J., Lees M. & Sloane-Stanley G. H. (1957). *J. biol. Chem.* **226**, 497.
Harrison H. E. & Harrison H. C. (1960). *Am. J. Physiol.* **199**, 265.
Harrison H. E. & Harrison H. C. (1961). *Am. J. Physiol.* **201**, 1007.
Hokin L. E. & Hokin M. R. (1959). *J. biol. Chem.* **234**, 1387.
Hokin L. E. & Hokin M. R. (1963). *Ann. Rev. Biochem.* **32**, 553.
Johnston J. M. & Bearden J. H. (1960). *Archs Biochem. Biophys.* **90**, 57.
Kowarski S. & Schachter D. (1969). *J. biol. Chem.* **244**, 211.
Michalska L. (1968). *Acta Biol. Med. Soc. Sc. Gedan.* **13**, 49.
Neville E. & Holdsworth E. S. (1968). *Biochim. biophys. Acta* **163**, 362.
Schachter D., Dowdle E. B. & Schenker H. (1960a). *Am. J. Physiol.* **198**, 263.
Schachter D., Dowdle E. B. & Schenker H. (1960b). *Am. J. Physiol.* **198**, 275.
Schachter D., Kowarski S. & Reid P. (1967). *J. clin. invest.* **46**, 1113.
Schachter D. & Rosen S. M. (1959). *Am. J. Physiol.* **196**, 357.
Solomon A. K., Lionetti F. & Curran P. H. (1959). *Nature, Lond.* **178**, 582.
Strickland K. P., Thompson R. H. S. & Webster G. R. (1956). *J. Neurolog. Neurosurg. Psychiat.* **19**, 12.
Thompson V. W. & DeLuca H. F. (1964). *J. biol. Chem.* **239**, 984.
Wasserman R. H. & Taylor A. N. (1968). *J. biol. Chem.* **243**, 3987.
Woolley D. W. & Campbell N. K. (1962). *Biochim. biophys. Acta* **57**, 384.

WBUDOWYWANIE [^{32}P]ORTOFOSFORANU DO FOSFOLIPIDÓW JELITA CIENKIEGO U SZCZURÓW

Streszczenie

1. Aktywność wbudowywania $^{32}P_i$ do fosfolipidów oraz absorpcję Ca^{2+} wzdłuż całej długości jelita cienkiego badano u szczurów jedno-, dwumiesięcznych i dorosłych.

2. Aktywność wbudowywania $^{32}P_i$ była dwu- do trzykrotnie wyższa u szczurów jednomiesięcznych w porównaniu z dwumiesięcznymi i dorosłymi.

3. U szczurów jednomiesięcznych istnieją wyraźne różnice w aktywności wbudowywania $^{32}P_i$ wzdłuż przebiegu jelita cienkiego. Najwyższy stopień wbudowywania wykazuje odcinek odpowiadający 1/3 odległości od odźwiernika.

4. Najwyższą aktywność wbudowywania wykazywała frakcja lecytynowa, a w dalszej kolejności sfingomielina, lizolecytyna i kefaliny.

5. Różnice odcinkowe we wbudowywaniu $^{32}P_i$ wydają się zależeć od wielkości puli metabolicznej fosforanu i innych prekursorów fosfolipidów.

6. Mechanizm wchłaniania Ca^{2+} nie wydaje się zależeć wprost od aktywności przemiany fosfolipidów.

Received 3 July, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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MODIFICATION OF CHROMATIN BY TRYPSIN

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The selective character of degradation of chromatin-bound histones by trypsin was established. The order of degradation of particular histones was found to be as follows: *f1*, *f3*, *f2a2* and *f2a1*. Histone *f2b* was most resistant to trypsin. Degradation of chromatin-bound histones is accompanied by changes in solubility of chromatin in 0.15 M-NaCl, changes of melting profiles and the transformation of chromatin from spherical into fibrillar form. An expansion of the model of chromatin structure of Zubay is proposed.

Chromatin, the form of genetic material characteristic of higher organisms, is composed of DNA and histones, and of smaller amounts of non-histone proteins and RNA. Recent studies (Zubay, 1964; Pardon, Wilkins & Richards, 1967; Bekhor, Kung & Bonner, 1969) have shown that chromatin possesses a precisely defined structure; however, the details and the role of particular components in this structure are not as yet clear. This is especially true of the size and shape of chromatin particles and of the spatial arrangement of individual components. According to the electron microscope studies of Ris (1967), chromatin occurs as fibrils of various diameter (60 - 250 Å), whereas Itzhaki & Rowe (1969) suggest that chromatin has the form of approximately spherical particles of 1 - 2 μ diameter. The latter authors have also demonstrated that treatment of chromatin with proteolytic enzymes causes the conversion of particles into an amorphous network and single strands. These results point to the vital structural role of chromosomal proteins. Zubay (1964) has postulated that the maintenance of chromatin structure is determined mainly by histones, which form intramolecular cross-links. Therefore the structural changes observed in the course of proteolysis of chromatin may be ascribed to the elimination of cross-linkage.

The purpose of this study was to investigate the relation between the degree of degradation of histones in chromatin by trypsin and the resulting structural changes of chromatin.

MATERIALS AND METHODS

Preparation of chromatin. Calf thymus was obtained from the slaughterhouse immediately after killing of the animal, frozen in solid CO_2 and stored at -20° until use. Chromatin was prepared by the method described by Marushige & Bonner (1966) for the isolation of chromatin from rat liver. For each preparation about 15 g of thymus was used, and the chromatin preparations were stored on ice, but not more than for one week. For experiments, samples of the preparation were dissolved in 0.01 M-tris-HCl, pH 8.0.

Preparation of histones. Total histones were prepared from chromatin either by the method of Fambrough & Bonner (1966) or Johns, Phillips, Simson & Butler (1961). Both methods yield histone preparations giving the same electrophoretic patterns.

Digestion of chromatin. A sample of chromatin solution (20 - 50 extinction units at 260 nm) was homogenized by hand in a Potter-Elvehjem glass-teflon homogenizer with trypsin (1×cryst., Koch-Light Lab., Colnbrook, Bucks., England) solution (25 μg/ml) to obtain a final concentration of 0.2 to 1.8 μg of enzyme per 1 ml, and incubated at 0° or 20°C for 10 to 100 min.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed according to Panyim & Chalkley (1969). To the solution of native or digested chromatin, 6 N-HCl was added at 0°C to the final concentration of 0.3 N. After 2 h the precipitate was removed by centrifugation and the clear supernatant containing no detectable amounts of DNA, was used for electrophoresis and gel filtration.

Gel filtration. Gel filtration of acid extracts of native and degraded chromatin which was found to be free of DNA, was carried out on Sephadex G-75 columns (75×2 cm, V_t=80 ml) using 0.01 N-HCl saturated with chloroform as eluent.

Solubility in 0.15 M-NaCl. The solution of native or digested chromatin, 1 ml, was added to 10 ml of cold (0°C) solution of 0.15 M-NaCl in 0.01 M-tris-HCl, pH 8.0, in a centrifuge tube, mixed and centrifuged for 5 min at 15 000 *g*. In the supernatant, the extinction at 260 nm was measured. The per cent of solubility was calculated on the basis of extinction of the supernatant and extinction of the sample used for digestion.

Electron microscopy. Electron microscopy was performed in the JSM-2 (Japan Electron Optics Laboratory Co, Japan) scanning electron microscope at accelerating voltages of 12, 15 and 25 kV and specimen angle of 45°. Initial magnifications were in the range of 1000 - 10 000.

A drop of native or digested chromatin, diluted with 0.01 M-tris-HCl, pH 8.0, to final concentrations of 0.004 and 0.0006% DNA, was placed on the specimen mount and dried at 0°C in vacuum. The sample was then shadowed with graphite and gold at different angles and analysed in the microscope.

Melting profiles. Melting profiles of chromatin were determined at 260 nm with Zeiss VSU-1 (Jena, German Democratic Republic) spectrophotometer in 0.25 mM-EDTA solution, pH 8.0.

Determination of DNA and RNA. DNA and RNA were fractionated by the Schmidt-Tannhauser procedure as modified by Ts'o & Sato (1959). DNA was determined by diphenylamine method of Burton (1956) and RNA by orcinol method of Dische & Schwartz (1937) using calf thymus DNA (Koch-Light Lab., Colnbrook, Bucks., England) and yeast RNA (Merck AG, Darmstadt, West Germany) as standards, respectively.

Determination of histones and non-histone proteins in chromatin. Histone was extracted with 0.25 N-HCl for 30 min at 0°C and assayed by the method of Lowry, Rosebrough, Farr & Randall using total calf thymus histone preparation as standard. After removal of nucleic acids by heating in 10% trichloroacetic acid at 95°C for 10 min, the non-histone protein was determined on the 0.25 N-HCl-precipitable material by the same method using bovine serum albumin (Michrome, Gurr, London, England) as standard.

RESULTS AND DISCUSSION

The preparations of calf thymus chromatin were found to contain on average 40.5% DNA, 43.1% histones, 12.1% non-histone proteins and 4.3% RNA, on dry weight basis. The weight-ratios of the particular components calculated from the above data are as follows: DNA 1.00, histones 1.06, non-histone proteins 0.3, and RNA 0.11.

The spectral properties of the chromatin preparations in 0.01 M-tris-HCl, pH 8.0, corresponded to the content of nucleic acids and proteins: the calculated ratios of extinction at 280 nm to 260 nm and 230 nm to 260 nm were 0.601 and 0.721, respectively (uncorrected for turbidity).

Polyacrylamide gel electrophoresis of acid extracts of native chromatin (Fig. 1a) revealed only five bands corresponding exactly to the five histone fractions: *f1*, *f3*, *f2b*, *f2a2* and *f2a1* [nomenclature according to Phillips & Johns (1965)].

Melting curves of chromatin, expressed as per cent of denaturation (Fig. 2), showed three distinct steps equivalent to the following melting temperatures (T_m): 67°, 76° and 85°C.

Analyses in the scanning electron microscope showed that native chromatin existed in the form of approximately spherical particles (Fig. 3) interlaced by fine strands.

The effect of low concentrations of trypsin on histones in chromatin was studied by polyacrylamide gel electrophoresis and gel filtration on Sephadex G-75 of acid extracts of chromatin after digestion with different amounts of enzyme. For comparison, similar analyses were performed on total histones isolated from calf thymus. It was found that the susceptibility to trypsin of histones in chromatin was much lower than that of free histones. Furthermore, free histones were degraded non-selectively, whereas in chromatin, as it was shown by electrophoresis (Fig. 1b-d), the degree of degradation of particular histones was markedly different. The most sensitive to trypsin was histone *f1* and the most resistant, histone *f2b*. The difference in the digestion of the remaining histones was less significant. However, the results

of several experiments have permitted to establish the following order of degradation: *f 3*, then *f 2a2* and *f 2a1*. In Fig. 5, the elution diagrams from Sephadex G-75 columns of free and chromatin-bound histones after degradation with 0.2 μg

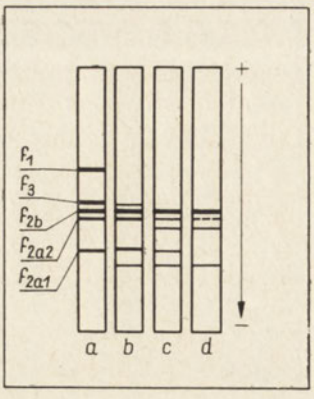

Fig. 1

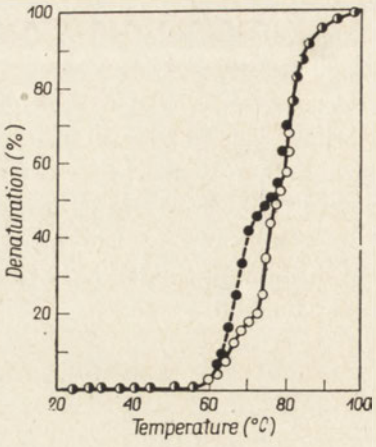

Fig. 2

Fig. 1. The electrophoretic patterns of acid extracts of native and trypsin-degraded chromatin. *a*, Native chromatin; chromatin after treatment with: *b*, 0.2 μg trypsin/ml; *c*, 0.4 μg trypsin/ml; *d*, 0.8 μg trypsin/ml. Digestion at 0°C for 30 min.

Fig. 2. The melting curves of native and trypsin-degraded chromatin in 0.25 mM-EDTA, pH 8.0. ○, Native chromatin, ●, chromatin after digestion with 1.8 μg trypsin/ml for 20 min at 20°C (40% soluble in 0.15 M-NaCl).

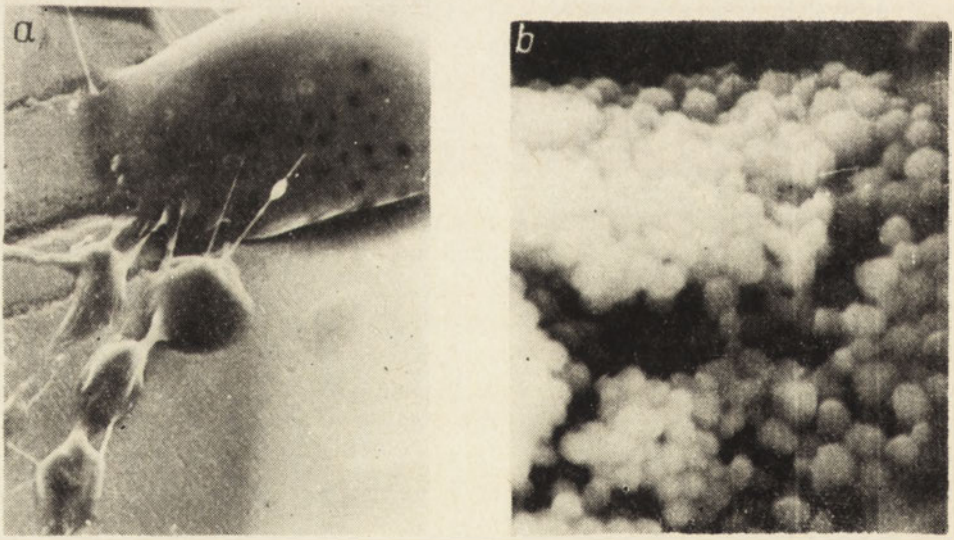

Fig. 3. Electron micrograms of native chromatin; *a*, magnification ×2310 (15 kV), chromatin concentration 0.004% DNA; *b*, magnification ×10 000 (25 kV), chromatin concentration 0.0006% DNA.

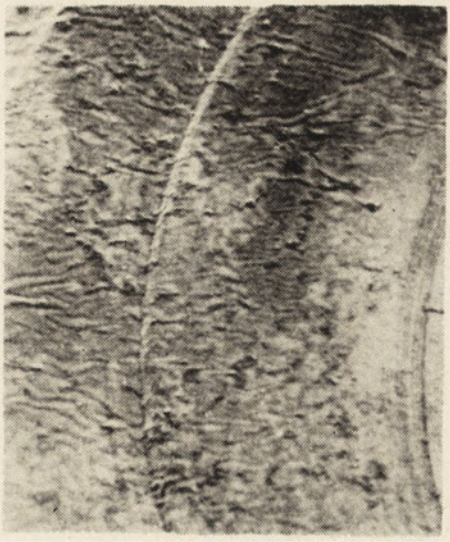

Fig. 4. Electron microgram of chromatin after digestion by trypsin. Magnification ×1700 (12 kV), chromatin concentration 0.004% DNA (chromatin 84% soluble in 0.15 M-NaCl).

trypsin/ml for 30 min at 20°C are presented. The digestion of histones in chromatin was confined to the proteolysis of only a limited number of peptide bonds.

Since the physico-chemical properties of chromatin, such as solubility in salt solution, melting temperature, viscosity etc., are determined to a large extent by histones, the modification of these proteins by trypsin treatment should induce changes in these properties. In fact, trypsin digestion of chromatin converted it from the insoluble to the 0.15 M-NaCl soluble form (Fig. 6). Similarly, there was a proportionality between the degree of solubilization of chromatin and trypsin concentration. Apart from this, digestion caused changes in melting profiles of chromatin (Fig. 2). In particular, the form of chromatin melting at 76° disappeared, while the amount of chromatin melting at 67° grew correspondingly.

Observation in the scanning electron microscope revealed that digestion by trypsin caused clearly visible changes in gross conformation of chromatin particles (Fig. 4). As can be seen, chromatin modified by trypsin did not occur as spherical particles but rather in a membrane-like form. In terms of structure such microscopic observations may be interpreted as a transition of chromatin from spherical into fibrillar form.

As it has been shown by Zubay & Wilkins (1964) and Ohba (1966), native chromatin possesses a characteristic microstructure maintained by histones, which form intramolecular cross-links of DNA molecules. The results presented in this paper are consistent with such a view of the role of histones in chromatin structure and allow a more precise localization of particular histone components in chromatin. Figure 7 presents the proposed spatial relations between DNA and particular histones in chromatin, which is an expansion of the model of Zubay (1964).

In this expanded model histones *f 3*, *f 2a2* and *f 2a1* form the bridges postulated by Zubay. This follows from their mode of degradation by trypsin and from the fact that the degradation of these histones caused a significant increase in the solu-

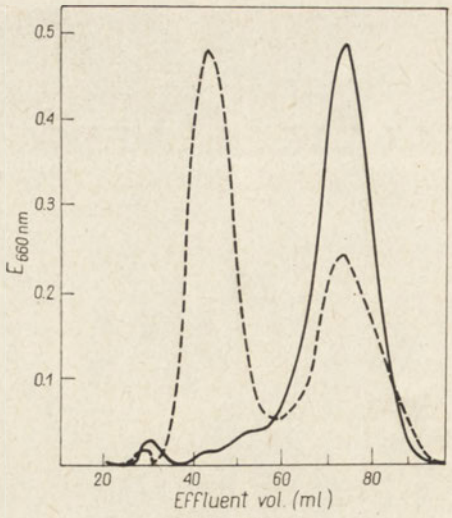

Fig. 5

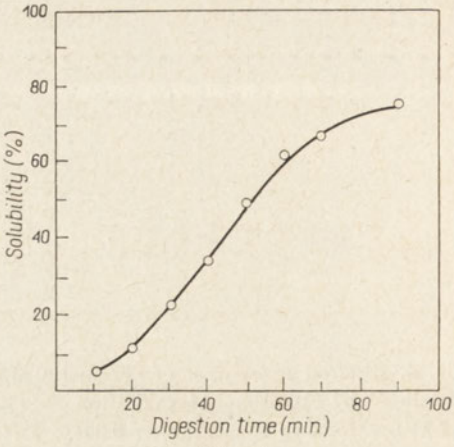

Fig. 6

Fig. 5. The elution diagrams from Sephadex G-75 column of products of degradation by trypsin of (—) free and (- - -) chromatin-bound histones (chromatin 98.2% soluble in 0.15 M-NaCl). The conditions of degradation by trypsin were the same for both free and chromatin-bound histones. The effluent was collected in 2.5 ml fractions. From each fraction, 1 ml was taken for determination of protein and the product of protein hydrolysis, according to Lowry *et al.* (1951). The extinction was measured at 660 nm.

Fig. 6. Solubility of chromatin in 0.15 M-NaCl in the course of digestion by trypsin (0.27 μg/ml, 20°C).

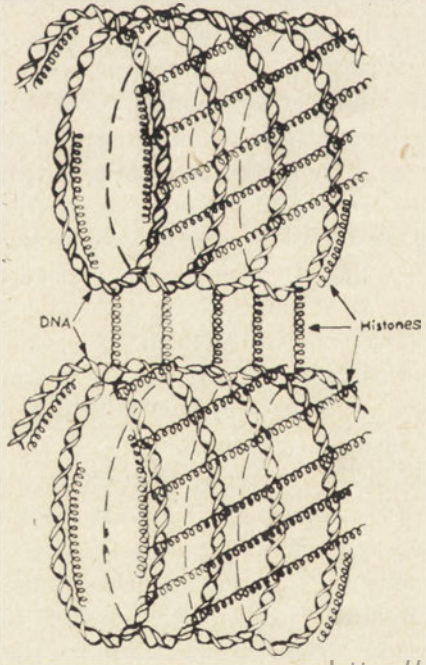

Fig. 7. The scheme of spatial interrelations of DNA and histones in chromatin [an expansion of the model of Zubay (1964)]. For details see text.

bility of chromatin in 0.15 M-NaCl. Histone *f 2b* does not form cross-links in chromatin, but rather is situated along DNA molecules. Such a proposition is supported by the fact that histone *f 2b* remained intact even after much more extensive trypsin treatment. Histone *f 1* occupies a unique position in chromatin structure. This is suggested by the high sensitivity of this histone to trypsin treatment, by the ease with which this histone can be extracted from chromatin (Lucy & Butler, 1955), and by the fact that selective extraction of this histone did not increase chromatin solubility in 0.15 M-NaCl (unpublished results). It is therefore suggested that histone *f 1* forms cross-links between different fragments of DNA super-coils.

The elimination of histone bridges in chromatin by trypsin digestion (partial degradation of histones *f 1*, *f 3*, *f 2a2* and *f 2a1*) causes pronounced changes in its structure. These changes are indicated by the transition from three-step to two-step melting profiles and especially by the conversion of spherical chromatin particles into a fibrillar form, which is seen as a membrane-like structure in the scanning electron microscope. Such a structural conversion could be equivalent to the transition of chromatin from dense to diffuse form observed by Littau, Burdick, Allfrey & Mirsky (1965). Since RNA synthesis proceeds in diffuse chromatin (Littau, Allfrey, Frenster & Mirsky, 1964), such structural transitions could account for the regulatory role of histones in the transfer of genetic information.

The authors are grateful to Prof. Dr. Irena Chmielewska, Director of the Institute of Biochemistry, Warsaw University, for her encouragement and critical review of the manuscript, and to Prof. Dr. eng. S. T. Jaźwiński, Director of the Institute of Science of Materials and Engineering, Warsaw Polytechnic, for making available the electron microscope and for interpretation of micrograms. The authors also wish to thank Mr. M. Łuszczyk for excellent technical assistance.

REFERENCES

Bekhor I., Kung G. M. & Bonner J. (1969). *J. molec. Biol.* **39**, 351.
Burton K. (1956). *Biochem. J.* **61**, 473.
Dische Z. & Schwartz K. (1937). *Mikrochim. Acta* **2**, 13.
Fambrough D. M. & Bonner J. (1966). *Biochemistry* **5**, 2563.
Itzhaki R. F. & Rowe A. J. (1969). *Biochim. biophys. Acta* **186**, 158.
Johns E. W., Phillips D. M. P., Simson P. & Butler J. A. V. (1961). *Biochem. J.* **80**, 189.
Littau V. C., Allfrey V. G., Frenster J. H. & Mirsky A. E. (1964). *Proc. natn. Acad. Sci. U.S.A.* **52**, 93.
Littau V. C., Burdick C. J., Allfrey V. G. & Mirsky A. E. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1204.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.
Lucy J. & Butler J. A. V. (1955). *Biochim. biophys. Acta* **16**, 431.
Marushige K. & Bonner J. (1966). *J. molec. Biol.* **15**, 160.
Ohba Y. (1966). *Biochim. biophys. Acta* **123**, 76.
Panyim S. & Chalkley R. (1969). *Archs Biochem. Biophys.* **130**, 337.
Pardon J. F., Wilkins M. H. F. & Richards B. M. (1967). *Nature, Lond.* **215**, 509.
Phillips D. M. P. & Johns E. W. (1965). *Biochem. J.* **94**, 127.

Ris H. (1967). In *Regulation of Nucleic Acid and Protein Biosynthesis* (V. V. Koningsberger & L. Bosch eds.) p. 11. Elsevier, Amsterdam.

Ts'o P. O. P. & Sato C. S. (1959). *Exp. Cell Res.* **17**, 227.

Zubay G. (1964). In *The Nucleohistones* (J. Bonner & P. O. P. Ts'o, eds.) p. 95. Holden-Day, San Francisco.

Zubay G. & Wilkins M. H. F. (1964). *J. molec. Biol.* **9**, 246.

MODYFIKACJA CHROMATYNY DZIAŁANIEM TRYPSYNĄ

Streszczenie

Wykazano różnice w podatności na degradację trypsyną poszczególnych histonów w chromatynie: najszybciej degradowany jest histon *f1*, następnie histony *f3*, *f2a2* i *f2a1*, natomiast histon *f2b* jest najbardziej oporny na działanie enzymu. Degradacji histonów towarzyszą zmiany rozpuszczalności chromatyny w 0,15 M-NaCl, krzywych topnienia oraz zmiany formy kulistej w formę fibrylarną. Sprecyzowano lokalizację przestrzenną poszczególnych histonów w modelu struktury chromatyny Zubaya.

Received 17 July, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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LYSINE-RICH HISTONE IN SULPHOSALICYLIC ACID EXTRACT FROM MUSCLES

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1. The fraction of sulphosalicylic acid (SSA) soluble muscle proteins which on paper electrophoresis exhibited the highest cathodic mobility, was found to originate from nuclei; fractions with lower mobility originated from mitochondria and cytoplasm.
2. SSA-soluble proteins isolated from muscle contained a fraction which on paper-, starch-gel and polyacrylamide-gel electrophoresis corresponded to the lysine-rich histone fraction of calf thymus.

It has been found in this laboratory that the proteins isolated by the tannin--caffeine procedure from SSA[1] extracts of various animal tissues, exhibit on paper electrophoresis considerable heterogeneity and contain fractions of high cathodic mobility (for ref. see Maskos & Mejbaum-Katzenellenbogen, 1968; recent data: Wilimowska-Pelc & Mejbaum-Katzenellenbogen, 1969; Wieczorek & Wilusz, 1970). It has been also demonstrated (Maskos & Mejbaum-Katzenellenbogen, 1968; Wieczorek & Mejbaum-Katzenellenbogen, 1968) that the most cathodic fraction of SSA-soluble proteins of thymus and kidney corresponds to the lysine-rich histone fraction.

As there are few reports (Schwartz, 1965a,b) in the literature concerning muscle histones, we have undertaken the isolation and localization in subcellular fractions of lysine-rich histones and other SSA-soluble proteins of skeletal and cardiac muscles.

MATERIALS AND METHODS

Tissues. The experiments were performed on skeletal muscles of rabbit and rat taken immediately after killing of the animal, and ox heart and calf thymus obtained from the slaughterhouse within the first hour after the animal's death, and cooled to 0°C. The tissues, except when otherwise noted, were homogenized at 2° C in an apparatus of the Waring blendor type.

[1] Abbreviation used: SSA, sulphosalicylic acid.

Very lysine-rich histones (F_1) from calf thymus were isolated by the procedure I of Johns (1964) or according to Kinkade & Cole (1966).

SSA-soluble proteins from muscles were isolated from the extract by the tannin--caffeine procedure (Mejbaum-Katzenellenbogen, Łomako & Maskos, 1968).

Isolation of glycogen-bound protein. Amylolysis of glycogen and isolation of the protein were carried out as follows: to a 20% solution of glycogen in 6.7 mM -NaCl - 20 mM-phosphate buffer, pH 6.9, salivary amylase was added and the mixture was left for several hours at room temperature with occasional stirring. The degradation of glycogen was followed by determination of maltose (Noelting & Bernfeld, 1948) and reducing sugars (Park & Johnson, 1949). The amylolysis was stopped by adding cold 0.45 M-SSA to a final concentration of 0.15 M. The mixture was centrifuged at 1400 *g* for 20 min at 2°C. The supernatant was filtered and the SSA-soluble proteins were concentrated by the tannin-caffeine procedure.

Subcellular fractions from rat skeletal muscle were obtained according to Martonosi & Feretos (1964). Differential centrifugation was performed on a K-50 centrifuge and VAC 40 ultracentrifuge (H. Janetzky, Engelsdorf, Leipzig, German Democratic Republic). Rat muscle (100 g) was homogenized for 2 min with four volumes of 0.1 M-KCl - 5 mM-histidine solution. Myofibrils together with nuclei were sedimented at 1000 *g* (20 min), mitochondria at 8000 *g* (20 min), microsomes at 90 000 *g* (60 min); the final supernatant was considered the cytoplasmic fraction. The particulate fractions were separately suspended in the above KCl-histidine solution at a volume half that of the respective supernatant, and centrifuged again: myofibrils with nuclei at 1500 *g* for 30 min, mitochondria at 15 000 *g* for 30 min, and microsomes at 105 000 *g* for 90 min. The nuclear-myofibrillar fraction was washed three more times. From the obtained subcellular fractions (except the microsomal one), proteins were extracted with SSA. The nuclear-myofibrillar fraction was treated with 200 ml of 0.15 M-SSA and homogenized for 3 min. The mitochondrial fraction was treated with 15 ml of 0.15 M-SSA and homogenized in a Potter--type glass homogenizer. To the cytoplasmic fraction, an equal volume of 0.3 M-SSA was added. The samples were left for 15 min at 2°C, then centrifuged, and from the supernatants protein was isolated by the tannin-caffeine procedure.

Myofibrils free from nuclei were obtained from rabbit skeletal muscle according to Perry (1952).

Column chromatography. SSA-soluble proteins from rabbit skeletal muscle were fractionated on CM-cellulose column according to Rzeczycki, Grudzińska, Hillar & Wszelaki-Lass (1963). The protein, 100 mg, dissolved in 8 ml of 0.05 M-sodium acetate buffer, pH 4.4, was applied to the column (1 × 20 cm) and eluted with a KCl concentration gradient from 0 to 0.6 M in the above acetate buffer. Fractions of 3 ml were collected at intervals of 10 min. In each fraction, protein was determined by the tannin method and spectrophotometrically at 235 nm in quartz cuvettes of 1 cm light-path. The fractions corresponding to individual protein peaks were pooled, dialysed at 2°C against water for 48 h and then freeze-dried.

Analytical methods. Protein was determined by the turbidimetric tannin method (Mejbaum-Katzenellenbogen, 1955).

Paper electrophoresis was carried out on Whatman no. 1 paper strips (4×30 cm) at pH 4.4 in citrate-phosphate buffer (McIlvaine, 1921) in a Carl Zeiss (Jena) apparatus for 9 h at 220 V. The electrophoretograms were stained with Amido Black 10 B according to Grassmann & Hanning (1952).

Starch-gel electrophoresis was carried out according to Hnilica, Edwards & Hey (1966), using 12% gel in 0.01 M-HCl - 0.6 mM-$AlCl_3$. Protein, 1 - 2 mg, dissolved in 0.01 M-HCl, was adsorbed on a piece of Whatman no. 1 paper (5×10 mm) and inserted into the gel 3 - 4 cm from the anodic end. The electrophoresis was run for 24 h at 30 mA. The gel was stained with 0.1% of Amido Black 10 B dissolved in methanol - water - acetic acid (5 : 5 : 2, by vol.), then destained with the above solvent mixture.

Polyacrylamide-gel electrophoresis was carried out according to Reisfeld, Lewis & Williams (1962). The separation was performed at pH 4.3 in 15% gel, in glass tubes (0.5×7 cm), 30 - 100 µg of protein dissolved in 50 µl of 20% sucrose being applied. The electrophoresis was run at 6 - 8 mA per one tube for 40 min. The protein was stained with a 1% solution of Amino Black in 7% acetic acid.

Reagents. Salivary amylase was prepared according to Fischer & Stein (1961). Glycogen was isolated from rabbit muscle homogenized with two volumes of cold 10% trichloroacetic acid for 3 min; after centrifuging, glycogen was precipitated from the supernatant by adding 2 volumes of ethanol. β-Alanine was from Reanal (Budapest, Hungary); starch from Koch-Light (Colnbrook, Bucks., England); histidine and 3,5-dinitrosalicylic acid from Schuchardt (Münich, West Germany); tannin was an U.S.S.R. product, lot no. 750666; Amido Black was from Grübler (Leipzig, German Democratic Republic); polyacrylamide, N,N′-methylene-bisacrylamide and N,N,N′,N′-tetramethylenediamine from Serva (Heidelberg, West Germany); Whatman CM-cellulose CM 11, 0.6 m-equiv./g, was from Balston (Maidstone, Kent, England). Other chemicals were reagent grade products of Polish origin.

RESULTS

Skeletal muscles of rabbit and rat, and ox cardiac muscle contained rather small amounts of SSA-soluble proteins (0.007%, 0.05% and 0.032%, respectively). The sulphosalicylic-acid extracts gave with iodine a positive reaction for glycogen.

The glycogen isolated from skeletal muscles contained 0.6% of protein, which was released on digestion by salivary amylase. This protein, when subjected to paper electrophoresis at pH 4.4, gave a pattern similar to that obtained from SSA-soluble proteins from muscles (Fig. 1). It was found that the addition of a tenfold excess (by weight) of glycogen to a solution of thymus lysine-rich histone or to the SSA-soluble proteins from muscle, protected these proteins against precipitation by 20% trichloroacetic acid. Thus it appears that glycogen, when present in the SSA extract, adsorbs the proteins and may interfere with their isolation by preventing their precipitation by high concentration of trichloroacetic acid. On the other hand, glycogen has no effect on precipitation of SSA-soluble proteins by tannin but interferes with regeneration of protein by caffeine.

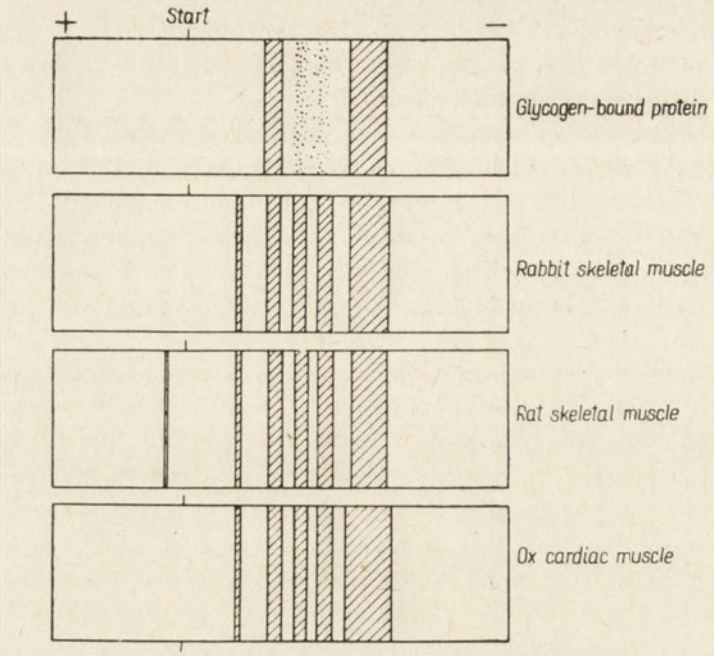

Fig. 1. Paper electrophoresis at pH 4.4 of the protein isolated from glycogen-amylolysis products and of SSA-soluble proteins from the indicated sources. Conditions as described in Methods.

The SSA-soluble proteins from skeletal and cardiac muscles, isolated by our procedure, were separated on paper electrophoresis at pH 4.4 into five or six fractions (Fig. 1). The fastest-moving cathodic fraction which accounted for 50% of the applied protein, corresponded probably to the very lysine-rich histone obtained from other tissues.

SSA-soluble proteins from rabbit skeletal muscle were compared on paper electrophoresis at pH 4.4 (Fig. 2A) and starch-gel electrophoresis at pH 2.3 (Fig. 2B) with very lysine-rich histone obtained from calf thymus by the method of Kinkade

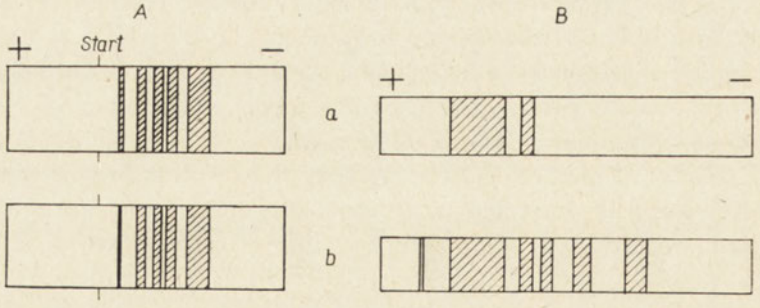

Fig. 2. Paper electrophoresis (*A*) and starch-gel electrophoresis (*B*) of: *a*, SSA-soluble proteins from rabbit muscle; *b*, calf thymus lysine-rich histone, obtained according to Kinkade & Cole (1966). Conditions as described in Methods.

& Cole (1966). On paper electrophoresis the lysine-rich histone and SSA-soluble muscle proteins showed the same degree of heterogeneity, being separated each into five fractions, whereas on starch-gel electrophoresis the SSA-soluble muscle proteins separated into six fractions and the lysine-rich histone into two only.

To study the origin of the individual electrophoretic fractions, the SSA-soluble proteins were isolated separately from muscle subcellular fractions and submitted to paper electrophoresis (Fig. 3). The nuclear-myofibrillar fraction gave one band

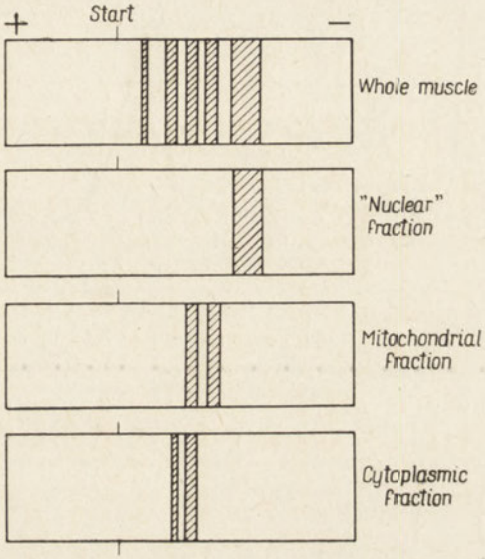

Fig. 3. Paper electrophoresis of SSA-soluble proteins isolated from whole rat muscle and from the subcellular fractions. Condition s as described in Methods.

corresponding to the fastest-moving fraction of SSA-soluble proteins from whole muscle. This fraction originated from nuclei as the separately isolated myofibrils, free from nuclei, contained no SSA-soluble proteins. The protein bands obtained from the mitochondrial and cytoplasmic fractions, corresponded to the fractions of smaller cathodic mobility.

Chromatographic separation on CM-cellulose at pH 4.4 of SSA-soluble proteins from rabbit skeletal muscle, is presented in Fig. 4. Five fractions were obtained, of which only proteins of peaks *III* and *V* were submitted to electrophoresis and compared with SSA-soluble proteins from subcellular muscle fractions. It was found that the proteins from peak *V* had the same mobility as those from the nuclear--myofibrillar fraction, whereas the proteins of peak *III*, as those of the mitochondrial and cytoplasmic fractions.

On polyacrylamide-gel electrophoresis, the proteins of peak *V* gave one very distinct band and three very slight ones (Fig. 5). The main band corresponded to the main fraction of very lysine-rich histone isolated from thymus after Johns (1964). It should be added that histone F_1 exhibited high heterogeneity, being separated into seven bands.

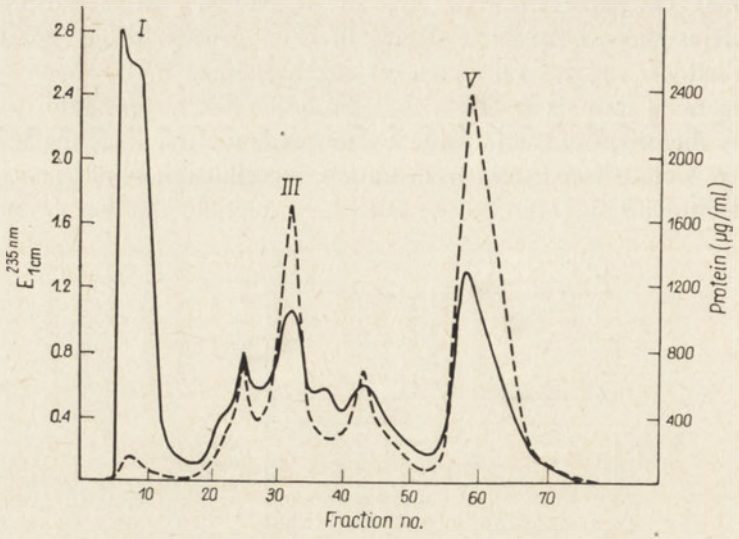

Fig. 4. Separation of SSA-soluble proteins of rabbit muscle by CM-cellulose chromatography. The elution was performed with increasing concentration of KCl up to 0.6 M in 0.05 M-acetate buffer, pH 4.4, and fractions of 3 ml were collected. - - -, Protein estimated by the tannin method; ———, absorption at 235 nm.

Fig. 5. Polyacrylamide-gel electrophoresis of *a*, peak *V* of SSA-soluble proteins of rabbit muscle (see Fig. 4); *b*, the lysine-rich histone isolated according to Johns (1964).

DISCUSSION

SSA-soluble muscle proteins, when submitted to starch-gel electrophoresis, were found to contain a fraction of the same mobility as the very lysine-rich histone (F_1) of calf thymus. The SSA-soluble proteins from skeletal muscle exhibited under these conditions greater heterogeneity (separation into six fractions) than histone F_1 from thymus obtained according to Kinkade & Cole (1966). However, it should be noted that in thymus cells the nuclei may account for as much as 60% of the cell mass, whereas in other tissues they correspond to 10 - 18%. Thymus, due to its small content of the cytoplasm, is a most convenient source of histones almost free from other proteins.

On the other hand, the comparison on polyacrylamide-gel electrophoresis of the most basic fraction of SSA-soluble proteins from skeletal muscle obtained by CM-cellulose chromatography, with the lysine-rich histone prepared by procedure I of Johns (1964) has demonstrated considerable heterogeneity of the latter preparation. The commonly used prolonged washing of the whole tissue or of the isolated nuclei may lead to proteolytic degradation of histone, and at the same time does not assure complete removal of cytoplasmic proteins. Kinkade & Cole (1966) have demonstrated that rapid freezing of fresh thymus followed by extraction with cold 10% trichloroacetic acid, protects the lysine-rich histone from degradation. The preparations obtained by their method and by the procedure of De Nooij & Westenbrink (1962) were less degraded than those from the tissue washed with saline.

Johns & Forrester (1969) have recently reported that native deoxyribonucleoprotein isolated from calf thymus, and also reconstituted deoxyribonucleohistone, absorb cytoplasmic proteins in a medium of 0.14 M-NaCl. These proteins can be easily removed by washing with 0.3 - 0.35 M-NaCl, but under these conditions the lysine-rich histone may be also released.

The presence of glycogen in dilute acid extracts, e.g. trichloroacetic acid or SSA, interferes with isolation of muscle proteins by preventing their precipitation with trichloroacetic acid. This observation does not confirm the suggestion of Roe, Bailey, Gray & Robinson (1961) that there are no bonds between protein and glycogen resistant to cold trichloroacetic acid. Moreover, in the present experiments it was found that glycogen obtained from muscle by trichloroacetic acid extraction, contained some protein which could be released only after amylase digestion. This glycogen-bound protein was found to correspond to thymus lysine-rich histone.

Bustin & Cole (1968) studied lysine-rich histone of calf thymus, mammary gland and liver of rabbit, and liver of chicken, using trichloroacetic acid extraction. The elution profiles from Amberlite IRC 50 columns of histones isolated from glycogen-poor tissues, thymus and mammary gland, were similar, four peaks being usually obtained. On the other hand, the elution profiles of histones isolated from liver, a tissue rich in glycogen, were quite different and possessed only one or two peaks. The authors interpret these results as an indication of organ specificity of lysine-rich histones, not taking into account the possibility of adsorption of histone by glycogen.

This work was supported by a grant from the Committee of Science and Technique through the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

REFERENCES

Bustin M. & Cole R. D. (1968). *J. biol. Chem.* **243**, 4500.
De Nooij E. H. & Westenbrink H. G. K. (1962). *Biochim. biophys. Acta* **62**, 608.
Fischer E. H. & Stein E. A. (1961). *Biochem. Prep.* **8**, 27.
Grassmann W. & Hanning K. (1952). *Z. physiol. Chem.* **290**, 1.

Hnilica L. S., Edwards L. J. & Hey A. E. (1966). *Biochim. biophys. Acta* **124**, 109.
Johns E. W. (1964). *Biochem. J.* **92**, 55.
Johns E. W. & Forrester S. (1969). *Eur. J. Biochem.* **8**, 547.
Kinkade J. M. & Cole R. D. (1966). *J. biol. Chem.* **241**, 5790.
Martonosi A. & Feretos R. (1964). *J. biol. Chem.* **239**, 648.
Maskos K. & Mejbaum-Katzenellenbogen W. (1968). *Acta biochim. Polon.* **15**, 205.
McIlvaine T. C. (1921). *J. biol. Chem.* **49**, 183.
Mejbaum-Katzenellenbogen W. (1955). *Acta biochim. Polon.* **2**, 279.
Mejbaum-Katzenellenbogen W., Łomako J. & Maskos Ch. (1968). *Arch. Immunol. Terap. Dośw.* **16**, 817.
Noelting G. & Bernfeld P. (1948). *Helv. chim. Acta* **31**, 286.
Park J. T. & Johnson M. J. (1949). *J. biol. Chem.* **181**, 149.
Perry S. V. (1952). *Biochem. J.* **51**, 495.
Reisfeld R. A., Lewis U. J. & Williams D. E. (1962). *Nature, Lond.* **195**, 281.
Roe J. H., Bailey J. M., Gray R. R. & Robinson J. N. (1961). *J. biol. Chem.* **236**, 1244.
Rzeczycki W., Grudzińska A., Hillar M. & Wszelaki-Lass E. (1963). *Acta biochim. Polon.* **10**, 49.
Schwartz A. (1965a). *J. biol. Chem.* **240**, 939, 944.
Schwartz A. (1965b). *Biochim. biophys. Acta* **100**, 202.
Wieczorek E. & Mejbaum-Katzenellenbogen W. (1968). *Acta biochim. Polon.* **15**, 382.
Wieczorek E. & Wilusz T. (1970). *Acta physiol. Polon.* **21**, 227.
Wilimowska-Pelc A. & Mejbaum-Katzenellenbogen W. (1969). *Acta Polon. pharm.* **26**, 609.

HISTONY BOGATE W LIZYNĘ W WYCIĄGACH SULFOSALICYLOWYCH Z MIĘŚNI

Streszczenie

1. Wykazano, że frakcja rozpuszczalnych w kwasie sulfosalicylowym (SSA) białek z mięśni, która w elektroforezie bibułowej wykazywała największą ruchliwość, jest pochodzenia jądrowego, natomiast frakcje o mniejszej ruchliwości pochodzą z mitochondriów i cytoplazmy.

2. Stwierdzono, że białka rozpuszczalne w SSA, izolowane z mięśni, zawierają frakcję, która w elektroforezie bibułowej oraz w żelu skrobiowym i poliakrylamidowym odpowiada frakcji histonów bogatych w lizynę, otrzymanej z grasicy cielęcej.

Received 28 July, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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THE UPTAKE OF CHOLESTEROL *IN VITRO* BY HUMAN TERM PLACENTAL TISSUE

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1. The cholesterol uptake by the placenta is correlated with the rate of entrance and accumulation within the tissue. 2. The distribution between subcellular fractions of placental tissue is not uniform. The highest accumulation of cholesterol is reached in mitochondrial and microsomal fractions. 3. Oxygen consumption by the tissue is not correlated with cholesterol uptake. 4. The inhibitors of oxidation and glycolysis are without effect on the cholesterol uptake by human placenta.

The synthesis of cholesterol from acetate and mevalonate in human term placenta *in vitro* has been demonstrated by Zelewski & Villee (1966); the synthesis was, however, very small, and as little as 0.004% of acetate radioactivity and 0.1% of mevalonate activity has been recovered in cholesterol. Cholesterol is the precursor of progesterone in placenta but its conversion in perfused human placenta amounts only to 0.1% (Solomon, 1960), which does not account for the high production of progesterone at late pregnancy. On the other hand, Morrison, Meigs & Ryan (1965) have demonstrated that cholesterol incubated with a suspension or extract of acetone--dried mitochondria of human placenta, is converted in 13 - 32% into pregnenolone and progesterone. Therefore it seems possible that cholesterol uptake by the placenta and its accumulation in subcellular fractions is one of the rate-limiting factors of progesterone biosynthesis.

Taking this into account, a study was undertaken concerning the uptake of free cholesterol by the placenta and its distribution in subcellular fractions. The effect of some metabolic inhibitors was also investigated.

MATERIALS AND METHODS

Reagents. [4-^{14}C]Cholesterol, spec. act. 55.8 mCi/milimole (Radiochemical Centre, Amersham, Bucks, England); silica gel (E. Merck A. G., Darmstadt, West Germany); $NADP^+$ disodium salt, glucose-6-phosphate disodium salt, and glucose--6-phosphate dehydrogenase (Sigma Chem. Co., St. Louis, Mo., U.S.A.). All other chemicals used were analytical grade products of Polish origin.

Tissue preparation. Human term placenta obtained immediately after delivery was transferred to ice. The blanched areas were isolated taking care to exclude gross connective tissue, maternal decidua and chorionic plate. After blotting and washing in cold 0.9% NaCl - 0.25 M-sucrose solution, relatively blood-free samples of chorionic villi were obtained. For experiments, the tissue was cut into small fragments, weighing about 0.1 g.

Cholesterol metabolism in fresh mitochondria. Fresh placental mitochondria were prepared according to Ryan (1959). The incubation was carried out in 25 ml Erlenmayer flask at 30°C in a total volume of 3 ml, for 2 h with air as the gas phase. The incubation medium contained [4-^{14}C]cholesterol, in amounts indicated in the table, 2 mM-$MgCl_2$ - 1 mM-Na_2HPO_4 - 50 mM-tris buffer, pH 7.2, and one of the following components: (*a*), NADPH-generating system: 10 µmols of $NADP^+$, 60 µmols of glucose-6-phosphate and 1.5 units of glucose-6-phosphate dehydrogenase; (*b*), 30 mM-glucose; (*c*), 30 mM-citrate; (*d*), 30 mM-succinate; (*e*), no additions. Controls were carried out with heated mitochondria. The flask contained about 10 mg protein of mitochondria, intact or sonicated (3 min at 16 Kc; MSE disintegrator.) The reaction was stopped by adding 5 mg of cholesterol dissolved in ethyl acetate. The ethyl acetate extract was collected, the solvent evaporated under reduced pressure and the residue submitted to thin-layer chromatography (t.l.c.) on silica gel in a system of benzene - ethyl acetate (5:1, v/v). The cholesterol zone was scraped from the plate and eluted with chloroform. The eluate was concentrated to a small volume and rechromatographed in light petroleum (b.p. 40 - 60°C) - diethyl ether - acetic acid (75:25:2, by vol.). In the obtained purified cholesterol fraction, radioactivity and cholesterol were determined.

Cholesterol uptake by the placenta. Two grams of tissue fragments were incubated at 30°C in 3 ml of a medium consisting of 0.25 mM-sucrose - 50 mM-tris buffer, pH 7.4 (further called the sucrose-tris medium), and 0.08 µCi of [^{14}C[cholesterol. After incubation, the tissue was separated from the medium on a Buchner funnel under suction, and washed several times with 0.25 M-sucrose until no radioactivity appeared in the effluent; then the tissue was dissolved in 6 N-KOH. In a part of the solution protein was determined, and the remaining part was extracted with ethyl acetate. From the extract, cholesterol was isolated by t.l.c. and purified by rechromatography as described above, then specific radioactivity of cholesterol was determined. In control experiments, the tissue was homogenized with ethyl acetate in an MSE homogenizer after 1 min incubation.

To test the effect of inhibitors and the consumption of oxygen, experiments were carried out in Warburg vessel; 0.5 g of tissue fragments was incubated for 1 h at 30°C in 3 ml of a medium (further called the saline medium) composed of 15 mM-KCl, 2 mM-EDTA, 5 mM-$MgSO_4$, 50 mM-tris buffer, pH 7.4, and 0.045 µCi of [^{14}C] cholesterol, with or without inhibitor added. The oxygen consumption was measured by the direct Warburg technique with air as the gas phase. CO_2 was absorbed with 0.2 ml of 40% KOH. After incubation the tissue was washed, dissolved in KOH, then protein was determined and cholesterol extracted and purified as described above.

The uptake and subcellular distribution of cholesterol. Six grams of tissue fragments were incubated at 30°C in 9 ml of sucrose-tris or the above saline medium, with 0.085 μCi of [^{14}C]cholesterol. Then the tissue was washed and homogenized for 2 min with 0.25 M-sucrose in a Potter-Elvehjem glass homogenizer provided with a teflon pestle, to give a 30% homogenate; this was centrifuged at 600 *g* for 10 min, and from the supernatant the mitochondrial fraction was collected at 8000 *g* for 10 min, and then the microsomal fraction at 100 000 *g* for 30 min; the supernatant represented the cytoplasmic fraction. The mitochondrial and microsomal fractions were suspended in 0.25 M-sucrose. Samples from each fraction were used for determination of protein, cholesterol and radioactivity.

Cholesterol absorption by subcellular fractions. The 30% homogenate in 0.25 M-sucrose was centrifuged at 600 *g* for 10 min. To 130 ml of the supernatant, 10 ml of 0.25 M-sucrose containing 0.4 μCi of [^{14}C]cholesterol was added dropwise. Following incubation at 30°C for different time intervals, samples were withdrawn and submitted to differential centrifugation as described above; in the subcellular fractions, protein, cholesterol and radioactivity were determined.

Radioactivity measurement. Samples were dissolved in 10 ml of scintillation fluid (4 g of 2,5-diphenyloxazole and 0.2 g of *p*-bis-2(5-phenyloxazolyl)benzene per 1 litre of toluene), and the radioactivity measured in a liquid scintillation counter USB-II (Biuro Urządzeń Techniki Jądrowej, Warszawa, Poland). Corrections were made for background (80 c.p.m.) and self-absorption determined almost every day. The efficiency of the counter was 50%, as calculated from counts on an external standard. The samples were counted at least at the level of five times the background. The error of counting was less than 10%.

Thin-layer chromatography was carried out on silica gel, rhodamine 6 G being used for staining; cholesterol spots were identified by ultraviolet absorption.

Protein was determined by the biuret method (Gornall, Bardawill & David, 1949), and cholesterol colorimetrically with the Lieberman-Burchard colour reagent (Schoenheimer & Sperry, 1934).

RESULTS

To check whether placental mitochondria are able to metabolize cholesterol, freshly isolated or sonicated mitochondria were incubated with [^{14}C]cholesterol and different substrates. It was found (Table 1) that the specific radioactivity recovered was the same as in the control experiments when the incubation was carried out with heated mitochondria. This indicates that, under the conditions applied, mitochondria do not participate in cholesterol metabolism.

The uptake of [^{14}C]cholesterol by the placenta was appreciable, and it was time-dependent for the first 2 h of incubation (Fig. 1). Changes in the distribution in subcellular fractions of the cholesterol absorbed by the tissue, are presented in Fig. 2. The radioactivity increased with the time of incubation in all fractions but it was several times higher in the mitochondrial and microsomal fractions than in the cytoplasmic one. On the other hand, when the homogenate was incubated with

labelled cholesterol and the radioactivity measured in the separated subcellular fractions, an increase in the content of [^{14}C]cholesterol was observed in the microsomal and mitochondrial fractions, simultaneously with a sharp decrease in the cytoplasmic fraction (Fig. 3).

The effect of various metabolic inhibitors on the uptake of cholesterol by the placental tissue, is presented in Table 2. Amytal, KCN, NaF and DNP, separately or together, had no effect on the uptake of cholesterol by the tissue.

The results of the experiments in which the incubation was carried out parallelly at 0° and 30°C (Table 3) indicate that the uptake and subcellular distribution of cholesterol were temperature-dependent. The effect of amytal and KCN on the

Table 1

The metabolism of [^{14}C]*cholesterol by fresh placental mitochondria*

Intact or sonicated mitochondria were incubated with NADPH-generating system, glucose, citrate, succinate or without any substrate, and [^{14}C]cholesterol (60×10^3 c.p.m. in expts. I, II and III, and 15×10^3 c.p.m. in expt. IV). For details see Methods. The control contained heated mitochondria. The results are expressed as c.p.m. per 1 mg of cholesterol.

Addition to the incubation medium	Mitochondria			
	intact		sonicated	
	Expt. I	Expt. II	Expt. III	Expt. IV
NADPH	9 250	10 900	11 700	3 100
Glucose	9 400	11 600	12 000	3 000
Citrate	9 200	11 300	11 200	3 600
Succinate	9 700	11 800	11 400	3 100
None	9 900	9 500	11 000	3 000
Control (heated mitochondria)	10 800	10 700	11 500	3 300

Table 2

Effect of inhibitors on cholesterol uptake by placental tissue

Placental tissue, 0.5 g, was incubated at 30°C with 0.045 μCi of [^{14}C]cholesterol in the saline incubation medium, with the addition of 3 mM-amytal, 3 mM-KCN, 15 mM-NaF and 0.3 mM-DNP. The incubation time was 60 min, except in the first experiment (1 min).

Addition	Experiment				Experiment			
	I	II	III	IV	I	II	III	IV
	c.p.m. per 1 mg protein				% of the control			
None (incubation 1 min)	3	8	2	3	6	8	2	7
None (control)	47	104	89	44	(100)	(100)	(100)	(100)
Amytal	66	111	91	58	140	107	120	131
KCN	50	123	97	46	106	118	109	104
NaF	44	113	90	44	94	109	101	100
DNP	52	77	80	44	110	74	90	100
Amytal+KCN+NaF+DNP	50	83	103	39	106	80	116	89

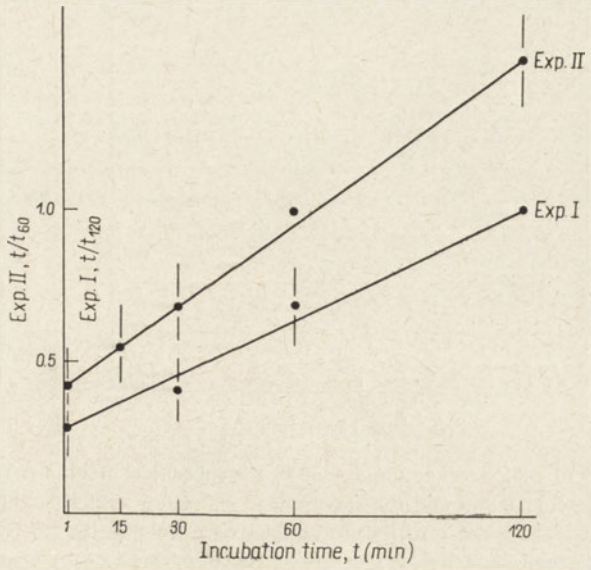

Fig. 1. Time-course of cholesterol uptake by the placenta. Tissue fragments were incubated with [^{14}C]cholesterol at 30°C as described in Methods. The results are expressed as: *I*, the ratio of c.p.m. per 1 mg of protein at a given time *t*, to that at 120 min; *II*, the ratio of c.p.m. per 1 mg of cholesterol at time *t* to that at 60 min. Each point represents the mean from 4 - 6 experiments, and the bars the S.D. values.

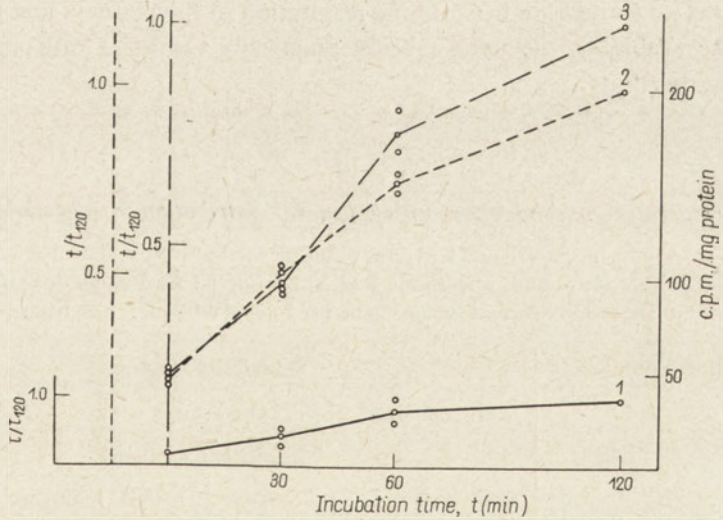

Fig. 2. Time-course of cholesterol distribution in subcellular fractions. The placental tissue was incubated at 30°C with [^{14}C]cholesterol, as described in Methods. Then the tissue was homogenized and the subcellular fractions isolated. The accumulation of cholesterol is expressed both as c.p.m. per 1 mg of protein, and in relation to the uptake after 120 min of incubation. Each point represents the mean from four experiments. — — —, Mitochondria; - - -, microsomes; ———, cytoplasm.

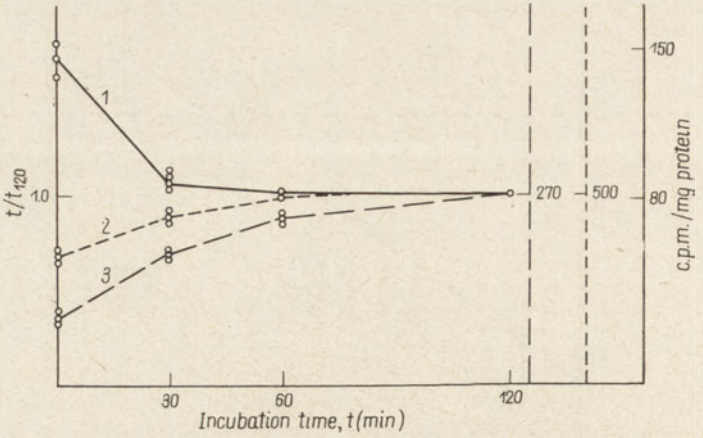

Fig. 3. Time-course of cholesterol accumulation by the subcellular fractions. The homogenate was centrifuged at 600 *g* for 10 min and the supernatant incubated with [^{14}C]cholesterol at 30°C, as described in Methods, then the subcellular fractions were isolated by differential centrifugation. The results are expressed as in Fig. 2. — — —, Mitochondria; - - -, microsomes; ———, cytoplasm.

distribution in subcellular fractions of the accumulated cholesterol, is also presented in Table 3. KCN had no effect. The amount of cholesterol after incubation with amytal was somewhat higher in mitochondria and microsomes than the corresponding control values, but the differences were not statistically significant.

There was no correlation between the respiration of the placenta and the cholesterol uptake (Table 4), the uptake being practically the same with and without succinate as substrate.

Table 3

Effect of temperature and inhibitors on cholesterol distribution in subcellular fractions

Six grams of tissue were incubated for 2 h at 30°C or 0°C in sucrose-tris or saline medium containing 0.085 µCi of [^{14}C]cholesterol and, where indicated, amytal or KCN. The control sample was incubated for 1 min. The results are expressed in c.p.m. per 1 mg of protein, ±S.D. from 4 experiments.

Incubation conditions		Subcellular fraction					
Temperature (°C)	Addition	Mitochondria		Microsomes		Cytoplasm	
		Incubation medium					
		Sucrose	Saline	Sucrose	Saline	Sucrose	Saline
0	None (control, 1 min)	15± 9	23± 13	14±10	24± 8	5± 3	11± 4
0	None	62±20	75± 30	47±30	75±40	16± 7	26±12
30	None	150±11	270± 34	195±81	300±60	28±10	31±12
30	3 mM-Amytal	240±44	335±100	261±80	370±80	25±12	23±15
30	3 mM-KCN	—	230± 50	—	250±30	—	18±15

Table 4

Effect of amytal and KCN on cholesterol uptake and oxygen consumption by placental tissue

The tissue, 0.5 g, was incubated in saline medium containing 0.045 μCi of [^{14}C]cholesterol with and without 30 mM-succinate as substrate. The accumulation of cholesterol is expressed in c.p.m. per 1 mg of protein, and the oxygen consumption as μl of O_2 per 500 mg fresh tissue weight.

Inhibitor	Without substrate		With succinate	
	Cholesterol	Oxygen	Cholesterol	Oxygen
None	54	38	54	185
3 mM-Amytal	60	10	66	182
3 mM-KCN	56	5	57	3

DISCUSSION

In the presented experiments, at 30°C an initial rapid uptake of cholesterol was observed, followed by a slower, time-dependent uptake (Fig. 1). The first step could be a result of strong cholesterol adsorption on the tissue surface, while the second step could be accounted for by cholesterol transport across the cell membrane. Consistent with this point of view is the cholesterol uptake by placental tissue at 0°C, which amounted to about 1/3 of the uptake observed at 30°C (Table 3). Here again the uptake at 0°C could be due to strong cholesterol adsorption on the tissue surface, as cholesterol was not removed by any of the washing procedures used. The increased uptake at higher temperature could result from cholesterol transport across the cell membrane.

The amount of the accumulated cholesterol increased with time both in the whole tissue and in the mitochondrial and microsomal fractions without reaching a plateau even after two hours, in contrast to the cytoplasmic fraction in which the concentration of cholesterol changed during the first 60 min of incubation and remained constant during the second hour (Fig. 2). These results could be explained as a limitation within the cell of cholesterol accumulation by the cytoplasmic fraction.

When the mechanism responsible for the transport of cholesterol across the cell membrane was excluded by using homogenate for incubation, a similar but faster rise of cholesterol accumulation in mitochondrial and microsomal fractions was observed while the cholesterol in the cytoplasmic fraction was falling down. The cholesterol accumulation expressed as c.p.m. per 1 mg. protein was much higher in the microsomal than in the mitochondrial fraction, in contrast to the distribution in intact tissue, where the accumulation in microsomes was somewhat lower than in mitochondria. This indicates that the pattern of cholesterol distribution within the intact cell is in some way dependent on compartmentation, whereas when the cell structure has been destroyed by homogenization, the distribution of cholesterol between the individual subcellular fractions is a physical process, possibly dependent on the lipid composition of the respective fractions (Parsons & Yano, 1967).

It has been demonstrated that under the conditions applied in our experiments there was practically no metabolism of cholesterol in fresh placental mitochondria.

Inhibitors of oxidation and glycolysis had no effect on the uptake of cholesterol by the placenta, and no correlation was found between cholesterol uptake and oxygen consumption. This indicates that the uptake does not depend on energy derived from glycolytic or oxidative processes. These results differ from those of Jensen (1969) concerning the uptake of cholesterol by intima media of rabbit aorta. He found that KCN increased, and the inhibitors of glycolysis lowered the uptake, and concluded that glycolysis provides the immediate energy required for cholesterol uptake. On the other hand, Rothblat, Hartzel, Mialhe & Kritchevsky (1966) demonstrated that cholesterol uptake by tissue culture cells of lymphoblasts from a murine leukemia consists in non-enzymic adsorption which is temperature and concentration dependent. Rothblat, Buchko & Kritchevsky (1968) demonstrated also that the uptake of free cholesterol by tissue culture cells is influenced by the concentration and kind of phospholipid added to the delipidated blood serum which was used as carrier medium for cholesterol.

In our previous experiments we were able to demonstrate in placental tissue a high incorporation of acetate and mevalonate into lanosterol but only a small conversion to cholesterol. In the present experiments we have shown a high accumulation of cholesterol in the microsomal fraction, in which the last steps of conversion occur. This conversion to cholesterol may be too small to account for the large amounts of progesterone and pregnenolone synthesized by the term placenta. Nevertheless, preformed cholesterol is also available from the maternal blood and may serve as the main precursor for the synthesis of progesterone. Our results support the suggestion that a simple diffusion mechanism is involved in the cholesterol transport across the cell membrane. High accumulation of cholesterol in mitochondria observed in the present studies makes this compound an effective substrate of progesterone biosynthesis in the tissue. This supports the view that the distribution and accumulation of cholesterol in subcellular fractions of placental tissue may be one of the limiting factors in the conversion of cholesterol to progesterone.

REFERENCES

Gornall A. G., Bardawill C. J. & David M. M. (1949). *J. biol. Chem.* **177**, 751.

Jensen J. (1969). *Biochim. biophys. Acta* **183**, 204.

Morrison G., Meigs R. A. & Ryan K. J. (1965). *Steroids*, suppl. II **4**, 177.

Parsons D. F. & Yano Y. (1967). *Biochim. biophys. Acta* **135**, 362.

Rothblat G. H., Buchko M. K. & Kritchevsky D. (1968). *Biochim. biophys. Acta* **164**, 327.

Rothblat G. H., Hartzel R. W., Mialhe H. & Kritchevsky D. (1966). *Biochim. biophys. Acta* **116**, 133.

Ryan K. J. (1959). *J. biol. Chem.* **234**, 268.

Schoenheimer R. & Sperry W. M. (1934). *J. biol. Chem.* **106**, 745.

Solomon S. (1960). In *The Placenta and Fetal Membranes* (C. A. Villee, ed.) p. 201. Williams & Wilkins Co., Baltimore.

Zelewski L. & Villee C. A. (1966). *Biochemistry* **5**, 1805.

WNIKANIE CHOLESTEROLU DO TKANKI ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Pobieranie cholesterolu ze środowiska inkubacyjnego jest związane z jego wnikaniem i akumulacją w tkance łożyskowej.

2. Rozmieszczenie cholesterolu we frakcjach subkomórkowych nie jest jednolite. Najwyższe nagromadzenie cholesterolu wykazano dla frakcji mitochondrialnej i mikrosomalnej.

3. Nagromadzenie cholesterolu w tkance łożyskowej nie jest skojarzone z ilością zużytego tlenu.

4. Inhibitory łańcucha oddechowego i glikolizy są bez wpływu na proces wnikania i akumulacji cholesterolu.

Received 30 July, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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PARTICIPATION OF PROTEIN IN THE STRUCTURE OF DEOXYRIBONUCLEIC ACID

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1. The effect of time and composition of the phenol mixture on the extraction of calf thymus DNA, was studied. 2. The obtained DNA preparation contained residual protein which could be removed only by treatment with a chloroform - octanol - 8-hydroxyquinoline mixture; this, however, lead to denaturation of DNA. 3. In the DNA preparation containing 0.26% of protein, the presence of magnesium, calcium, aluminium, iron and copper was spectrographically detected. 4. The possible bonds between protein and DNA have been discussed.

Deoxyribonucleic acid isolated by the commonly used procedures is known to contain trace amounts of protein which is firmly bound with DNA. Occurrence of amino acids in DNA hydrolysates has been reported for DNA from various sources (Balis, Salser & Elder, 1964; Salser & Balis, 1967, 1969). This seems to indicate that amino acids, peptides or proteins participate in the structure and function of DNA. The protein found in DNA preparations is not a histone (Butler, Phillips & Shooter, 1957; Champagne, Mazen & Pouyet, 1964), and is commonly called the residual protein.

It has been demonstrated (Butler *et al.*, 1957) that DNA preparations with a high content of residual protein are more heterogeneous with respect to the distribution of sedimentation coefficient that the DNA containing little protein, and that proteolytic enzymes lower the sedimentation heterogeneity of calf thymus DNA. Also the effect of chymotrypsin treatment on the molecular weight of the DNA isolated from fish sperm, calf thymus and rat spleen, points to the possibility that DNA molecules are held together by protein residues (Hermans, 1959).

Deoxyribonucleoproteins in concentrated NaCl solutions are to a large extent dissociated, and the protein component can be easily removed (Hammarsten, 1924; Signer & Schwander, 1949). Another, very gentle and efficient deproteinization procedure utilizes chloroform which forms with protein loose molecular bonds (Sevag, Lackman & Smolens, 1938). Marko & Butler (1951) and Kay, Simmons & Dounce (1952) have introduced detergents which, forming stable complexes with

proteins due to the action of polar groups, lead to dissociation of nucleoprotein and precipitation of protein. Kirby (1956) described the isolation of DNA from animal tissues by extraction of the homogenate with 90% phenol. There are many modifications of this method which is considered the most suitable procedure for the preparation of high-molecular DNA with the lowest content of residual protein.

In the present work, the possibility was studied of obtaining from calf thymus a DNA preparation free of protein; possible bonds between protein and DNA have also been discussed.

MATERIALS AND METHODS

Preliminary experiments. Two grams of minced calf thymus was homogenized in a glass Potter-type homogenizer with 14 ml of one of the five aqueous solutions described in Table 1. Then the homogenate was transferred to a 30 ml tube stoppered with a glass stopper, 4 ml of phenol was added, and DNA extracted at room temperature on a shaker WU-2 (Spółdzielnia Pracy "Mechanika Precyzyjna", Warszawa, Poland). At determined time intervals, the sample was centrifuged at 1000 *g* for 15 min to separate the two phases; from the aqueous phase 0.05 or 0.1 ml was withdrawn for DNA estimation. Then to the sample 0.05 or 0.1 ml of the appropriate aqueous solution was added and the extraction was continued. The efficiency of DNA extraction after 103 h and the amount of protein present in the DNA preparations obtained with different solvent mixtures, is shown in Table 1. The lowest protein content was obtained with the extraction mixture *V*, which was further used for preparation of DNA.

Preparation of calf thymus DNA by the modified phenol method. The modification of the method of Kirby (1956) consisted in applying a longer time of extraction, adding a detergent and 8-hydroxyquinoline to the extraction mixture, and deproteinization prior to and following incubation with ribonuclease.

The minced thymus (50 g) was homogenized for several seconds at a moderate speed in a Pragomix (Electro-Praha, Prague, Czechoslovakia) homogenizer with 1000 ml of an aqueous solution (pH 7.2) of 0.3 M-sodium trichloroacetate (Na-TCA) containing 1% of sodium dodecyl sulphate. The homogenate was extracted, with shaking, for 48 h at room temperature with 500 ml of phenol containing 0.3 M-Na--TCA (4:1, v/v) and 0.1% of freshly crystallized 8-hydroxyquinoline. Then the phases were separated by centrifuging at 1000 *g* for 30 min at 15°C. After separation of the aqueous phase containing the nucleic acids, to the residue a fresh portion of the organic phase (250 ml) and aqueous phase (500 ml) were added; the extraction was continued for 24 h and the phases were separated by centrifugation. The aqueous extracts were combined (about 1400 ml) and agitated for 3 h with 400 ml of the Sevag deproteinization mixture (chloroform - 1-octanol, 24:1, v/v). The deproteinization was repeated four times, for 30 min each. The DNA was precipitated by

injecting, by means of a syringe, the aqueous extract into two volumes of cold 95% (w/v) ethanol. The excess of ethanol was removed by pressing the precipitated DNA against the side of the beaker. DNA was then suspended in 1000 ml of 0.1×SSC[1] and left standing until DNA was dissolved (about 48 h); then 10×SSC solution was added in such an amount as to obtain a concentration of 1×SSC. To this solution, pancreatic ribonuclease (1 mg/ml in 0.15 M-NaCl - 0.1 M-EDTA, pH 8) was added to a final concentration of 20 μg/ml. Prior to being added, the ribonuclease was heated for 10 min at 80°C to inactivate deoxyribonuclease. The mixture was incubated overnight at 4°C, and the solution deproteinized by the Sevag technique until no protein appeared at the interphase.

DNA was precipitated again with two volumes of ethanol and dissolved in 1000 ml of 0.1×SSC solution. Then 100 ml of 3 M-sodium acetate containing 0.1 mM-EDTA was added, and DNA precipitated by dropwise addition of 550 ml of propanol. The dissolution and precipitation with propanol was repeated three times. The final DNA sediment was dissolved in 1000 ml of 0.1×SSC, then 10×SSC was added to a final concentration of 1×SSC. The solution was deproteinized again by the Sevag procedure until E_{260}/E_{230} reached a value of 2.32.

Analytical methods. Total phosphorus was estimated by the method of Fiske & Subbarow (1925), nitrogen according to Kjeldahl, and DNA by the method of Burton (1956). The total amount of DNA in thymus was determined according to Schneider (1946). Hyperchromicity was estimated after thermal denaturation. The E(P) extinction coefficient was estimated in 0.14 M-NaCl. RNA was determined according to Mejbaum (1939), and protein by the micromethod of Lowry, Rosebrough, Farr & Randall (1951). The amounts of metals were determined in a Hilger E-478 spectrograph with quartz optics, over the range of 250 - 370 nm. The samples (30 - 50 mg) were excited in an arc of direct current of 6 A, until full evaporation, from spectrally pure carbon electrodes (Ringsdorfwerke RWO, Ringsdorf, German Democratic Republic). Dimensions of the crater were: diameter 3 mm, depth 6 mm. The plates ORWO-WU2 (VEB Filmfabrik, Wolfen, German Democratic Republic) were exposed twice. The arc was generated in a BIG-300 generator.

Reagents. Sodium trichloroacetate was prepared by mixing stoicheiometric amounts of trichloroacetic acid and sodium hydroxide and adjusting the mixture to pH 7. Dodecyl sulphate, sodium salt (Schuchardt, München, West Germany) was crystallized twice from boiling ethanol and washed with ether. 8-Hydroxyquinoline was of Polish origin; it was crystallized twice from boiling chloroform. Pancreatic ribonuclease (EC 2.7.7.16) crystallized five times was a product of Koch-Light (Colnbrook, Bucks., England). Bovine serum albumin (British Drug Houses, Poole, Dorset, England) was dialysed against 0.01 M-EDTA and water. Phenol, a commercial preparation, was distilled twice. Other chemicals were reagent grade products of Polish origin.

[1] 1×SSC is a solution of 0.15 M-NaCl - 0.015 M-sodium citrate, pH 7.0.

RESULTS

The content of DNA in calf thymus, determined by the method of Schneider (1946), was found to be 2.07 g/100 g of fresh tissue. This value was taken as reference in calculating the yield of DNA extraction from the tissue.

The composition of five solutions used for extracting DNA by the phenol method, is presented in Table 1. As the curves illustrating the dependence of the yield on the extraction time were found to be asymptotic (Fig. 1), the extraction was not continued longer than for 103 h. The yield was the greatest when the extraction mixture contained only phenol and sodium trichloroacetate (mixture *III*) but the DNA preparation contained also the greatest amount of protein. The extraction of DNA

Table 1

Extraction of DNA from calf thymus by the Kirby phenol method

The tissue, 2 g, was extracted with the indicated mixture (pH 7.2) for 103 h. Then the phases were separated by centrifugation and 5 ml of the aqueous phase was dialysed for 48 h against five changes of water at 4°C, then protein and DNA were determined. The total content of DNA in thymus was 2.07 g/100 g of tissue.

Composition of the extraction mixture			DNA extracted (g/100 g of tissue)	Yield (%)	Protein content (g/100 g of DNA)
No.	Organic phase (4 ml)	Aqueous phase (14 ml)			
I	Phenol	0.3 M-NaCl	no separation of phases		
II	Phenol	Water	0	0	
III	Phenol	0.3 M-Na-TCA	1.75	85	6.9
IV	Phenol	1% SDS in 0.3 M-Na-TCA	1.2	60	3.2
V	0.1% 8-hydroxyquinoline in phenol	1% SDS in 0.3 M-Na-TCA	1.2	60	1.4

Table 2

Characteristics of the DNA preparation obtained from calf thymus

N (%)	15.78	E(P)	6450
P (%)	9.39	E_{260}/E_{230}	2.32
N/P ratio	1.68	E_{260}/E_{280}	1.90
RNA	none	Hyperchromicity following thermal denaturation (%)	38
Protein (%)	0.26		

Content of metals (mg atoms/g atom P-DNA)				
Mg	Ca	Al	Cu	Fe
0.82	0.37	0.25	0.04	0.03

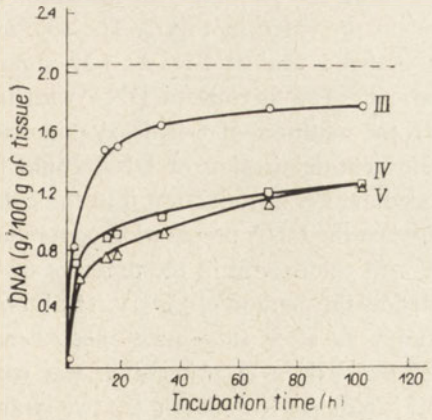


Fig. 1

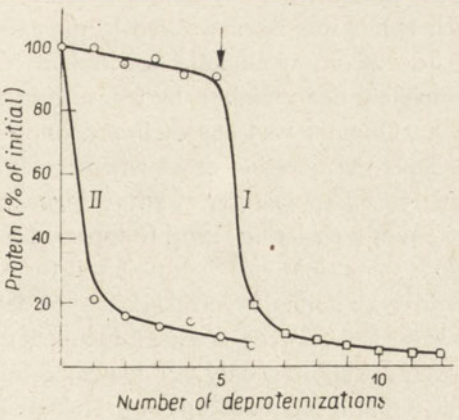


Fig. 2

Fig. 1. Effect of time on the extraction of DNA from calf thymus. Two grams of tissue was extracted with mixtures *III*, *IV* or *V* (see Table 1), as described in Methods. The dashed line indicates the content of DNA in the tissue (2.07 g/100 g).

Fig. 2. The removal of protein from a mixture of DNA and bovine serum albumin by the Sevag procedure, and the effect of 8-hydroxyquinoline. *I*, To 25 ml of a solution containing 8.8 mg of DNA (pH 7), 2 ml (8.8 mg) of bovine serum albumin dialysed against 0.01 M-EDTA and water, was added. This mixture was deproteinized with shaking for 10 min by the Sevag technique, with 5-ml portions of chloroform - 1-octanol (24:1, v/v). Then samples were withdrawn, the phases separated by centrifugation and in the aqueous phase protein was determined. After the fifth deproteinization, to the chloroform - 1-octanol mixture 0.1% 8-hydroxyquinoline was added (shown by arrow). *II*, Bovine serum albumin (27 ml. 8.8 mg of protein) was parallelly submitted to deproteinization.

with mixtures *IV* and *V* amounted only to 60%. The content of protein in the preparation was the lowest with the extraction mixture *V*, which contained sodium dodecyl sulphate and 8-hydroxyquinoline. The DNA preparations obtained with mixtures *IV* and *V* had a much greater viscosity than those extracted with mixture *III*; this may indicate much smaller degradation of the former DNA preparations during the extraction procedure. It should be noted that when water without any additions was applied as the inorganic phase (mixture *II*), no DNA was found in the aqueous phase; on the other hand, when the aqueous phase contained 0.3 M-NaCl (mixture *I*), viscosity of the solution was so high that separation of the phases proved impossible.

As the content of protein in DNA was the smallest when both a detergent and a chelating agent were present, mixture *V* was used for extraction of larger amounts of DNA. By repeated extraction, it was found possible to increase the yield up to about 90% of the DNA present in fresh tissue. The characteristics of the obtained preparation are presented in Table 2.

The obtained DNA preparation, containing 0.26% of protein, was found by spectrographic analysis to contain magnesium, calcium, aluminium, iron and copper. As these metals are rather strong chelating agents, it may be assumed that the re-

sidual protein is firmly bound by chelating bonds. The possibility of formation of such bonds was demonstrated in the experiment presented in Fig. 2. The solution of bovine serum albumin which had been dialysed against EDTA, was almost completely deproteinized by chloroform treatment. The mixture of DNA with the serum albumin was deproteinized only after the addition of 8-hydroxyquinoline.

Thus the question arose whether complete deproteinization of DNA could be achieved by application of an analogous procedure, i.e. introduction into the chloroform of a chelating agent. It appeared that, when the DNA preparation containing 0.26% of protein was deproteinized for 48 h with chloroform in the presence of 8--hydroxyquinoline, no protein could be detected by the method of Lowry. The DNA, however, underwent considerable denaturation, as after subsequent heat denaturation it showed only 9% hyperchromicity. From these experiments it was concluded that a small amount of protein (0.26%) is required to maintain the two-strand structure of calf thymus DNA.

DISCUSSION

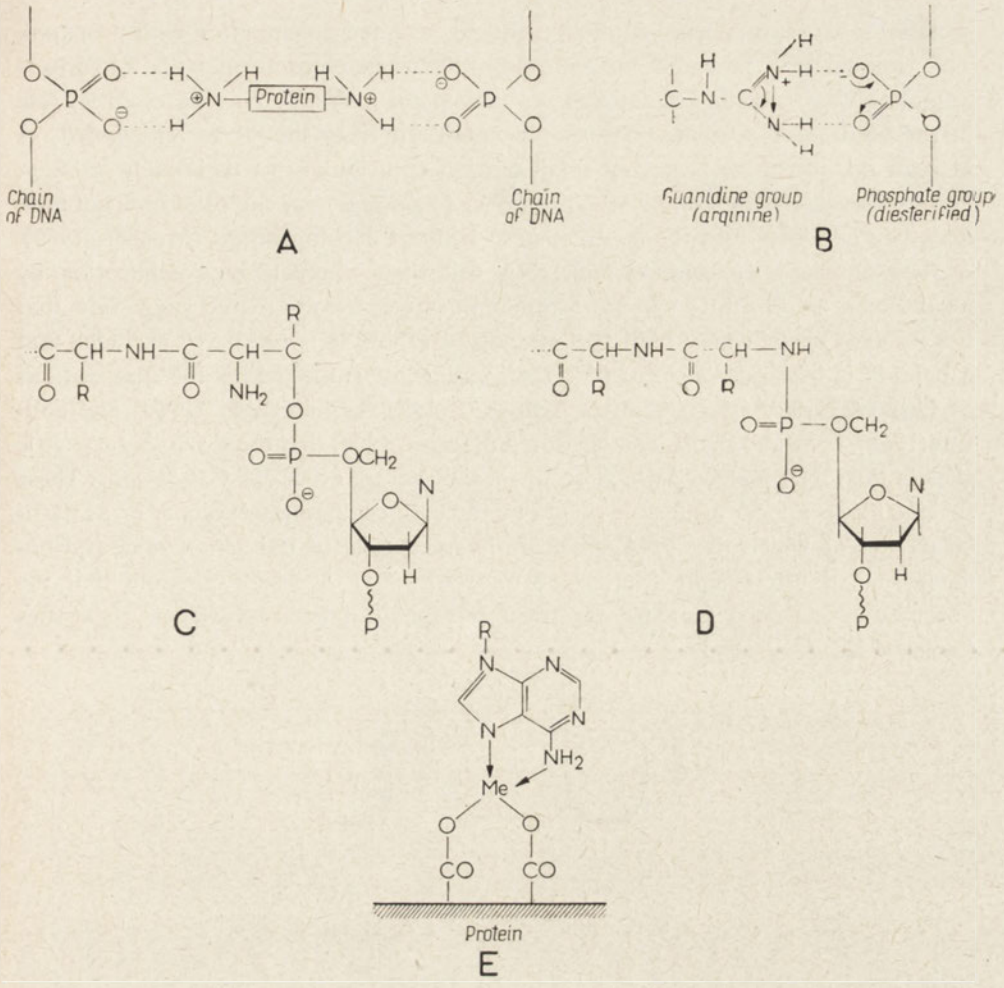
DNA is practically insoluble in phenol but, as demonstrated by Kirby (1956), the DNA-protein complex can be solubilized. In this case, the dissociation of deoxyribonucleoprotein would occur in the organic phase. When a chelating agent, 8-hydroxyquinoline, which forms stable complexes with at least 43 metals, is present in the organic phase, metal ions involved in DNA-protein binding may be removed, resulting in dissociation of DNA from protein. DNA passes into the aqueous phase, and the protein dissolved in the phenol undergoes denaturation and in part may pass to the water-saturated phenol phase. The solubility of protein in phenol is difficult to explain. May be it should be ascribed to the strong affinity of phenol for amide bonds (Dawydoff, 1953).

Trichloroacetate present in the aqueous phase has a threefold role: it raises the ionic strength of the solution; it precipitates protein; and it acts as an additional complexing ion which enhances DNA extraction. The anions which are most widely used together with the phenol procedure of DNA preparation, are *p*-aminosalicylate, trichloroacetate and benzoate, their complexing capacity decreasing in that order. If trichloroacetate is replaced by the same concentration of chloride ion, DNA is not released from the tissue nucleoprotein.

Dodecyl sulphate lowers the amount of protein in the aqueous phase. Its effect may be due to a very strong binding with guanidine groups of arginine, which leads to dissociation of nucleoprotein and precipitation of protein.

The mechanism of extraction of DNA from tissues by a two-phase system is complex. The following stages of this process should be considered: (*1*), disruption of bonds between DNA and protein; (*2*), passing of DNA to the aqueous phase; (*3*), passing of protein to the organic phase.

The removal of protein is essential for obtaining pure DNA preparations. Therefore it is necessary to include in the extraction mixture the agents which would assure the disruption of all types of DNA-protein bonds.



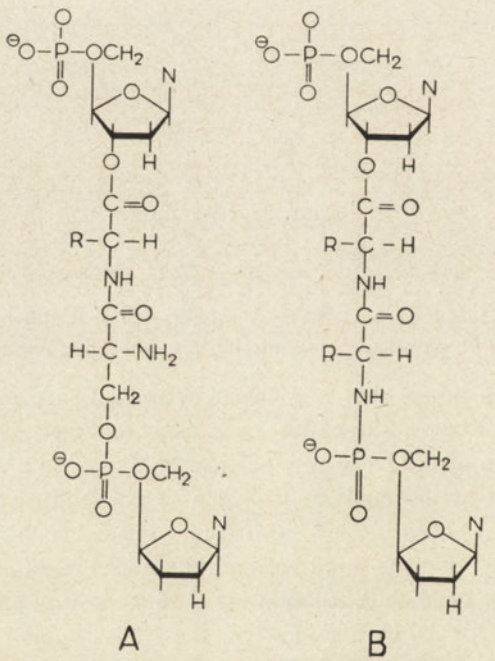
Scheme 1. Possible linkages between DNA and protein. A and B, different types of electrostatic and hydrogen bonds; C, phosphoester bonds; D, phosphoamide bonds; E, chelating bonds.

Different types of bonds that may occur between DNA and protein are shown in Scheme 1: *A*, Electrostatic bonds; negatively charged phosphate groups are linked with amino groups of basic amino acids. *B*, Hydrogen bonds between nitrogen of nitrogen bases or amino acids and oxygen of phosphate groups. *C* and *D*, Covalent bonds; *O*-phosphoserine has been found in acid hydrolysates of DNA and it has been concluded that the bond between the nucleic acid and peptide is of phosphoester character (Bendich & Rosenkranz, 1965). Also a phosphoamide binding has been proposed for DNA-peptides, on the basis of their stability in alkaline medium (Drygin, Bogdanov & Prokofiev, 1966). *E*, The binding through a chelating linkage with a divalent metal.

The results presented in this paper do not support the binding of residual protein through electrostatic, hydrogen or covalent bonds, as it proved impossible to remove the residual protein by several-fold deproteinization with a chloroform-

-octanol mixture, by phenol extraction and by repeated precipitation with propanol. The residual protein could be removed only when chloroform with 8-hydroxyquinoline was applied. Thus it appears that metal ions may play an essential role in the binding of protein. Five- or six-membered rings should be anticipated, as in such systems there is a very small tension contributing to their high stability. Six-membered chelate complexes are known to possess very high thermal stability, and they can pass into the gaseous state without decomposition (Moeller, 1959).

The observed denaturation of DNA following complete deproteinization by means of a chloroform - 8-hydroxyquinoline solution leads to the suggestion that the residual protein may bind two strands of DNA. It is not be to excluded that a peptide is incorporated into the DNA strand, as suggested by the observations of Lesko & Emery (1966), Sadron, Pouyet, Freund & Champagne (1965) and Sadron (1968). It has been also demonstrated (Sadron, 1968) that there are characteristic sites in the DNA molecule which occur on the average every 500 000 daltons. These specific places appear as flexible points by the light-scattering technique, or as particular sites at which the DNA molecule is susceptible to the action of deoxyribonuclease and ultrasounds (Bernardi & Sadron, 1964). It seems possible that the observed phenomena may be explained by chemical heterogeneity at these sites (Scheme 2).



Scheme 2. Possible modes of peptide incorporation into DNA chain: A, through phosphoester binding; B, through phosphoamide binding.

The author wishes to express his sincere gratitude to Professor Dr. Wanda Mejbaum-Katzenellenbogen for valuable suggestions and continued interest in the

course of this work and preparation of the manuscript. Thanks are also due to Dr. A. Idzikowski, Head of the Spectrographic Laboratory at the Institute of Technology in Wrocław, for making available the apparatus for spectrographic determinations. This work was supported by the Committee of Science and Technique through the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

REFERENCES

Balis M. E., Salser J. S. & Elder A. (1964). *Nature, Lond.* **203**, 1170.
Bendich A. & Rosenkranz C. (1965). *Nukleinovyje Kisloty*, p. 245. Mir, Moskwa.
Bernardi G. & Sadron C. (1964). *Biochemistry* **3**, 1411.
Burton K. (1956). *Biochem. J.* **62**, 315.
Butler J. A. V., Phillips D. M. & Shooter K. W. (1957). *Archs Biochem. Biophys.* **71**, 423.
Champagne M., Mazen A. & Pouyet J. (1964). *Biochim. biophys. Acta* **87**, 682.
Dawydoff W. (1953). *Fawerfosch. Text Tech.* **4**, 412.
Drygin J. F., Bogdanov A. A. & Prokofiev M. A. (1966). *Chimia Prirodn. Sojed.* **3**, 218.
Fiske C. H. & Subbarow Y. (1925). *J. biol. Chem.* **66**, 375.
Hammarsten E. (1924). *Biochem. Z.* **144**, 383.
Hermans J. (1959). *Biochim. biophys. Acta* **32**, 504.
Kay E. R., Simmons N. S. & Dounce A. L. (1952). *J. Am. chem. Soc.* **74**, 1724.
Kirby K. S. (1956). *Biochem. J.* **64**, 405.
Kirby K. S. (1959). *Biochim. biophys. Acta* **36**, 117.
Lesko S. A. Jr. & Emery A. J. Jr. (1966). *Biochem. biophys. Res. Commun.* **23**, 707.
Lowry O. H., Rosebrough N. Y., Farr A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.
Marko A. M. & Butler G. C. (1951). *J. biol. Chem.* **190**, 165.
Mejbaum W. (1939). *Hoppe-Seyler's Z. physiol. Chem.* **258**, 117.
Moeller T. (1959). *Chemia nieorganiczna dla zaawansowanych*, p. 210. PWT, Warszawa.
Sadron C. (1968). *Bull. Soc. Chim. biol.* **50**, 16.
Sadron C., Pouyet J., Freund A. M. & Champagne M. (1965). *J. Chim. Phys.* **10**, 1187.
Salser J. S. & Balis M. E. (1967). *Biochim. biophys. Acta* **149**, 220.
Salser J. S. & Balis M. E. (1969). *J. biol. Chem.* **244**, 822.
Schneider W. J. (1946). *J. biol. Chem.* **164**, 747.
Sevag M. G., Lackman D. B. & Smolens J. (1938). *J. biol. Chem.* **124**, 425.
Signer R. & Schwander H. (1949). *Helv. chim. Acta* **32**, 853.

UDZIAŁ BIAŁKA W STRUKTURZE KWASU DEZOKSYRYBONUKLEINOWEGO

Streszczenie

1. Przebadano wpływ czasu oraz czynników chemicznych na proces ekstrakcji fenolem DNA z grasicy cielęcej oraz na usuwanie białka resztkowego.

2. Usunięcie resztek białka z preparatu DNA jest możliwe przy zastosowaniu mieszaniny chloroformu i oktanolu z dodatkiem 8-hydroksychinoliny, jednakże proces ten prowadzi do denaturacji DNA.

3. Spektrograficznie wykazano, że w preparacie DNA o zawartości białka 0,26% są obecne metale wielowartościowe — magnez, wapń, glin, żelazo i miedź.

4. Przedyskutowano możliwe typy wiązań mogących łączyć białko z kwasem dezoksyrybonukleinowym.

Received 3 August, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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THE EFFECT OF MAGNESIUM IONS ON THE INTERACTION OF HISTONES WITH DEOXYRIBONUCLEIC ACID

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1. The interaction of whole histone and its fractions with a native DNA preparation containing only 0.26% of residual protein and trace amounts of polyvalent metals, was studied. In the absence of Mg^{2+} ion there were considerable differences in binding of the lysine-rich fraction in comparison with other histone fractions. 2. In the preparation of deoxyribonucleoprotein from calf thymus, in which the protein to DNA ratio was 1.5, appreciable amounts of polyvalent metals were present. The compact, condensed structure of the deoxyribonucleoprotein was dependent on Mg^{2+} concentration in the medium. 3. In aqueous medium at pH 7, the ratio of protein to DNA in the reconstituted nucleohistone corresponded to the composition of the natural deoxyribonucleoprotein only in the presence of Mg^{2+} ion over the concentration range of 1 - 100 mM. 4. A model of nucleohistone is proposed in which cation bridges bind together the parallelly oriented DNA strands, decreasing the electrostatic repulsion between DNA molecules.

Divalent metal ions are known to affect the structure of nucleoproteins. Compact deoxyribonucleoprotein can be obtained only in the presence of divalent cations, whereas swelling occurs after their removal or when their concentration becomes very low, less than 0.5 mM-Me^{2+}.

Numerous authors have studied the interaction of the two main components of chromatin, namely histones and DNA (Bonner & Ts'o, 1964; Busch, 1965; Olins, 1969; Agrell, 1969; Sluyser & Snellen-Jurgens, 1970; Johns & Forrester, 1970). In the present work, studies were undertaken on the effect of magnesium ion on the binding of histones with highly purified DNA. The DNA used for the experiments was a preparation containing a very low amount of residual protein (0.26%) and almost free of contaminating polyvalent metals; it was obtained by the phenol method of Kirby as modified by Maskos (1971).

MATERIALS AND METHODS

Preparation of deoxyribonucleoprotein. This was done according to Mirsky & Pollister (1946) using calf thymus gland as starting material.

Preparation of histones. Whole histone was prepared by the method of Davison, James, Shooter & Butler (1954) in the modification of Phillips & Johns (1959), and the fractions of lysine-rich histone (F_1), moderately lysine-rich histones (F_{2a} and F_{2b}), and arginine-rich histone (F_3), by procedure I of Johns (1964). Preparations of the histones were dissolved in acidified water, filtered through sintered Jena glass funnels no. 4, and precipitated by adding 15 volumes of cold acetone. After 1 h the mixtures were centrifuged for 10 min at 1000 *g*. The sediments were washed six times with acetone and dried in a vaccum desiccator over P_2O_5 and KOH. Before the experiments, the histone preparations were dissolved in water and dialysed for 24 h against 0.01 M-EDTA (pH 7), and then for 48 h against three changes of bidistilled water. The solutions of histones were adjusted to a concentration of 1 mg/ml, pH 7. The preparations of histone fractions were checked by starch-gel electrophoresis after Johns, Phillips, Simson & Butler (1961), and the patterns obtained were in agreement with those presented by the above authors.

Preparation of DNA. Deoxyribonucleic acid from calf thymus was prepared by the phenol method of Kirby as modified by Maskos (1971); it contained 0.26% of protein and metal ions (for detailed analysis see Maskos, 1971). DNA solution in water (pH 7) was prepared at a concentration of 0.2 mM-P-DNA, which corresponded to 66.4 μg DNA/ml.

Analytical methods. Concentration of protein in histone solutions was determined by the turbidimetric tannin micromethod (Mejbaum-Katzenellenbogen, 1955). Protein in deoxyribonucleoprotein preparation was estimated according to Lowry, Rosebrough, Farr & Randall (1951), whole histone being used as standard.

DNA was determined according to Burton (1956) or by measurement of extinction at 260 nm in a VSU2-P spectrophotometer (Carl Zeiss, Jena, German Democratic Republic), the same results being obtained with either method. The metals in DNA and deoxyribonucleoprotein were assayed spectrographically in a Hilger E-478 spectrograph (Hilger & Watt, London, England) with quartz optics, over the spectrum range 250 - 370 nm.

Precipitation of DNA by histones. Various amounts of histone solutions (1 mg/ml) were added to a 2.5 ml sample of DNA solution. A characteristic gelatinous sediment of deoxyribonucleohistone was then formed. The whole was vigorously stirred for 10 min using an Unipan mixer (Warszawa, Poland), then the sample was centrifuged for 30 min at 15 000 rev/min at 4°C in an Unipan centrifuge, type 310, with an angular rotor. In the supernatant the extinction at 260 nm was measured. The degree of DNA precipitation was calculated taking into account the changes in volume due to introducing the histone solution. The extinction at 260 nm of a DNA solution to which histone was not added, was taken as 100.

The effect of Mg^{2+} *on the DNA precipitation by histones.* To samples of DNA solutions containing different amounts of magnesium sulphate (0.1 - 600 mM), histone solutions were added in such amounts which without Mg^{2+} gave 50% precipitation of DNA (the protein : DNA ratio in the sample was for F_1 0.8, for F_{2a}, F_{2b} and whole histone 1.2, and for F_3 1.4). The samples were vigorously stirred, centrifuged, and in the supernatants the extinction at 260 nm was measured.

The effect of Mg^{2+} *on the solubility of deoxyribonucleoprotein.* The preparation from calf thymus, 0.2 g, was suspended in 100 ml of water. After 12 h of swelling, a 4-ml sample was centrifuged for 1 h at 15 000 rev/min, and the extinction of the supernatant was measured at 260 nm. The remaining part of the nucleohistone gel (96 ml) was dialysed at 4°C against 5 litres of 1 mM-$MgSO_4$, pH 7. After 24 h, a white fibrous sediment of deoxyribonucleoprotein appeared. A 4-ml sample of the solution was withdrawn for E_{260} measurement, and the remainder of the dialysis residue was again dialysed for 24-h periods, successively against the following solutions: 1 mM-$MgSO_4$, pH 7 (twice); 1 mM-EDTA, pH 7 (three times); 1 mM-$MgSO_4$ (three times) and then for 48 h against 1 mM-EDTA. Following each dialysis, a 4-ml sample was withdrawn, centrifuged, and the extinction of the supernatant was measured, using as a blank the same solution as used for dialysis..

The effect of phosphate and pH *on formation of insoluble DNA-histone complexes.* To study the effect of pH values, such amounts of histones were taken which precipitated a half of DNA from the solution. To 2.5-ml samples of DNA solutions, 0.5 ml of Sörensen 0.066 M-Na,K-phosphate buffer was added and then an appropriate amount of histone solution. The experiments were carried out over a pH range from 5 to 8. The extinction at 260 nm of supernatants was read against phosphate buffer.

RESULTS

The ratio of protein to DNA in the native deoxyribonucleoprotein preparation obtained from calf thymus, amounted to 1.5. However, to precipitate 1 mg of pure DNA preparation, as much as 2 4 mg of whole histone was required (Fig. 1, Table 1); the same amount of protein was necessary in the case of histones F_{2a} and F_{2b}, whereas

Table 1

Precipitation of DNA by whole histone and its fractions

Fraction	Protein : DNA ratio at maximum precipitation	DNA precipitation (%)	Protein : DNA ratio at 50% precipitation
Whole histone	2.4	96.0	1.25
F_1	1.5	95.4	0.80
F_3	2.7	98.3	1.40
F_{2a}	2.4	97.5	1.23
F_{2b}	2.4	100.0	1.20

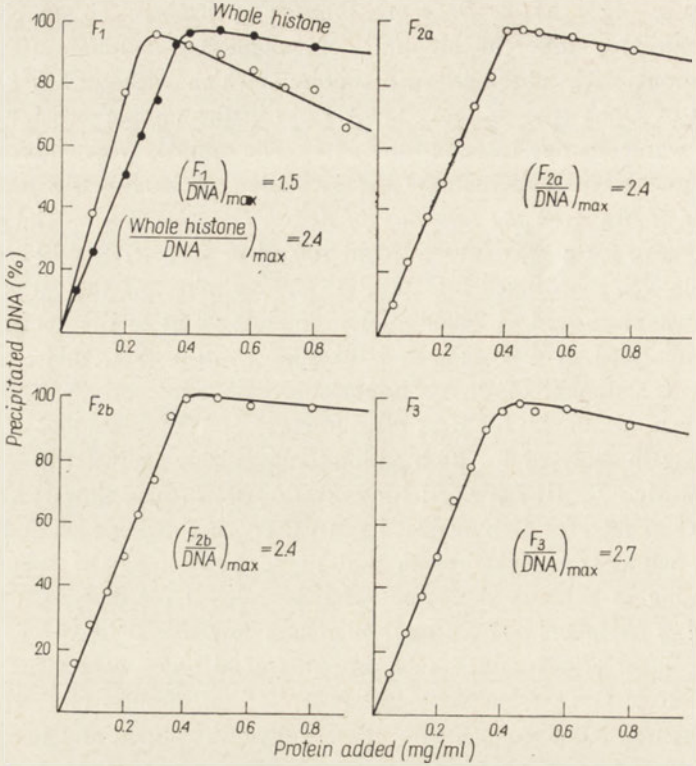


Fig. 1. Precipitation of DNA by ●, whole histone and ○, the indicated DNA fractions. The concentration of DNA was 0.2 mM-P-DNA.

for histone F_3 as much as 2.7 mg. The lowest amount was required with histone F_1 which, when added at a weight ratio of 1.5, precipitated 95.4% of the DNA. In no case, except with histone F_{2b}, a complete precipitation was achieved. When the amount of protein exceeded the value at which the maximum precipitation of DNA occurred, a solubilization of the nucleohistone was observed (Fig. 1). However, in comparison with the DNA : F_1 complex, the dissolution of nucleohistones formed with other histone fractions was much smaller. At a F_1 : DNA ratio of 6, only 62% of DNA was present in the precipitate; with other fractions, at the same ratio, about 90% was present in the form of insoluble nucleohistone.

To study the effect of Mg^{2+} concentration on formation of the insoluble form of the nucleohistone, histone was added in such an amount as to cause precipitation of 50% of the DNA when no Mg^{2+} was present. The addition of Mg^{2+} up to a concentration of 0.1 mM was without effect (Fig. 2). Higher amounts increased the binding of DNA. Complete precipitation of DNA occurred when the concentration of magnesium ions exceeded 1 mM, whereas when the Mg^{2+} concentration reached a value of 100 mM (I 0.4), a rapid dissociation of the complex occurred. The dissociation took place most readily in the case of the DNA complex with the lysine-rich histone fraction F_1. In a 200 mM-$MgSO_4$ solution (I 0.8), the DNA was fully released.

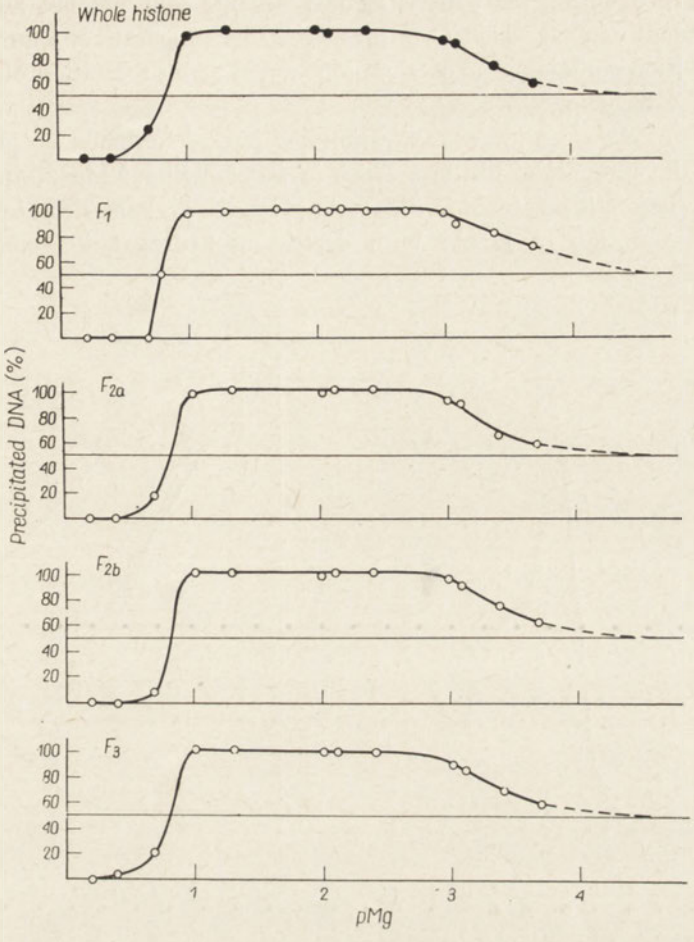


Fig. 2. Effect of Mg^{2+} concentration on precipitation of DNA by whole histone and its fractions. The histones were added in the amounts causing precipitation of 50% of the DNA (see Table 1). The concentration of $MgSO_4$ ranged from 0.1 to 600 mM, and is expressed as $pMg = -\log Mg^{2+}$.

Full dissociation of the DNA-F_3 complex was obtained only at a $MgSO_4$ concentration as high as 600 mM (I 2.4). The other two histone fractions, F_{2a} and F_{2b}, as well as the whole histone, formed complexes which underwent complete dissociation in 400 mM-$MgSO_4$ (I 1.6).

The preparation of deoxyribonucleoprotein from calf thymus was found by spectral analysis to contain rather considerable amounts of polyvalent metals (calcium, magnesium, aluminium, iron and copper). This preparation was used to study the effect of Mg^{2+} ion on the solubility of deoxyribonucleoprotein (Fig. 3). Swollen, partly dissolved deoxyribonucleoprotein was precipitated completely when dialysed against Mg^{2+} solution. Removal of Mg^{2+} by means of dialysis against EDTA caused swelling and dissolution of the deoxyribonucleoprotein. It should be noted that the dialysis against EDTA resulted in the removal not only of the

Mg^{2+} introduced during the dialysis against $MgSO_4$ solution, but also possibly of some divalent cations which were present in the preparation. Further dialysis against a solution containing magnesium ion caused again formation of condensed deoxyribonucleoprotein.

The effect of hydrogen ion concentration and phosphate buffer on precipitation of DNA by the amounts of histones which in water, without Mg^{2+}, at pH 7 gave a 50% precipitation, is presented in Fig. 4. At pH 5, the arginine-rich histone caused complete precipitation of DNA. With decreasing hydrogen ion concentration,

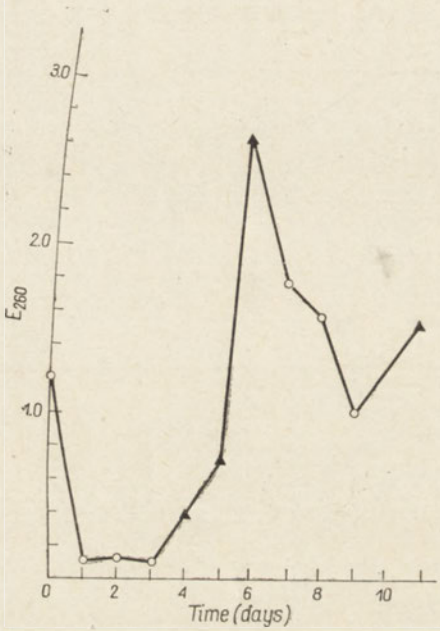


Fig. 3

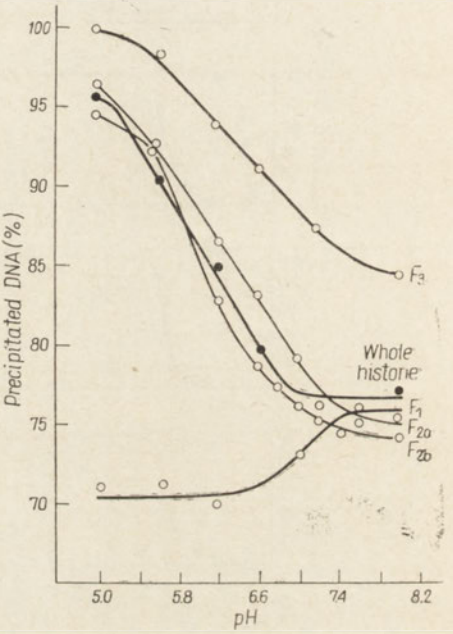


Fig. 4

Fig. 3. Effect of Mg^{2+} on the solubility of deoxyribonucleoprotein from calf thymus. The preparation, 200 mg in 100 ml of water, was dialysed against 1 mM-$MgSO_4$ and subsequently against 1 mM-EDTA, 1 mM-$MgSO_4$ and 1 mM-EDTA. At 24 h intervals, 4-ml samples were withdrawn, centrifuged, and in the supernatant the extinction at 260 nm was measured.

Fig. 4. Effect of pH and phosphate ion on the interaction between DNA and histones. The his tones were added in the amount which in the absence of phosphate, at pH 7, caused 50% precipitation of DNA (see Table 1).

the amount of the precipitated DNA decreased, 85% of precipitation being obtained at pH 8. The moderately lysine-rich histone fractions F_{2a} and F_{2b} and the whole histone behaved in a similar way. At the lowest pH value about 95% of DNA precipitated, and at pH 8, 75%. In the case of the lysine-rich fraction, over the pH range 5 - 6.2, the extent of formation of the insoluble form of the nucleohistone was unaltered, about 70% of DNA being precipitated. At pH values exceeding 6.2, the precipitation slightly increased and at pH 8 it attained the values obtained with the moderately lysine-rich fraction and whole histone.

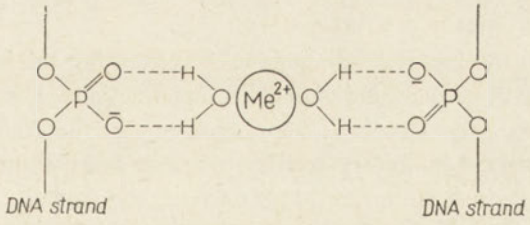
DISCUSSION

If it is assumed that histones are bound by salt-type interactions to all phosphate groups of DNA, and that there is on the average one basic amino acid per four amino acid residues, then steicheiometric binding of histone to DNA should require a histone : DNA weight ratio of 1.3 - 1.4 (Phillips, 1962). In the present work a similar ratio (1.5) was obtained only in the case of the lysine-rich histone, whereas fractions F_{2a}, F_{2b}, F_3 and whole histone were required in higher amounts (histone: : DNA ratio of 2.5).

Wilkins (1959) suggested that histone bridges between DNA molecules are unstable in solutions of low ionic strength as a consequence of electrostatic repulsion between DNA strands. The existence of strong electrostatic repulsion between DNA molecules (even when they are situated rather far apart) at low ionic strength and in the absence of divalent cations, is in agreement with the theoretical studies concerning the electrostatic field surrounding cylindrical polyions (Katchalsky, 1964).

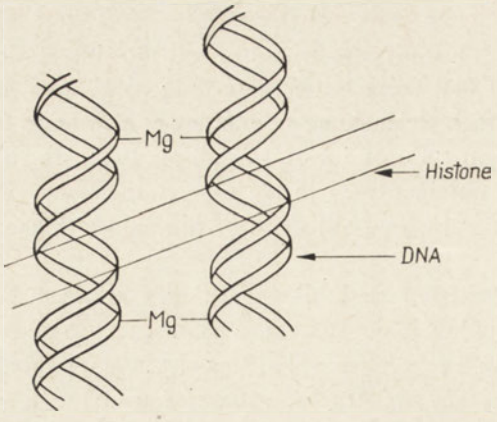
Monovalent cations (Na^+, K^+ or NH_4^+) have little effect on the structure of nucleoproteins; they are linked by rather weak bonds with phosphate groups or form a rather loose, non-bound atmosphere of counterions in the surrounding solution (Katchalsky, 1964).

Mg^{2+} promoted the formation of compact long fibres of nucleohistone in the presence of an amount of protein half that required for formation of the condensed form of the nucleohistone. The fact that in the presence of divalent cations a much smaller amount of protein was required for binding of DNA (the histone : DNA ratio being close to that prevailing in native nucleoprotein), could be connected with a large decrease of electrostatic repulsive forces. The presence of magnesium ions permits close association and tight packing of DNA molecules. Formation of long fibres would be the natural consequence of parallel arrangement of DNA molecules. This arrangement could promote the cross-linking of those fragments of the DNA to which Mg^{2+} ions are bound, leading to formation of large deoxyribonucleoprotein aggregates. Magnesium ions forming cation bridges between phosphate groups of adjacent DNA chains would lead to aggregation, as proposed in



Scheme 1

Schemes 1 and 2. This suggestion is in agreement with rentgenographic studies of intact calf thymocytes which suggest a model of chromosomes in which histone bridges join the parallelly oriented DNA chains into large three-dimensional aggregates (Wilkins, 1959; Wilkins, Zubay & Wilson, 1959; Luzatti & Nicolaieff, 1959).



Scheme 2

In the present work, the transition from the condensed fibrillar form of deoxyribonucleoprotein to a form of swollen gel, was achieved by removing the magnesium ions. If the differences between the compact and swollen chromatin depend on the mode of cross-linking with histones, then the factors that regulate the cross--linking are of biological importance. In agreement with the proposed model (Schemes 1 and 2), electrostatic repulsion between adjacent DNA molecules may be one of the factors involved in the transition of heterochromatin into euchromatin; the repulsive forces may be increased in the chromosomal region by removal from the DNA of polyvalent metal ions. This could take place e.g. through trapping and binding of magnesium ions by active sites of nucleic acid polymerases.

In the light of the presented experiments it seems possible to get a closer insight into the processes occurring in chromosomes. The swellings appearing in various places along the chromosomes could be due to a relaxation of the chromosome structure. As mentioned above, if the electrostatic repulsive forces between the DNA molecules are involved in formation of a compact nucleoprotein structure, then the local swelling of the chromosome could be a consequence of the removal of metal ions, and even small changes in the environment could result in considerable changes in chromosome structure.

There are still in the literature discrepancies concerning the inhibition of RNA and DNA synthesis *in vitro* by individual histone fractions. For instance, Allfrey, Littau & Mirsky (1963) and Hindley (1963) reported that the arginine-rich fractions (F_3) exhibited the highest inhibitory activity, whereas other authors (Hnilica & Billen, 1964; Wood, Irvin & Holbrook, 1968) found the highest inhibition by the lysine-rich fraction (F_1). If the decrease in the synthesis of nucleic acids *in vitro* results from aggregation and precipitation of DNA, then the reported differences would be mainly due to the differences in the experimental conditions concerning the histone : DNA ratios, ionic media and methods of histone preparation. In our experiments, in the presence of phosphate ions, the arginine-rich fraction (F_3) had the highest capacity to precipitate DNA, whereas in the absence of phosphate

the lysine-rich fraction (F_1) gave the highest precipitation (Figs. 4 and 1). It should be noted that those authors who reported that fraction F_3 inhibited the synthesis of nucleic acid to the greatest extent, carried out their experiments using phosphate buffer. On the other hand, when glycine buffer was used, fraction F_1 appeared to be the strongest inhibitor (Hnilica & Billen, 1964).

Hindley (1963), using a histone : DNA ratio of 2.5, found that the arginine-rich fraction was the most potent inhibitor of the DNA-dependent RNA synthesis. In the present work it was found that at this ratio the arginine-rich histone precipitated DNA almost completely, whereas the nucleohistone formed from the lysine-rich histone underwent partial solubilization when the protein : DNA ratio exceeded 1.5 (Fig. 1).

This work was supported by the Committee of Science and Technique through the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

REFERENCES

Agrell J. P. S. (1969). *Biochim. biophys. Acta* **186**, 226.
Allfrey V. G., Littau V. C. & Mirsky A. E. (1963). *Proc. natn. Acad. Sci. U.S.A.* **49**, 414.
Bonner J. & Ts'o P. O. P. (1964). *The Nucleohistones*. Holden-Day, San Francisco.
Burton K. (1956). *Biochem. J.* **62**, 315.
Busch H. (1965). *Histones and other Nuclear Proteins*. Academic Press, New York.
Davison P. F., James D. W. F., Shooter K. V. & Butler J. A. V. (1954). *Biochim. biophys. Acta* **15**, 415.
Hindley J. (1963). *Biochem. biophys. Res. Commun.* **12**, 175.
Hnilica L. S. & Billen D. (1964). *Biochim. biophys. Acta* **91**, 271.
Johns E. W. (1964). *Biochem. J.* **92**, 55.
Johns E. W. & Forrester S. (1970). *Biochim. biophys. Acta* **209**, 54.
Johns E. W., Phillips D. M. P., Simson P. & Butler J. A. V. (1961). *Biochem. J.* **80**, 189.
Katchalsky A. (1964). *Biophys. J.* **4**, 9.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.
Luzatti V. & Nicolaieff A. (1959). *J. molec. Biol.* **1**, 127.
Maskos K. (1971). *Acta biochim. Polon.* **18**, 57.
Mejbaum-Katzenellenbogen W. (1955). *Acta biochim. Polon.* **2**, 279.
Mirsky A. E. & Pollister A. W. (1946). *J. gen. Physiol.* **30**, 117.
Olins D. E. (1969). *J. molec. Biol.* **43**, 439.
Phillips D. M. P. (1962). *Progr. Biophys. Biophys. Chem.* **12**, 211.
Phillips D. M. P. & Johns E. W. (1959). *Biochem. J.* **72**, 538.
Sluyser M. & Snellen-Jurgens N. H. (1970). *Biochim. biophys. Acta* **199**, 490.
Wilkins M. H. F. (1959). *Nucleoproteins*, p. 45. R. Stoops, New York.
Wilkins M. H. F., Zubay G. & Wilson H. R. (1959). *J. molec. Biol.* **1**, 179.
Wood W. G., Irvin J. L. & Holbrook D. J. (1968). *Biochemistry* **7**, 2256.

WPŁYW JONÓW MAGNEZOWYCH NA INTERAKCJĘ HISTONÓW Z KWASEM DEZOKSYRYBONUKLEINOWYM

Streszczenie

1. Przebadano interakcję histonu całkowitego i jego frakcji z natywnym DNA zawierającym minimalną ilość białka resztkowego (0.26%) i maksymalnie uwolnionym od zanieczyszczeń metalami wielowartościowymi. Stwierdzono, że w nieobecności Mg^{2+} występują istotne różnice w wiązaniu frakcji bogatej w lizynę w porównaniu do pozostałych frakcji histonów.

2. W preparacie dezoksyrybonukleoproteidu grasicy cielęcej, w którym stosunek białka do DNA wynosił 1.5, wykazano obecność znacznych ilości metali wielowartościowych. Stwierdzono, że zwarta, skondensowana struktura dezoksyrybonukleoproteidu uzależniona jest od stężenia jonów magnezowych.

3. Wykazano, że w środowisku wodnym o pH 7 stosunek białka do DNA w sztucznym nukleohistonie odpowiada składowi naturalnego dezoksyrybonukleoproteidu tylko w obecności jonów magnezowych w zakresie stężeń 1 - 100 mM.

4. Zaproponowano model nukleohistonu, w którym mostki kationowe łączą równolegle zorientowane cząsteczki DNA, zmniejszając elektrostatyczne odpychania łańcuchów sąsiednich cząsteczek DNA.

Received 3 August, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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ARGINASE ISOENZYMES IN LIVER AND KIDNEY OF SOME MAMMALS

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1. In the liver of all mammals studied, two arginase isoenzymes (A_1 and A_3) resolved by DEAE-cellulose chromatography, are present. Isoenzyme A_1 is the main one in man, dog, cat, rat and rabbit, whereas isoenzyme A_3, in horse, ox, calf and pig. 2. In the kidney, also two isoenzymes (A_1 and A_4) are present. In all animals fraction A_4 represents 88 - 97% of the whole enzyme activity. 3. It seems that the pattern of occurrence of particular isoenzymes in liver and kidney of mammals is tissue--dependent rather than species-dependent.

Of all the arginases present in various tissues, the most extensively studied are those from mammalian liver. All arginase preparations from liver have been found to possess the same molecular weight, 120 000 - 136 000. The amino acid composition of liver arginases of horse (Greenberg, Bagot & Roholt, 1956), rat (Hirsch-Kolb & Greenberg, 1968) and calf (Grassmann, Hörmann & Jankowsky, 1958) was similar. The enzyme contained rather high amount of lysine, glycine and arginine, and little or no sulphur amino acids. Notwithstanding the similarity in amino acid composition, these arginases differed in their behaviour on electrophoresis. The purified enzyme from rat liver migrated in polyacrylamide gel at pH 8.3 toward the cathode, whereas the enzymes of horse and calf migrated on paper at pH 8.6 toward the anode. These differences might be related to the differences in protein conformation. From the studies on rat liver arginase (Hirsch-Kolb & Greenberg, 1968) it is known that the enzyme molecule in the presence of 8 M-urea dissociates into four inactive subunits of molecular weight 30 000, which, like the native arginase, migrate toward the cathode.

In our previous work we have demonstrated in the rat the presence of arginase isoenzymes, in liver of A_1 and A_3, and in kidney of A_1 and A_4 (Gąsiorowska, Porembska, Jachimowicz & Mochnacka, 1970). All these isoenzymes had the same molecular weight, 120 000. We have also found that fractions A_1, obtained both from liver and kidney, migrated on starch-gel electrophoresis at pH 8.3 toward the cathode, whereas fractions A_3 and A_4, toward the anode (Porembska, Jachimowicz

& Gąsiorowska, 1971). The reported in the literature different behaviour of arginases from liver of various animals in the electric field may be due to the isolation by the respective authors of different arginase isoenzymes.

There are numerous reports in the literature concerning the activity of arginase in the liver of various animals. However, it is difficult to compare the results obtained in different laboratories, as the determinations of enzyme activity were carried out under different conditions. On the other hand, arginase activity in kidney has been studied in a few animals only.

The aim of the present work was to compare the arginase activity in liver and kidney of animals belonging to ten species of mammals, and to study the quantitative distribution patterns of the arginase isoenzymes.

MATERIALS AND METHODS

Tissue extracts. Liver and kidney of cat, dog, guinea-pig, rabbit and rat were isolated immediately after killing of the animal by bleeding. Liver and kidney of horse, sheep, ox and calf were obtained in the slaughterhouse as soon as possible after killing of the animal, and transported to the laboratory in ice. Samples of human liver and kidney were taken within 6 - 10 h after death of people killed in traffic accidents.

The tissues were washed thoroughly with cold 5 mM-$MnCl_2$ - 5 mM-tris-HCl buffer, pH 7.5, to remove blood, and then homogenized with 5 vol. of the above buffer three times in a teflon-glass Potter-type homogenizer for 1 min at 1900 rev/min. The homogenate was left for an hour at 0 - 4°C with gentle stirring and then kept at -10°C for 24 h. Following thawing, the homogenate was centrifuged at 6000 rev/min at 4°C, and the sediment extracted once more as described above. In the combined extracts, the content of protein and arginase activity were determined.

Separation of arginase isoenzymes. To concentrate the proteins in the extract, ammonium sulphate was added to 0.9 saturation (at which all arginase activity undergoes precipitation). The mixture was left for 1 h in the refrigerator, then centrifuged at 2°C; the sedimented protein was dissolved in 5 mM-$MnCl_2$ - 5 mM--tris-HCl buffer, pH 8.3, to obtain a concentration of 40 - 60 mg/ml; if a smaller volume of the buffer was used, the less soluble isoenzyme was sometimes not dissolved. The solution was dialysed overnight against the same buffer, then the slight inactive precipitate formed was centrifuged off at 12 000 rev/min for 15 min at 2°C, and the clear supernatant was submitted to DEAE-cellulose column chromatography. If the chromatography was to be carried out on CM-cellulose, the protein precipitated by ammonium sulphate was dissolved in 5 mM-$MnCl_2$ - 5 mM-tris-HCl buffer of pH 7.5, and dialysed against this buffer.

The chromatography of the extract was carried out on columns (1×18 cm) of DEAE-cellulose equilibrated with 5 mM-tris-HCl buffer, pH 8.3, or of CM-cellulose equilibrated with 5 mM-tris-HCl, pH 7.5. The amount of protein applied to the column was in the case of liver 80 - 150 mg, and in the case of kidney, taking into account lower activity of the enzyme, 300 - 400 mg. The protein was eluted

with 30 ml of the solution used for equilibration, and then with a linear KCl concentration gradient, up to 0.3 M, in the same buffer. Fractions of 5 ml were collected at a flow rate of 1 - 1.5 ml/min, and in appropriate portions protein and arginase activity were determined.

Arginase assays. These were carried out as described previously (Gąsiorowska *et al.*, 1970) in a medium containing 50 mM-glycine buffer, pH 9.5, 5 mM-$MnCl_2$, 25 mM-arginine (pH 7.5) and arginase source. The applied amounts of protein were within the range at which the reaction rate was linear with protein concentration and linear with time.

Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Reagents were the same as used in the previous work (Gąsiorowska *et al.*, 1970).

RESULTS

Under the conditions applied in the present work for the extraction of liver and kidney homogenates, practically all arginase activity passed into solution and the activity present in the discarded tissue debris did not exceed 5 - 7%. From 1g of liver, after two extractions with a total of 10 ml of the buffer, 120 - 160 mg of protein was extracted in the case of man, dog, guinea-pig, rabbit, rat and cat, and about half as much, 62 - 74 mg, in the case of pig, calf, horse and ox. On the other hand, in analogous preparations from kidney of all animals studied, the amount of extracted protein was nearly the same, 30 - 50 mg.

The highest arginase activity in liver (Table 1), expressed in μmols of ornithine formed per 1 g of tissue per 1 min, was found in rat and dog; lower activity, about 1/3

Table 1

Arginase activity in liver and kidney of some mammals

The presented results are mean values from three different liver or kidney extract preparations. Limit values are given in parentheses.

Animal	Liver	Kidney	Ratio of liver activity to kidney activity
	Activity (μmols/min/g of fresh tissue)		
Man	50 (49 - 51)	0.4 (0.3 - 0.5)	125
Cat	65 (60 - 75)	1.45 (1.4 - 1.5)	44
Dog	302 (280 - 315)	0.23 (0.16 - 0.33)	1313
Rabbit	52 (42 - 58)	0.35 (0.25 - 0.41)	149
Rat	290 (270 - 330)	6.0 (5 - 7)	48
Guinea-pig	98 (89 - 110)	0.145 (0.12 - 0.17)	700
Pig	44 (38 - 55)	1.55 (1.5 - 1.6)	22
Ox	104 (100 - 109)	0.33 (0.15 - 0.45)	315
Calf	27 (25 - 30)	0.19 (0.17 - 0.21)	142
Horse	31 (30 - 33)	0.17 (0.15 - 0.2)	182

of that in the rat, was found in the guinea-pig and ox. The activity of arginases from the liver of man, cat, rabbit and pig was of the same order and amounted to 1/6 that of rat or dog. The lowest activity was observed in the liver of calf and horse.

In kidney, the highest activity was found in rat, smaller in cat and pig, whereas in other animals the activity was much lower, 1/15 - 1/35 of that found in the rat. In all studied kidneys, the arginase activity was considerably lower than in liver of the same animal, by a factor from 22 in pig to as much as 1300, in the dog.

The results of separation of extracts from mammalian livers by chromatography on DEAE-cellulose, are presented in Fig. 1. In all cases, the distribution of protein fractions was similar, two peaks being obtained. The first sharp peak of the protein which was not adsorbed on DEAE-cellulose and was eluted with the buffer, contained 40 - 50% of the recovered protein; the second, broad peak of protein which was adsorbed on the column and eluted by a KCl concentration gradient, contained, correspondingly, 60 - 50% of the protein.

Similarly as in our previous experiments on the rat (Gąsiorowska *et al.*, 1970), in livers of all ten animal species studied two arginase fractions, A_1 and A_3, were obtained, which were separated by chromatography on DEAE-cellulose in an identical manner. It appears that these two arginase isoenzymes are characteristic of mammalian liver. On the other hand, the distribution of the activity between the two isoenzymes showed considerable differences (Table 2). In man, cat, dog, rabbit and rat, fraction A_1 contained 80 - 90% of the recovered arginase activity; in guinea-pig this value was somewhat lower, 70%, in pig and calf only 20 - 30%, and in horse and ox it was as low as 10 - 15%. Thus in livers of some mammals almost all arginase activity was found to be present in isoenzyme A_1, whereas in other, in isoenzyme A_3.

As fraction A_1 was not adsorbed on cationic DEAE-cellulose, it appears to have a charge less positive than fraction A_3, or even a negative charge. This was confirmed by chromatography on anionic exchanger using pig liver extract preparation in which 70% of the activity was present in fraction A_3. On CM-cellulose (Fig. 2), arginase activity was resolved into two peaks which also differed markedly in activity, but this time the fraction non-adsorbed on the column (which corresponded to the fraction A_3 from DEAE-cellulose) contained about 70% of the activity, whereas the adsorbed fraction (which corresponded to fraction A_1 from DEAE-cellulose), only 30%.

As the results obtained for the pig by chromatography on the anionic and cationic exchangers were found to be in very close agreement, it seems that quanti-

Fig. 1. DEAE-cellulose column chromatography of extracts from livers of various mammals. The extract (80 - 150 mg of protein) was applied to the column (1 × 18 cm); fractions of 5 ml were eluted at a rate of 1 - 1.5 ml/min. The conditions of preparation of the material and of chromatography were as described in Methods. ●, Arginase activity; ○, protein; — — —, KCl concentration gradient.

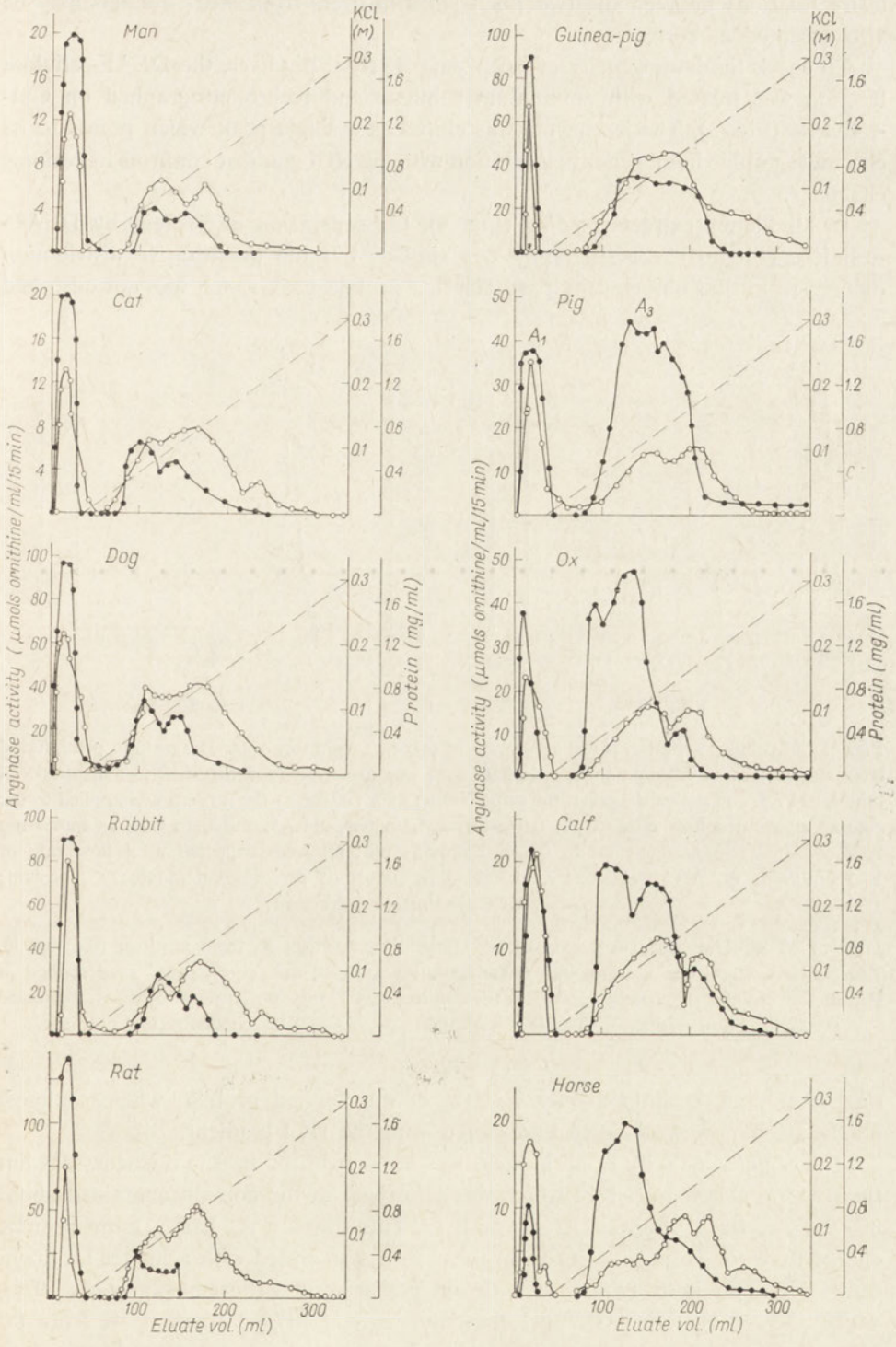

Fig. 1

tative relations between the two isoenzymes in livers from other animals may be also accepted as correct.

To check the homogeneity of isoenzyme A_1 from pig liver, the DEAE-cellulose fraction was treated with ammonium sulphate and rechromatographed on CM--cellulose (Fig. 3). The isoenzyme was eluted as a single peak which points to its chromatographic homogeneity; its elution with the KCl gradient confirms its cationic character.

In all kidney extracts studied (Fig. 4), the separation of protein by DEAE--cellulose column chromatography was similar, but the quantitative distribution differed from that observed in liver. The first protein peak which was not adsorbed

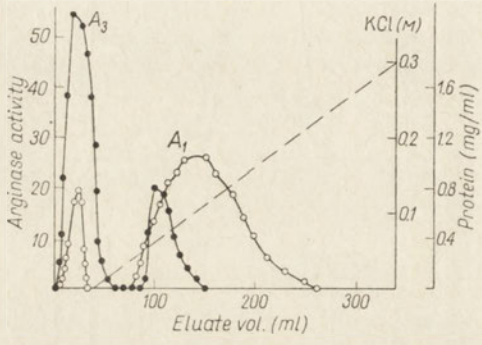

Fig. 2

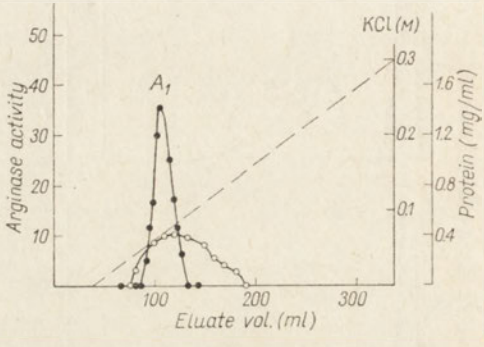

Fig. 3

Fig. 2. CM-cellulose column chromatography of proteins from pig liver. The protein precipitated from the extract at 0.9 ammonium sulphate sat., was dissolved in 5 mM-$MnCl_2$ - 5 mM-tris-HCl buffer, pH 7.5, and dialysed against the same buffer; then 100 mg of the protein was applied to the column and eluted first with 30 ml of 5 mM-tris-HCl buffer, pH 7.5, and then with an increasing KCl concentration gradient up to 0.3 M. Fractions of 5 ml were collected at a flow rate of 1 - 1.5 ml/min. ●, Arginase activity, expressed as μmols of ornithine/ml/15 min; ○, protein; — — —, KCl concentration gradient.

Fig. 3. CM-cellulose chromatography of DEAE-cellulose fraction A_1 from pig liver (see Fig. 1). The fraction, after precipitation with 0.9 ammonium sulphate sat., was treated as described in Fig. 2. The amount of protein applied to the column was 35 mg. ●, Arginase activity, expressed as μmols of ornithine/ml/15 min; ○, protein; — — —, KCl concentration gradient.

on the column, contained only 7 - 10% of the applied protein, whereas almost all the protein was adsorbed and eluted with the KCl gradient.

The arginase activity of all kidneys was also separated into two isoenzymes but the fraction eluted with KCl which was adsorbed on the column contained about 88 - 97% of the recovered enzyme activity. This fraction A_4 had a more positive charge than the isoenzyme A_3 from liver, as it was eluted with a higher KCl concentration. This is in agreement with our earlier results (Porembska *et al.*, 1971), which showed that on starch-gel electrophoresis at pH 8.3 fraction A_4 from rat kidney migrated faster toward the anode than fraction A_3 from rat liver.

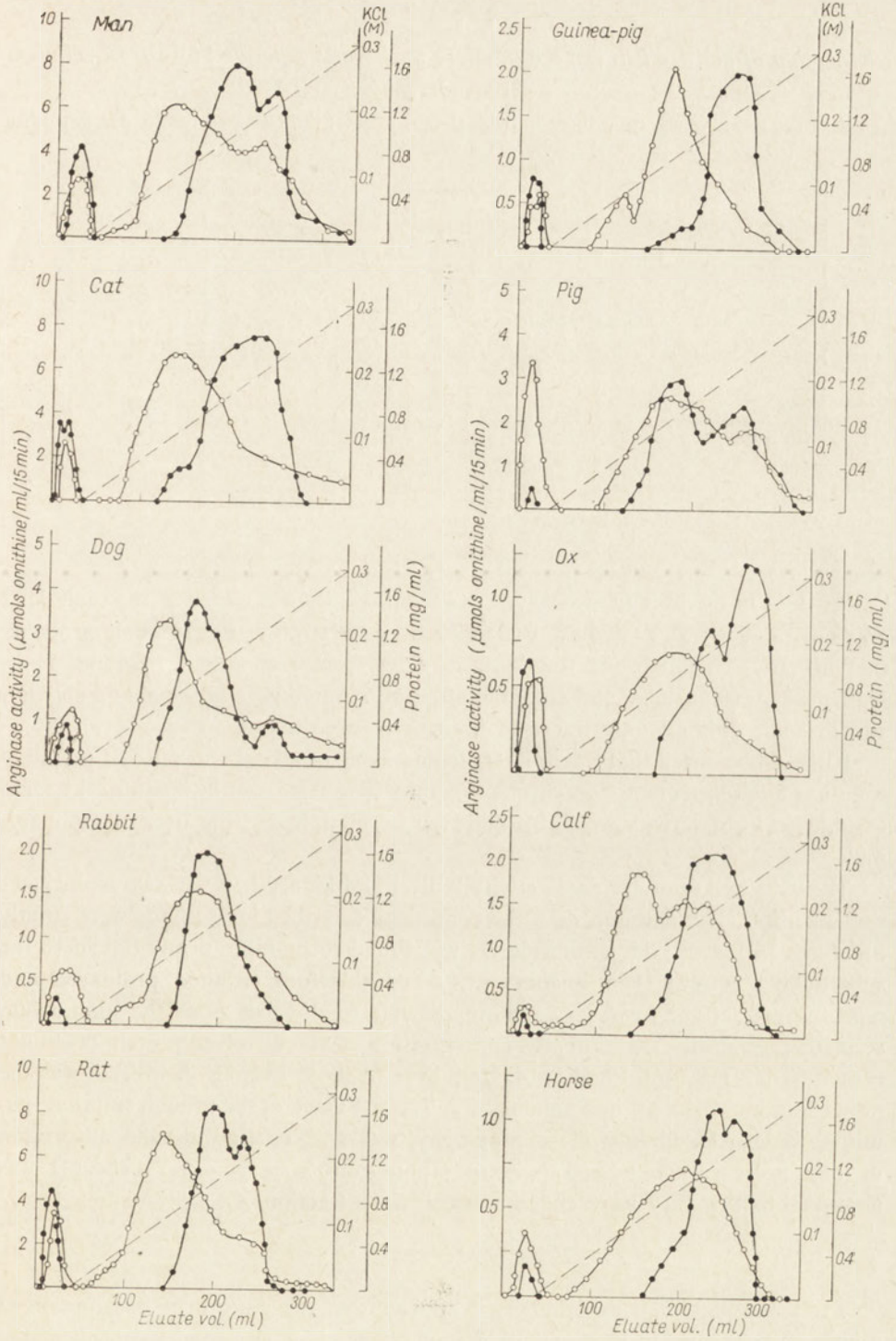

Fig. 4. DEAE-cellulose column chromatography of extracts from kidneys of various mammals. Conditions of chromatography as in Fig. 1; the amount of protein applied was 300 - 400 mg. ●, Arginase activity; ○, protein; — — —, KCl concentration gradient.

Table 2

Distribution of activity between the arginase isoenzymes separated by DEAE-cellulose chromatography

The data are mean values from three individual experiments; the obtained results did not differ by more than 5%.

Animal	Liver isoenzymes		Kidney isoenzymes	
	A_1	A_3	A_1	A_4
	Activity (%)			
Man	85	15	12	82
Cat	87	13	5	95
Dog	87	13	3	97
Rabbit	84	16	3	97
Rat	90	10	9	91
Guinea-pig	68	32	8	92
Pig	23	77	3	97
Ox	13	87	3	97
Calf	25	75	6	94
Horse	15	85	4	96

Whereas in the liver the isoenzyme A_3 in all species was eluted at the same KCl gradient, in the case of kidney some differences were observed. It seems probable that in various animals the fractions A_4 differ from each other. Fraction A_1 in kidney was very unstable and accounted for only a small part of the eluted activity, and in some animals only traces of its activity were found.

It should be noted that fractions A_4 from kidney of all animals studied probably were not homogeneous, as on chromatography they separated into two peaks each. Double peaks of activity were also observed in fraction A_3 from liver of some animals.

The distribution of arginase activity in liver and kidney between two isoenzymes was quantitatively reproducible if the conditions described in Methods were strictly observed. In kidney, the isoenzyme A_4 was found to be more firmly bound to the subcellular fractions than isoenzyme A_1 and therefore required prolonged and repeated extraction; it was also more difficult to dissolve from the ammonium sulphate precipitate. Therefore, the concentration of the dissolved protein could not exceed 60 mg per 1 ml. Fraction A_3 from liver, in those animals in which it was the minor fraction, behaved in a similar way. The pH value of the protein solution submitted to DEAE-cellulose chromatography, was also critical for the adsorption of these protein fractions and their separation from isoenzyme A_1. If the pH was not equal to 8.3, they were eluted together with fraction A_1.

DISCUSSION

From the presented experiments it appears that the pattern of distribution of arginase isoenzymes in liver and kidney of mammals is not species-dependent but rather tissue-dependent. The presence of isoenzymes A_1 and A_3 is characteristic

of liver, and isoenzymes A_1 and A_4, of kidney. In kidneys of all animals, the isoenzyme A_4 was the main one, whereas in the liver the quantitative relations between the two isoenzymes varied in different animals.

Isoenzymes A_1 had a positive charge, and isoenzymes A_3 and A_4, a negative one. In rat liver, the positively charged isoenzyme A_1 was the main one, and probably this is the isoenzyme which was purified by Hirsch-Kolb & Greenberg (1968); in the calf, the main isoenzyme was the negatively charged A_3 which probably was isolated by Grassmann *et al.* (1958). It seems that the isoenzymes which occurred in small amounts and had lower stability, had been lost during the purification procedure.

The relative content of isoenzymes A_1 and A_3 in the liver of animals from various mammalian species, seems to be of interest. In most of the species, the isoenzymes A_1 represented almost the whole arginase activity of the tissue. Only in the horse, ox and calf the isoenzyme A_3 was the main one. The factors responsible for the preponderance of the particular isoenzyme in liver are not clear but is seems tempting to speculate on the role of the diet, as in the herbivorous horse, calf and ox isoenzyme A_3 was the main fraction. This explanation, however, could not account for the results obtained with the rabbit which also is herbivorous but in which almost the whole arginase activity was found in the fraction A_1.

The excellent technical assistance of Mrs. Aleksandra Bakuła is gratefully acknowledged.

REFERENCES

Gąsiorowska I., Porembska Z., Jachimowicz J. & Mochnacka I. (1970). *Acta biochim. Polon.* **17**, 19.
Grassmann W., Hörmann H. & Jankowsky O. (1958). *Z. physiol. Chem.* **312**, 273.
Greenberg D. M., Bagot A. E. & Roholt O. A., Jr. (1956). *Archs Biochem. Biophys.* **62**, 446.
Hirsch-Kolb H. & Greenberg D. M. (1968). *J. biol. Chem.* **243**, 6123.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.
Porembska Z., Jachimowicz J. & Gąsiorowska I. (1971). *Bull. Acad. Polon. Sci. Ser. biol.* **19**, 27.

IZOENZYMY ARGINAZY W WĄTROBIE I NERCE RÓŻNYCH GATUNKÓW SSAKÓW

Streszczenie

1. W wątrobie badanych ssaków stwierdzono obecność dwu izoenzymów arginazy A_1 i A_3 identycznie zachowujących się podczas chromatografii na kolumnie z DEAE-celulozy. Izoenzym A_1 był główną frakcją arginazy w wątrobie człowieka, psa, kota, szczura i królika, natomiast A_3 w wątrobie konia, wołu, cielęcia i świni.

2. W nerce wykazano obecność również dwu izoenzymów arginazy A_1 i A_4. Izoenzym A_4 u wszystkich badanych zwierząt stanowił 88 do 97% całej aktywności enzymatycznej.

3. Wydaje się, że obraz izoenzymów arginazy w wątrobie i nerce zależy od tkanki a nie od gatunku zwierzęcia.

Received 5 August, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

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THE PROPERTIES OF CRYSTALLINE PHOSPHOPYRUVATE HYDRATASE FROM SWINE MUSCLE

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1. Swine muscle phosphopyruvate hydratase (EC 4.2.1.11) was purified and a crystalline preparation was obtained. 2. The enzyme is active only in the presence of divalent metal ions, Mg^{2+} being the best activator. 3. The molecular weight of the enzyme was found to be 90 000. K_m for 2-phosphoglycerate was 0.33 mM and for phosphoenolpyruvate 0.9 mM. 4. The optimum pH in the imidazole and phosphate buffers was 6.8.

Phosphopyruvate hydratase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11), formerly known as enolase, was discovered by Lohmann & Meyerhof (1934). In crystalline form it was obtained first by Warburg & Christian (1941) from brewer yeast as the inactive mercury complex. Federtchenko (1958), Czok & Bücher (1960) and Holt & Wold (1961) isolated the enzyme from rabbit muscle, Boser (1959) from potatoes, and Wood (1964) from ox brain. Saito (1967) described the purification and some biological properties of bacterial phosphopyruvate hydratase, and Baranowski, Wolna & Morawiecki (1968) obtained crystalline enzyme from human muscle.

In the present communication, the isolation of phosphopyruvate hydratase from swine muscle, its properties and activity in comparison with the enzyme isolated from other sources, are described.

MATERIALS AND METHODS

Protein estimation. Protein concentration was determined spectrophotometrically in Unicam SP-500 at 280 and 260 nm, and calculated according to Warburg & Christian (1941).

Phosphopyruvate hydratase assay. Enzyme activity was determined as described earlier (Baranowski *et al.*, 1968). The test sample contained: 50 mM-imidazole buffer, pH 6.8, 1 mM-sodium 2-phospho-D-glycerate, 3 mM-magnesium sulphate and 0.4 M-potassium chloride in a total volume of 3.0 ml. The reaction was followed by the measurement of extinction at 240 nm at 25° after addition of the enzyme.

The increase of 0.100 in extinction corresponded to the conversion of 0.226 μmol of substrate. One unit was defined as the utilization of 1 μmol of substrate per minute at 25°. The specific activity is expressed in units per milligram of protein.

Determination of the molecular weight. The molecular weight was determined by gel filtration on Sephadex G-100 and G-200 columns according to Andrews (1964) in tris-HCl buffer, pH 7.4; also thin-layer gel filtration on Sephadex G-150 superfine was applied according to Marek (1969). The following standards were used: phosphopyruvate hydratase from human muscle obtained by the method of Baranowski *et al.* (1968), glyceraldehyde-3-phosphate dehydrogenase from swine muscle prepared by the method of Elödi & Szörenyi (1956), bovine serum albumin and soybean trypsin inhibitor (Sigma, St. Louis, Mo., U.S.A.), chymotrypsinogen (Koch-Light, Colnbrook, Bucks., England), and ribonuclease A (Reanal, Budapest, Hungary).

Other reagents. 2-Phosphoglycerate was synthesized as described by Meyerhof & Kiessling (1957) or purchased, similarly as phosphoenolpyruvate, from the British Drug Houses (Poole, Dorset, England). Imidazole was recrystallized from benzene. Ammonium sulphate was recrystallized as described by Beisenherz *et al.* (1953).

In some experiments the enzyme protein was desalted by filtration on Sephadex G-25.

RESULTS

Isolation of the muscle phosphopyruvate hydratase

The process of purification is summarized in Table 1.

Step 1. The swine skeletal muscle immediately after killing of the animal was placed in an ice-bath, and further operations were carried out at 4°C. The muscle

Table 1

Summary of purification of phosphopyruvate hydratase from swine muscle

For the preparation 1 kg of swine muscle was used. For details see text.

Step	Total protein (g)	Activity (units/mg protein)	Recovery (%)
1. Extract	40.0	1.72	100.0
2. Ist ammonium sulphate fractionation	3.9	10.2	57.0
3. Heat treatment	2.0	19.2	55.5
4. Gel filtration on Sephadex G-50	0.73	42.5	44.0
5. Gel filtration on Sephadex G-100	0.45	60.0	39.0
6. IInd ammonium sulphate fractionation	0.34	66.0	33.5
7. Crystallization	0.29	70.0	29.0

was ground in a meat grinder and homogenized in the blendor type MM3 (WWP Niewiadów, Poland) with two parts (w/v) of glass-distilled water. The homogenate was stirred for 30 min and centrifuged at 2000 *g* for 20 min at 0°C.

Step 2. To the supernatant fluid, solid ammonium sulphate was added very slowly to a concentration of 2.4 M. The solution was left overnight at 4°C, then the precipitated protein was separated by centrifugation and discarded. To the clear solution, solid $(NH_4)_2SO_4$ was added to 2.75 M. After standing overnight, the precipitate was collected by centrifugation and dissolved in a small volume of 0.03 M-$MgSO_4$.

Step 3. The clear solution was prewarmed at 30°C for 30 min, heated at 55°C for 3 min and then cooled in an ice-bath. The precipitate of denatured protein was centrifuged and discarded.

Step 4. A sample of the clear supernatant (7 ml containing approximately 500 mg of protein) was applied on the top of a Sephadex G-50 column (2.5×50 cm). The protein was eluted with water, fractions of 3 ml being collected with a fraction collector. The protein and enzyme assays were performed on each fraction. The enzymically active fractions were collected, $MgSO_4 \cdot 7H_2O$ added to a concentration of 0.5%, and the sample concentrated by ultrafiltration in a dialysing tube (Union-carbide Corp., Chicago, U.S.A.) according to Rosenfeld (1963).

Step 5. The concentrated protein was applied to a Sephadex G-100 column and eluted with water.

Step 6. The enzymically active fractions were pooled and the protein precipitated between 2.4 - 3.2 M-ammonium sulphate concentration was collected by centrifugation.

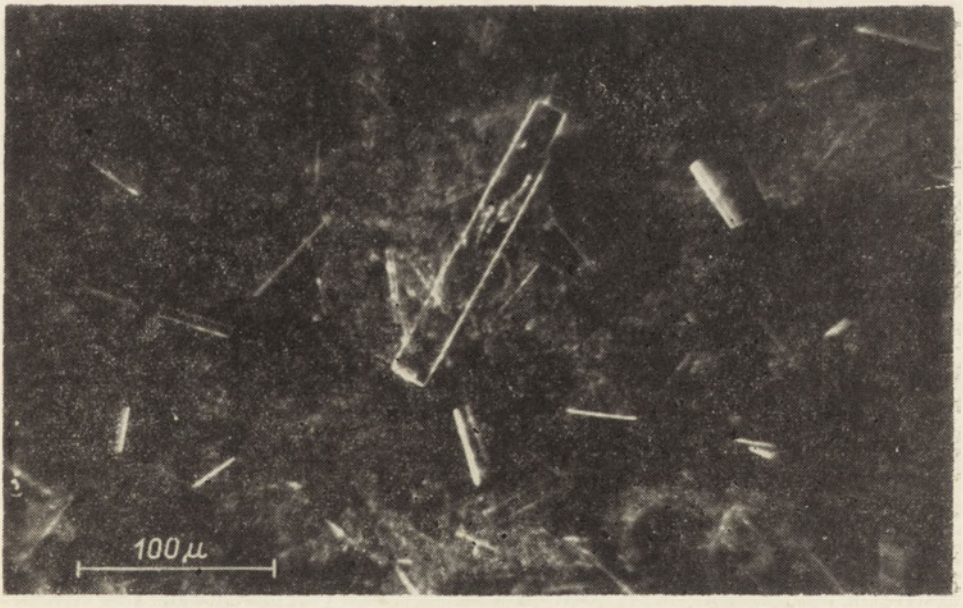

Fig. 1. Crystals of the phosphopyruvate hydratase from swine muscle.

Step 7. The precipitate was dissolved in a small volume of 0.05 M-imidazole buffer, pH 7.8, containing 0.5% of $MgSO_4$, and to the clear solution a saturated solution of ammonium sulphate was added until a slight turbidity appeared. After a few days the crystallization was complete. The enzyme crystallized in long rectangular plates shown in Fig. 1. Crystallization proceeded readily in about 5% solution of protein.

Properties of crystalline phosphopyruvate hydratase

The activity of the crystalline preparation was found to be 70 units/mg of protein.

The preparation, when submitted to gel filtration on Sephadex G-50, G-75 and G-100 columns, gave a single peak. Also in the ultracentrifuge, after 154 min centrifugation at 116 000 *g*, the enzyme gave a single symmetrical peak (Fig. 2).

The molecular weight of the enzyme calculated from the gel filtration on Sephadex G-100 or from the thin-layer gel chromatography was found to be 90 000.

The effect of pH on the rate of the enzymic reaction was measured in imidazole and phosphate buffers. As shown in Fig. 3, the optimum pH for both buffers was found to be the same, pH 6.8, but the activity in phosphate buffer was lower by about 40%.

Phosphopyruvate hydratase from different sources is known to act only in the presence of some divalent metal ion such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and other. The enzyme shows a rather broad metal-ion specificity, but Mg^{2+} is the best acti-

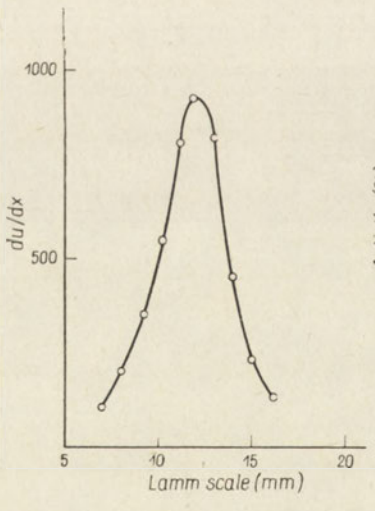

Fig. 2

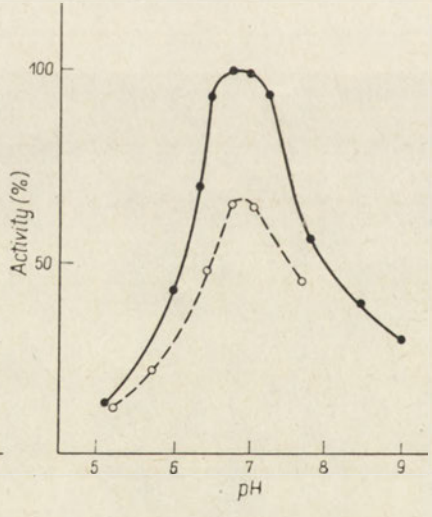

Fig. 3

Fig. 2. Sedimentation diagram of swine muscle phosphopyruvate hydratase after 154 min of sedimentation at 116 000 *g*.

Fig. 3. Effect of pH on the activity of phosphopyruvate hydratase from swine muscle. The reaction mixture and the conditions were as described in Methods, except the pH value and kind of buffer. ●, Imidazole buffer; ○, phosphate buffer.

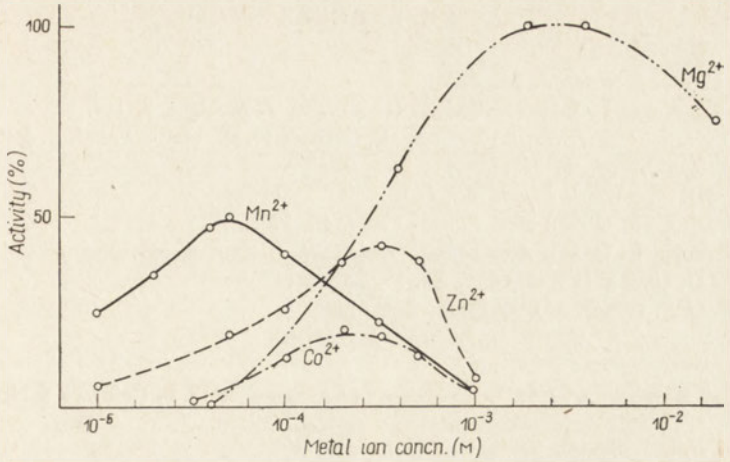

Fig. 4. Effect of the concentration of metal ions on the activity of phosphopyruvate hydratase from swine muscle. The assays were carried out as described in Methods.

vator. The enzyme from swine muscle was found to have similar properties. The enzyme was activated better by Mg^{2+} than by Mn^{2+}, Zn^{2+} or Co^{2+} (Fig. 4).

The Michaelis constants for 2-phosphoglycerate and phosphoenolpyruvate were 0.33 mM and 0.9 mM, respectively.

DISCUSSION

The methods that were used for purification of phosphopyruvate hydratase from rabbit muscle (Federtchenko, 1958; Czok & Bücher, 1960; Holt & Wold, 1961) could not be applied for the isolation of the enzyme from swine muscle. However, our procedure of purification of the enzyme from human muscles (Baranowski *et al.*, 1968) after only a slight modification was useful for the isolation of the swine enzyme. The modification consisted in the application of Sephadex G-50 instead of Sephadex G-75, and rechromatography on Sephadex G-100.

Sephadex should be prepared in water. The elution in a buffer did not lead to the separation of the enzyme from a coloured protein. The crystallization was successful only in a concentrated, about 5%, protein solution prepared in the imidazole buffer.

The presence of magnesium ions during all steps of purification prevented the loss of activity of the enzyme. Magnesium ions were necessary also for the crystallization.

Such properties of swine phosphopyruvate hydratase as Michaelis constants for substrates, activation by metal-ions, dependence on the kind of buffer and pH values, form of crystals and molecular weight, were similar to those of the enzyme from rabbit and human muscles.

The authors are indebted to Doc. Dr. A. Morawiecki for ultracentrifugation of enzyme.

REFERENCES

Andrews P. (1964). *Biochem. J.* **91**, 222.
Baranowski T., Wolna E. & Morawiecki A. (1968). *Eur. J. Biochem.* **5**, 119.
Beisenherz G., Boltze H. J., Bücher Th., Czok R., Garbade K. H., Meyer-Arendt E. & Pfleiderer G. (1953). *Z. Naturforsch.* **8**, 555.
Boser H. (1959). *Z. physiol. Chem.* **315**, 163.
Czok R. & Bücher Th. (1960). *Adv. Protein Chem.* **15**, 373.
Elödi P. & Szörenyi E. (1956). *Acta physiol. Acad. Sci. Hungar.* **9**, 339.
Federtchenko O. (1958). *Ukr. Biochim. Zh.* **30**, 552.
Holt A. & Wold F. (1961). *J. biol. Chem.* **236**, 3227.
Lohmann K. & Meyerhof O. (1934). *Biochem. Z.* **273**, 60.
Marek M. (1969). *Experientia* **25**, 136.
Meyerhof O. & Kiessling W. (1957). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 211. Academic Press, New York & London.
Rosenfeld A. (1963). *Biochim. biophys. Acta* **75**, 241.
Saito N. (1967). *J. Biochem., Tokyo* **61**, 59.
Warburg O. & Christian W. (1941). *Biochem. Z.* **310**, 384.
Wood T. (1964). *Biochem. J.* **91**, 453.

WŁASNOŚCI KRYSTALICZNEJ HYDRATAZY FOSFOPIROGRONIANOWEJ Z MIĘŚNI WIEPRZOWYCH

Streszczenie

1. Oczyszczono hydratazę fosfopirogronianową z mięśni wieprzowych stosując frakcjonowanie siarczanem amonu, kontrolowaną denaturację cieplną, chromatografię na żelu Sephadex G-50 i G-100 i krystalizację.

2. Enzym jest aktywny tylko w obecności jonów metali dwuwartościowych. Jony magnezu aktywują najsilniej.

3. Ciężar cząsteczkowy badanego enzymu wyznaczono na 90 000. Stała Michaelisa dla 2-fosfoglicerynianu wynosiła 0.33 mM, dla fosfoenolopirogronianu 0.9 mM.

4. Optimum pH w buforze imidazolowym i fosforanowym określono na 6.8.

Received 11 September, 1970.

A C T A B I O C H I M I C A P O L O N I C A
Vol. 18 1971 No. 1

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DEVELOPMENTAL CHANGES IN THE ACTIVITY OF GALACTOSE-1-PHOSPHATE URIDYLYLTRANSFERASE IN THE RAT

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Developmental patterns of galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) activity in liver, kidney, brain and blood are presented. Appreciable activity was found in liver, kidney and brain of young foetuses, and a distinct increase was observed in liver about 10 days after birth. In blood, the enzyme activity was unexpectedly high in foetuses and within the first days after birth, and then decreased with the age of animals. In kidney and brain the enzyme activity was practically unchanged in the course of pre- and postnatal growth.

Galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) is one of the enzymes responsible for the utilization of galactose. It catalyses the reversible reaction:

$$\alpha\text{-D-Galactose-1-P} + \text{UDPglucose} \rightleftharpoons \text{UDPgalactose} + \alpha\text{-D-glucose-1-P}.$$

In human tissues, in the pathological condition known as galactosemia, the lack of this enzyme leads to severe pathological changes (Kalckar, Anderson & Isselbacher, 1956), the biochemical mechanism of which is so far not known in detail. Since the importance of this enzyme became clear, attempts were made to find out whether any special variations in its activity take place in the course of ontogenesis. Changes in the activity due to the inductive action of galactose in the diet have also been demonstrated (Stifel, Herman & Rosensweig, 1968).

Age-dependent variations in the enzyme activity in rat liver have been studied by Isselbacher (1957) and Bertoli & Segal (1966) but their studies gave quite contradictory results. Isselbacher, who used the UDPglucose consumption test, found higher amounts of the enzyme in adult liver than in the newborn one, whereas Bertoli & Segal, who employed the assay with ^{14}C-labelled galactose-1-P, found higher activity in the liver of newborn animals.

In view of these discrepancies it was thought advantageous to reexamine the developmental changes in the activity of galactose-1-phosphate uridylyltransferase in various rat tissues, using a larger group of animals and the assay with [^{32}P]galactose-1-phosphate.

EXPERIMENTAL

Reagents. [^{32}P]α-D-Galactose-1-P was prepared as described previously (Sawicka & Chojnacki, 1969). The specific activity of the preparation was 5×10^5 - $2\times$ $\times10^6$ counts/min/μmol. UDPglucose was from Sigma Chemical Comp. (St. Louis, Mo., U.S.A.).

Animals. Rats of the Wistar strain, males and females, were bred in the local animal house on a standard LSM diet (Bacutil, Poland) and water *ad libitum*. The age of foetuses was estimated from the day of mating. The number of animals in a given age group studied and their weight are presented in Table 1. With the exception of the group of 7-day-old foetuses which were taken from one female, the animals in each group were from at least two litters.

Tissue preparations. The animals were killed by decapitation, the liver, kidney and brain were taken, and weighed samples of the tissues were homogenized for 2 min in 10 - 50-fold volumes of cold 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5, using a "Struers" MSE homogenizer. The homogenates were centrifuged at 4°C at 16 000 *g* for 20 min in a Sorvall centrifuge, and the clear supernatants were used for enzymic assays and protein determination.

Samples of blood from cervical vessels were collected into heparinized tubes. Haematocrit was estimated using an International Microcapillary Centrifuge MB and Microcapillary Reader. The enzymic assays were performed on haemolysates of the whole blood prepared by adding 9 vol. of water to the blood.

Enzyme assays. The activity of galactose-1-phosphate uridylyltransferase was assayed as described previously (Sawicka & Chojnacki, 1969). In the above tests the [^{32}P]UDPgalactose formed during the incubation of [^{32}P]galactose-1-P and

Table 1

Age and number of rats studied for galactose-1-phosphate uridylyltransferase activity

Animals			Number of individual experiments			
Age (days)	Number	Weight (g)	Liver	Kidney	Brain	Blood
Foetuses						
7	10	0.86	2*	—	2*	—
14	15	4.4 - 5.1	6*	6*	6*	6
After birth						
1	6	5.5 - 7.0	6	6	6	4
5	6	7.9 - 10.8	6	6	6	6
10	6	13.6 - 18.2	6	6	6	6
14	6	23.0 - 28.5	6	6	6	1
21	6	45.5 - 51.0	6	6	6	4
28	6	35.4 - 65.5	6	6	6	6
35	6	58.0 - 85.5	6	6	6	5
45	6	79.0 - 135.0	6	6	6	5
Adults	6	-	6	6	6	5

* Samples of studied tissues were pooled from 3 - 5 animals before homogenization.

UDPglucose with enzyme preparation is adsorbed on active charcoal and its radioactivity is estimated using a G-M tube and conventional ancillary equipment. For the incubation, tissue extracts from liver, kidney and brain were used in a volume of 100 µl of appropriately diluted 15 000 *g* supernatant corresponding to 0.1, 0.5 and 10 mg of fresh tissue, respectively. For blood estimation, 50 µl of 10% haemolysate was used. These amounts of the preparations were chosen for the sake of convenience, as in the quantitative assay involving 15 min of incubation they represented a similar magnitude of enzymic activity. The assay was performed in duplicate within 2 - 3 h after killing of the animal.

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

RESULTS

The activities of galactose-1-phosphate uridylyltransferase in extracts from liver, kidney and brain of rats of different age, calculated per 1 mg of extract protein, are presented in Fig. 1, in which the activity in the blood per 1 ml of packed blood cells is also included. Age-dependent changes in the enzyme activity were much greater in liver than either in kidney or in brain. In liver, appreciable activity was found in 7-day-old foetuses; it increased with age, and reached the maximum ten days after birth; then the activity decreased rapidly to a plateau, on the 21st day, which was on the level of the values observed for 7-day-old foetuses.

In kidney, the activity was practically unchanged over the whole period of development from young foetus to the adult animal.

In brain extract, the activity was very low, amounting to about 1/10 of the activity in liver of young foetus and adult rat, and was virtually unchanged over the whole period studied.

When the activity was expressed in units per 1 mg of fresh tissue, in liver, kidney and brain a similar pattern of age-dependent changes could be observed.

The enzymic activity in the blood was the highest in foetuses and till the fifth day after birth, then rapidly decreased; the lowest values were found in 45-day-old and adult rats.

DISCUSSION

The results presented in this paper are similar to the data obtained by Bertoli & Segal (1966). The developmental changes in galactose-1-phosphate uridylyltransferase activity in liver exhibited a similar pattern, the highest activity being found ten days after birth of the animals. Also the almost unchanged activity in kidney during development from foetus to the adult rat, was similar. However, it is not possible to compare directly the absolute values of enzyme activities in rat tissues obtained in the present work, with the results of Bertoli & Segal (1966) due to differences both in the preparation of material and assay conditions. Moreover, in the present work, the sex-dependent differences in enzymic activity were not taken into account as it was not possible to distinguish the sex in very young foetuses. As demonstrated by Bertoli & Segal (1966), the enzyme activity in males

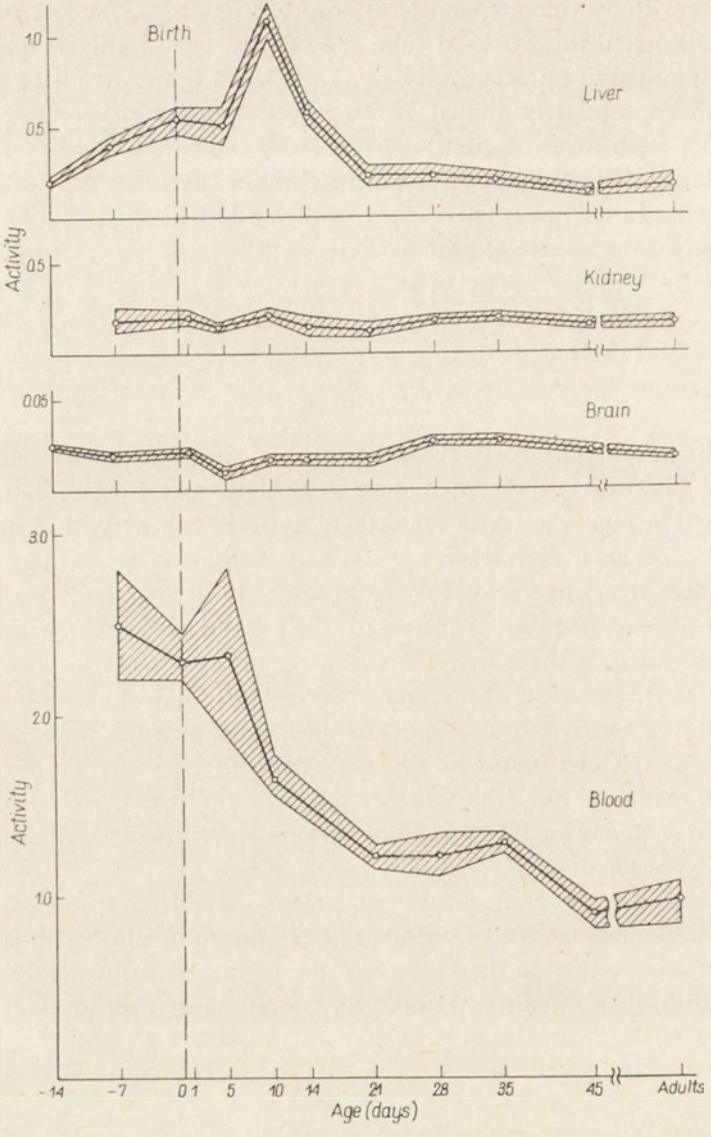

Fig. 1. Developmental changes in the activity of galactose-1-phosphate uridylyltransferase in rat liver, kidney, brain and blood. Each point represents the average values from 2 - 6 determinations (see Table 1). Standard errors of the mean are marked as shadowed areas. The enzymic activity in liver, kidney and brain is expressed in μmols of UDPgalactose synthesized per 1 mg of protein per hour, and in the blood in μmols of UDPgalactose synthesized per 1 ml of packed erythrocytes per hour.

was only 20% higher than in females. In our experiments the values of standard error of the mean were ±10 - 20%, which might have been due to these differences, but this does not seem to have obscured the pattern of age-dependent changes in enzyme activity.

The steady increase in enzyme activity in foetus liver appears to be due to maturation of the tissue, whereas the increase observed in 10-day-old animals could have been caused by adaptive changes in the liver due to the intake of lactose with milk. It should be noted that an increase in enzyme activity in gut following feeding a galactose-rich diet was demonstrated in rats by Stifel *et al.* (1968).

In the course of maturation of brain and kidney only slight fluctuations in enzyme activity were observed.

The unexpectedly high activity observed in the blood of foetuses and within the first few days after birth is probably due to the presence of young erythrocytes whereas the subsequent rapid decrease in activity may be related to the maturation of red blood cells. This finding clearly indicates that the determinations of galactose-1-P uridylyltransferase in whole blood do not reflect the changes in enzyme activity occurring in liver, which are both physiologically and quantitatively more important. It is evident that by estimating the enzymic activity in the blood, especially in early childhood, no insight may be gained into the capacity of the organism to metabolize galactose.

The expert technical assistance of Mrs. Barbara Łyczakowska is acknowledged.

REFERENCES

Bertoli D. & Segal S. (1966). *J. biol. Chem.* **241**, 4023.
Isselbacher K. J. (1957). *Science* **126**, 652.
Kalckar H. M., Anderson E. P. & Isselbacher K. J. (1956). *Biochim. biophys. Acta* **20**, 262.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.
Sawicka T. & Chojnacki T. (1969). *Clin. chim. Acta* **23**, 463.
Stifel F. B., Herman R. H. & Rosensweig N. S. (1968). *Science* **162**, 692.

ZMIANY ROZWOJOWE AKTYWNOŚCI URYDYLILOTRANSFERAZY GALAKTOZO-1-FOSFORANOWEJ U SZCZURA

Streszczenie

Zbadano zmiany aktywności urydylilotransferazy galaktozo-1-fosforanowej (EC 2.7.7.12) w tkankach szczura w rozwoju. Aktywność enzymu obserwuje się w wątrobie, nerce i mózgu już u wczesnych płodów. Aktywność enzymu w wątrobie wykazuje znaczny, krótkotrwały wzrost około 10 dnia po urodzeniu. Stwierdzono nadspodziewanie wysoką zawartość enzymu we krwi płodów oraz u zwierząt w ciągu kilku pierwszych dni po urodzeniu. W nerce i mózgu aktywność enzymu praktycznie nie ulega zmianom w czasie rozwoju płodowego i pozapłodowego.

Received 7 October, 1970.

Note added in proof: More accurate estimates of the age of foetuses may be obtained from their actual weight (cf. C.J. Goodner, M.J. Conway & J.H. Werbach, *Pediat. Res.* **3**, 121,1969).

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. I

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8. Nomenklatura, jednostki miar i ogólnie przyjęte skróty powinny być zgodne z tymi, którymi posługuje się Biochemical Journal. Również i inne liczne wskazówki zawarte w ,,Suggestions and instructions to authors" ogłaszanych w styczniowych numerach Biochemical Journal, mogą i powinny być wykorzystane przez autorów przesyłających swe prace do Acta Biochimica Polonica. Nomenklaturę enzymów i koenzymów należy stosować zgodnie z zaleceniami Enzymatycznej Komisji Międzynarodowej Unii Biochemicznej. Szczegóły tych zaleceń oraz ich uzasadnienie znajdują się w książce: Enzyme Nomenclature (Elsevier, Amsterdam 1965). Nowo-użyte w pracy skróty należy wyjaśnić. W tytule pracy i w streszczeniu należy stosować pełne nazwy a nie skróty.

9. Forma zewnętrzna maszynopisu. Prace należy przesyłać do redakcji w dwóch egzemplarzach pisanych na maszynie jednostronnie, z marginesem szerokości około 4 cm po lewej stronie i około 1 cm po prawej stronie, z podwójną interlinią oraz z numeracją stron. Pierwszy egzemplarz należy pisać na papierze białym. Jako nawiasu zwykłego należy używać znaków ( ) a nie / /. Na podobnych kartkach poza tekstem pracy należy umieścić: a) cytowaną literaturę, b) tablice, c) spis podpisów pod rysunkami, d) rysunki lub fotografie również w dwóch egzemplarzach, e) pełne imię i nazwisko autora(ów) oraz dokładny adres oraz skróconą wersję tytułu nie przekraczającą 50 miejsc literowych (,,żywą paginę"). W tekście należy zaznaczyć ołówkiem na marginesie w przybliżeniu miejsca, w których powinny być umieszczone tablice i rysunki. Prace wymagające wprowadzenia większych poprawek zostaną przepisane na koszt autora.

10. Rysunki i fotografie należy wykonać w postaci nadającej się do reprodukcji lub przerysowania; ich wymiary nie powinny przekraczać 15×20 cm. Na odwrocie każdego rysunku lub fotografii należy podać ołówkiem: nazwisko autora, pierwsze słowa tytułu pracy i kolejny numer rysunku. Należy unikać podawania na rysunkach objaśnień tekstowych. Na fotografiach nie należy dopisywać żadnych oznaczeń; jeśli są one konieczne, należy umieścić je na przypiętej do fotografii kalce technicznej. Nadmierna ilość rysunków może być wykonana wyłącznie na koszt autora.

11. Dla dokonania zmian uważanych przez Redakcję za celowe odsyła się autorowi jeden egzemplarz pracy. Po ostatecznym uzgodnieniu tekstu maszynopisu autor nie powinien wprowadzać żadnych zmian w korekcie (poza poprawieniem błędów drukarskich).

12. Autora obowiązuje jedna korekta autorska, którą należy zwracać Redakcji jak najszybciej, najdalej w ciągu trzech dni. Zaleca się wykonanie korekty ołówkiem kolorowym, barwy odmiennej od ołówka korektora. W razie nieobecności autor powinien wskazać osobę upoważnioną do przeprowadzenia korekty.

13. Autor otrzymuje bezpłatnie 25 egzemplarzy odbitek pracy. Żądanie większej ilości odbitek winno być wyrażone na piśmie jednocześnie ze złożeniem pracy w Redakcji. Koszt dodatkowych egzemplarzy ponosi autor. Odbitek recenzji książek redakcja nie wysyła.

14. W razie nie przyjęcia pracy do druku jeden jej egzemplarz pozostaje w aktach Redakcji.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 1

RECENZJE KSIĄŻEK

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS. Vol. X, part 4 (R. T. Holman, ed.) Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1969; str. 327 - 431, cena £4.75.

Kolejny tomik tego wydawnictwa zawiera trzy monografie. Zakres ich wybiega znacznie poza dziedzinę chemii lipidów. D. C. Malins i J. C. Wekell w artykule p.t. „Biochemia lipidów morskich organizmów" dają przegląd wszystkich klas lipidów występujących w tych obiektach oraz podają wiele ciekawych informacji o występowaniu i biosyntezie pewnych szczególnych związków lipidowych, występujących wyłącznie u ryb głębinowych. Informacje dotyczące różnic w składzie lipidowym omawianych obiektów posiadają głęboką podbudowę w rozważaniach autorów o ekologii, fizjologii i ewolucji tych organizmów.

Dwa następne artykuły to: „Glikosfingolipidy tkanek zwierzęcych" (E. Martensson) i „O cerebrozydach i gangliozydach" (E. Klenk). W wielu wypadkach pierwszy jest koreferatem drugiego lub odwrotnie. Autorzy zebrali i zawarli w swych artykułach wiele nowych danych dotyczących roli glikolipidów, ich biosyntezy i immunochemii oraz zaburzeń ich metabolizmu. Oba artykuły zawierają także cenne dane metodyczne dotyczące izolacji i oczyszczania tych związków. Niestety, redakcja nie uchroniła czytelnika przed rozgardiaszem nomenklaturowym. Dopuszczenie w dwu podobnych sąsiadujących monografiach dwu odmiennych systemów nomenklatury glikolipidów znacznie utrudnia lekturę.

Oba artykuły są, jak się wydaje, niepotrzebnie ukierunkowane wyłącznie na dziedzinę związków lipidowych; zrezygnowano w nich, niestety, z danych o glikoproteidowych substancjach zawierających te same sekwencje cukrowcowe. Mimo tych braków oba omawiane artykuły stanowią bardzo wyczerpujące opracowania tematu. Szczególnie monografia Martenssena wyróżnia się obszernością i przejrzystością.

Monografie zamieszczone w recenzowanym tomiku wykraczają swą tematyką poza chemię lipidów i winny znaleźć adresatów również wśród specjalistów z dziedziny ekologii, fizjologii, immunochemii i neurochemii.

Tadeusz Chojnacki

L. Szepesy, GAS CHROMATOGRAPHY. Akadémiai Kiadó, Budapest 1970. (Tłumaczenie z węgierskiego na język angielski przez E. D. Morgana) str. 384.

Technika chromatografii gazowej, której zasady zostały po raz pierwszy podane przez A. T. Jamesa i A. J. Martina w 1952 r. (Biochem. J. 50, 679, 1952), znalazła od kilkunastu lat szerokie zastosowanie w badaniach naukowych i w analizie przemysłowej zarówno do celów analitycznych, jak i preparatywnych. Technika ta charakteryzuje się wysoką czułością i krótkim czasem przebiegu analizy, przy czym do badania potrzeba zaledwie mikrogramowych ilości próbki; przeto w okresie rozwoju chromatografii gazowej ukazała się wielka liczba publikacji traktujących o jej zastosowaniu do badań analitycznych, fizykochemicznych, strukturalnych, do badania kinetyki i innych. Pełna automatyzacja urządzeń do chromatografii gazowej otworzyła dalsze możliwości stosowania tej metody;

Monografia Szepesy'ego w sposób syntetyczny i przystępny przedstawia podstawy teoretyczne metody, stosowaną aparaturę (uwzględniając najrozmaitsze techniki specjalne, jak np. programowanie zmiany temperatury podczas rozdziału czy też zmiany szybkości przepływu gazu nośnikowego), jak również zastosowanie chromatografii gazowej do rozwiązywania specjalnych problemów w badaniach naukowych i przemyśle.

Całość materiału książki podzielona jest na 11 rozdziałów, przy czym podstawy teoretyczne oraz zagadnienia techniczne zajmują ok. 2/3 jej objętości. Dla biochemików, farmaceutów i analityków szczególną wartość ma rozdział VIII, w którym autor podaje zestawienie piśmiennictwa źródłowego (1140 pozycji) dotyczącego analizy różnych związków organicznych, aminokwasów, cukrowców, związków siarkowych, halogenkowych i metaloorganicznych. Szkoda tylko, że w tym rozdziale brak omówienia krytycznego zastosowania chromatografii gazowej przy badaniu wspomnianych grup substancji chemicznych. Tym niemniej książka Szepesy'ego jest bardzo cennym wydawnictwem umożliwiającym zapoznanie się z podstawami tej najnowszej techniki analizy chemicznej o dużych możliwościach zastosowania w laboratoriach o różnym profilu badań.

Włodzimierz Ostrowski

RADIATION PROTECTION AND SENSITIZATION. Proceedings of the Second International Symposium on Radiosensitizing and Radioprotective Drugs, Istituto Superiore di Sanitá, Rome, 6 - 8 May 1969 (H. Moroson & M. Quintiliani, eds.) Taylor & Francis Ltd., London, 1970; str. 524+xvi, cena £8.00.

Wśród wielu zagadnień radiobiologicznych duże znaczenie praktyczne jak i teoretyczne zyskał w ciągu ostatnich kilkunastu lat problem substancji uwrażliwiających na promieniowanie i substancji chroniących przed szkodliwymi skutkami promieniowania. Temu problemowi poświęcona jest omawiana książka zawierająca referaty i doniesienia wygłoszone w czasie sympozjum, które odbyło się w Rzymie w maju 1969 r.

W pięciu artykułach przeglądowych i w sześćdziesięciu ośmiu oryginalnych pracach doświadczalnych zawarta jest bardzo duża ilość informacji o szerokim zakresie tematycznym: od teoretycznych rozważań na temat molekularnych mechanizmów uwrażliwiania na promieniowanie i ochronnego działania różnych środków, poprzez badania nad „reperacją" DNA pod wpływem substancji uwrażliwiających i ochronnych, aż do badań klinicznych nad zastosowaniem tychże substancji w radioterapii. Zamieszczone w książce prace doświadczalne są zgrupowane w pięciu następujących rozdziałach: 2. Procesy molekularne, 3. Ochrona i uwrażliwianie w pojedynczych komórkach, 4. Ochrona i uwrażliwianie w układach wielokomórkowych, 5. Biochemia i farmakologia związków chroniących i uwrażliwiających, oraz 6. Studia kliniczne.

Studiowanie omawianej książki pozwala zorientować się, że coraz większa liczba znanych związków uwrażliwiających na promieniowanie i chroniących przed szkodliwymi skutkami promieniowania to nie tylko ważny problem kliniczny (i ewentualnie wojskowy), ale także zagadnienie, które pozwala coraz głębiej i szerzej wniknąć w poznanie złożonego problemu reagowania żywej materii na promieniowanie, którego intensywność wciąż się zwiększa w otaczającym nas świecie.

Książka niewątpliwie zainteresuje nie tylko wszystkich zajmujących się radiobiologią, ale także liczną rzeszę biochemików i farmakologów zaangażowanych w badania nad wpływem środowiska na żywe ustroje.

Mariusz Żydowo

SCHERING SYMPOSIUM ON BIODYNAMICS AND MECHANISM OF ACTION OF STEROID HORMONES. Advances in Biosciences 2 (G. Raspé, ed.) Pergamon Press - Vieweg, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1969; str. 353, cena $10.

Kolejny tom tej serii wydawniczej stanowi zbiór referatów sympozjalnych przedstawionych przez wybitnych specjalistów z wielu aktualnie przodujących ośrodków badawczych na świecie. W piętnastu zamieszczonych referatach, opartych na dużym dorobku własnym autorów, oprócz

wielu nowych faktów doświadczalnych znajdujemy oryginalną interpretację wyników uwzględniającą biodynamikę procesów metabolicznych oraz mechanizm działania hormonów sterydowych na poziomie molekularnym. Obszerne sprawozdanie z wnikliwej, o charakterze roboczym, dyskusji po każdym referacie pozwala czytelnikowi prześledzić drogę prowadzącą do uzyskania wyników, jak i snuć przypuszczenia o dalszych postępach pracy doświadczalnej.

Tematy prac umieszczone w tej książce dają się zgrupować w następujące zagadnienia: 1. Automatyzacja analizy sterydów i jej zastosowanie do badań metabolitów, płynów ustrojowych i preparatów farmaceutycznych; 2. Regulacja metabolizmu w różnych tkankach; 3. Metabolizm estrogenów z uwzględnieniem molekularnych podstaw ich wiązania w komórce; 4. Biochemiczny mechanizm działania androgenów i antagonistów hormonów sterydowych.

Zagadnienia poruszone w części referatowej omawianej książki są uzupełnione materiałami z sesji dyskusyjnej toczącej się nad klinicznymi aspektami mechanizmu działania estrogenów, progestogenów, androgenów i kortykosterydów. Duży walor materiałów z tej dyskusji upatruje oceniający w tym, że pozwalają one zorientować się zarówno w aktualnej problematyce badawczej wielu badaczy zajmujących się mechanizmem działania hormonów sterydowych, jak i w trudnościach metodycznych i interpretacyjnych.

Książka ta jest cenną pozycją nie tylko dla wszystkich pracujących doświadczalnie w dziedzinie biochemii sterydów, lecz także zainteresuje biologów, fizjologów i lekarzy pracujących w klinice.

Leon Żelewski

SCHERING WORKSHOP ON STEROID METABOLISM "*IN VITRO VERSUS IN VIVO*". Advances in Biosciences 3 (G. Raspé, ed.) Pergamon Press - Vieweg, Oxford, London, Edinburgh New York, Sydney, Paris, Braunschweig, 1969; str. 217, cena $12.

Trzeci tom tej oryginalnej serii wydawniczej poświęcony jest referatom dyskusyjnym kolejnego sympozjum zorganizowanego przez A. G. Schering.

Dla wszystkich pracujących doświadczalnie w biochemii sterydów jest oczywiste, że wyników uzyskanych *in vitro* nie można przenieść bez wielu istotnych zastrzeżeń na cały organizm. Zdanie sobie sprawy z truizmu tego sformułowania w oparciu o wyniki doświadczalne zostało docenione przez organizatorów podejmujących temat sympozjalny o metabolizmie sterydów w układach izolowanych enzymów, struktur subkomórkowych, tkankach i narządach w odniesieniu do wyników uzyskanych na całym organizmie.

Przedstawione w takim ujęciu prace doświadczalne dotyczą następujących zagadnień: 1. Aktywność 11-β-hydroksysterydowej dehydrogenazy w poszczególnych tkankach a jej funkcja metaboliczna w całym organizmie; 2. Metabolizm estrogenów w organizmie człowieka i zwierząt doświadczalnych w odniesieniu do wyników uzyskanych w perfundowanym zespole łożyskowo-płodowym, izolowanych tkankach lub preparatach frakcji subkomórkowych; 3. Metabolizm hormonów sterydowych w tkance jajnikowej z uwzględnieniem efektów metabolicznych inhibitorów biosyntezy sterydów; 4. Metabolizm androgenów w hodowli tkankowej, perfundowanym narządzie oraz całym organizmie.

Studiowanie poszczególnych prac doświadczalnych, z dwudziestu przedstawionych na tym sympozjum, pozwala zdać sobie sprawę z tego, że doświadczenia przeprowadzone *in vitro* stanowią uzupełnienie wyników doświadczeń *in vivo*, a nie powinny pretendować do stworzenia całościowego ujęcia metabolizmu i mechanizmu działania hormonu sterydowego w organizmie. Wyniki uzyskane na uproszczonych modelach biologicznych, jakimi są perfundowany narząd, tkanka lub preparat subkomórkowy, można ogólnie scharakteryzować jako wiedzę o możliwościach i kierunkach metabolicznych istniejących potencjalnie w danej tkance. Warunki stworzone arbitralnie w doświadczeniach *in vitro* przez zniszczenie struktury komórkowej i subkomórkowej przy równocześnie zaburzonej współzależności regulacyjnej nie pozwalają wyciągać daleko idących

wniosków wyjaśniających funkcję określonej reakcji lub układu metabolicznego w całym organizmie. Błędem byłoby jednak sądzić, że autorzy referowanych na tym sympozjum prac nie docenili dużej wartości wyników uzyskanych w prawidłowo zaplanowanych i wykonanych oraz ostrożnie zinterpretowanych doświadczeniach *in vitro*. W wielu przedstawionych nowych faktach doświadczalnych stwierdzanych *in vitro* można znaleźć klucz do rozwiązania podstawowych zagadnień metabolicznych i regulacyjnych zachodzących w całym ustroju.

Kolejny, trzeci, tom z serii Advances in Biosciences jest pożyteczną pozycją dla biochemików pracujących doświadczalnie nad metabolizmem hormonów sterydowych, zainteresuje on również biologów, fizjologów oraz klinicystów.

Leon Żelewski