POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

Vol. 20 No. 2

QUARTERLY

ABPLAF 20(2) 113–206 (1973)

WARSZAWA 1973

POLISH SCIENTIFIC PUBLISHERS

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

Vol. 20 No. 2

CONTENTS

QUARTERLY

POLISH SCIENTIFIC PUBLISHERS
WARSZAWA 1973

ADDRESS

ul. Banacha 1, 02-097 Warszawa
(Zakład Biochemii A.M.)
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 1217+203 egz. Ark. wyd. 8,25 ark. druk. 6,5+0,25
Papier druk. sat. kl. III, 80 g. 70×100
Oddano do składania 13.II.73 r. Podpisano do druku 19.IV.73 r.
Druk ukończono w kwietniu 1973
Zam. 125 R-30 Cena zł 25.—

Warszawska Drukarnia Naukowa, Warszawa, Śniadeckich 8

ACTA BIOCHIMICA POLONICA

Vol. 20 1973 No. 2

URSZULA KOCEMBA-ŚLIWOWSKA and J. KAWIAK

LIBERATION OF CELL SURFACE MATERIAL BY THE INSOLUBLE TRYPSIN DERIVATIVE

Laboratory of Cytophysiology, Department of Biophysics, Postgraduate School of Medicine, ul. Marymoncka 99, 01-813 *Warszawa, Poland*

The column of insoluble derivative of trypsin and maleic acid-ethylene copolymer was used to liberate surface material from Ehrlich ascites tumour cells. Protein, hexosamine, fucose and sialic acid were released by passage through the column. The cell-free supernatant of the effluent contained diffusible peptides as well as hexosamine, fucose and sialic acid bound to non-diffusible substances. Molar ratios of hexosamine: fucose:sialic acid were about 4:2:1.

The fate of a tissue graft depends on the major histocompatibility antigens present on the surface of donor cells. A more precise study of the cell membrane antigens required a search for effective methods to solubilize them. Soluble material containing histocompatibility antigens was extracted with detergents from sarcoma cells (Hilgert *et al.*, 1969) and with chelating agents from mouse embryo tissue (Edidin, 1966); attempts were also made to utilize autolysis and the action of proteases on the isolated membrane fractions from ascites cells BP 8 or spleen cells of mouse (Nathenson & Davies, 1966; Shimada & Nathenson, 1969).

Since the material solubilized from cell membrane prepared by fractionation of homogenate may contain in addition to surface antigens intracellular components (Finean *et al.*, 1966), it seemed useful to design a method avoiding the disruption of cells. For this purpose, the action of the insoluble derivative of trypsin (IDT[1]) on the intact cell surface was investigated.

MATERIALS AND METHODS

Biological material. Ehrlich ascites tumour cells E_4 (Breguła, 1968) and mice of the A/KT strain were obtained through courtesy of Dr. Alina Czarnomska of the Institute of Oncology, Warsaw. Ascites cells were transplanted to 96 mice,

[1] Abbreviations used: IDT, the insoluble derivative of trypsin; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; IDT-TLCK, IDT inactivated by treatment with TLCK; TAME, *p*-toluenesulphonyl-L-arginine methyl ester.

males aged about 5 months. On the 7th day after inoculation the cells were collected by centrifugation from the peritoneal ascites fluid. On average, from one mouse 245×10^6 cells were obtained. The cells were washed three times with 2 ml of a saline solution containing in 100 ml 0.8 g of NaCl, 0.05 g of KCl, and 1 ml of 1.15 M-Na-phosphate buffer, pH 7.0, and finally were suspended in this solution (about 82×10^6 cells/ml).

Insoluble trypsin derivative. The water-insoluble derivative of trypsin (IDT) was prepared according to Levin *et al.* (1964) by coupling the enzyme to maleic acid anhydride-ethylene copolymer in 0.2 M-sodium phosphate buffer, pH 7.5, at 4°C. Sodium phosphate buffer was used, as in this medium larger particles of IDT are formed than in potassium phosphate buffer. The enzyme concentration in reacting mixture was 170 mg/ml and copolymer to enzyme weight ratio was 1:4. The IDT particles were collected on 45×45 μm nylon mesh and washed until trypsin activity disappeared in the effluent. The IDT preparation retained 12% of the free trypsin proteolytic activity tested with casein (Northrop *et al.*, 1948) and 70% of the esterolytic activity tested with TAME (Hummel, 1959). The trypsin was stably bound in the IDT and it was not released on storage between the experiments.

Inactive IDT was prepared by passing through the IDT column 1 mg of TLCK, the low-molecular-weight trypsin inhibitor (Shaw *et al.*, 1965); the inhibition of the proteolytic activity of IDT was complete and irreversible; no chymotrypsin proteolytic activity was observed.

Column filtration of cells. For the experiment, two columns (7×5 mm) were prepared; one of them consisted of IDT containing 2.5 mg of bound trypsin, and the second of the same amount of IDT inactivated by passing TLCK. From the freshly prepared ascites cells suspension, 5 ml was left standing, and a 5 ml portion was applied to either column; the flow rate was 0.25 ml/min. The cells in the effluent were counted, and their viability was evaluated by the trypan blue test (Kawiak *et al.*, 1965). The column was washed with 5 ml of saline solution used for suspension of cells. The effluent and washing were combined, and the cells were centrifuged off at 800 *g* for 10 min. One part of the supernatant was assayed directly and another was dialysed against three changes of 500 ml of water for 48 h at 4°C.

Analytical methods. Protein was determined according to Lowry *et al.* (1951), uronic acid after Bitter & Muir (1962), sialic acid by the thiobarbiturate method (Spiro, 1966), hexosamine by the method of Elson-Morgan as modified by Boas (1953), fucose after Dishe & Shettles (1948).

Spot tests. Samples were applied on glass-fibre paper and dried. To detect protein, they were treated with 20% aqueous sulphosalicylic acid solution, stained with 1% Amido Black in 12.5% trichloroacetic acid for 1 h, and destained with 7% acetic acid. For carbohydrates the PAS reaction (Zacharius & Zell, 1969) was applied, and for lipids staining with oil red (Uriel *et al.*, 1957).

Reagents. Maleic acid anhydride and ethylene copolymer, molar ratio 1:1 (EMA-21) was from Monsanto Comp. (St. Louis, Mo., U.S.A.). It was activated

at 105°C under vacuum for 24 h before use. Trypsin, 2×crystallized and lyophilized preparation was from Serva Finebiochemicals (Heidelberg, West Germany). Casein was from British Drug Houses Ltd. (Poole, England) and TAME from Koch-Light Ltd. (Colnbrook, Bucks, England). Trypsin inhibitor TLCK was from Cyclo Chem. Inc. (Los Angeles, Calif., U.S.A.). The following substances were used as standards: human serum albumin (Instytut Surowic i Szczepionek, Warsaw, Poland), D-glucuronic acid (A. G. Fluka, Buchs, Switzerland), *N*-acetylneuraminic acid (Koch-Light), D-glucosamine hydrochloride (T. Schuchardt, Munich, West Germany), D-fucose (Biochemical Res., Calif., U.S.A.). Amido Black 10 B was from Koch-Light Ltd., oil red O from E. Gurr Ltd. (London, England), trypan blue from Chemapol (Prague, Czechoslovakia). All other chemicals used were of A. R. purity.

RESULTS

The washed ascites cells suspended in saline solution on standing at room temperature were found to release increasing amounts of protein; therefore filtration through IDT and IDT-TLCK columns was always performed simultaneously, the difference between the two columns being taken as the effect of IDT.

The ascites cells applied to the IDT and IDT-TLCK columns were recovered in effluents almost completely. The trypan blue test demonstrated that filtration had little effect on survival of the cells; after filtration through IDT and IDT-TLCK columns 8 and 9%, respectively, of the cells were dead, whereas in the non-filtered sample, 5%. The IDT-treated cells were, however, more fragile than the IDT-TLCK-treated ones when histological fixation was done in unfavourable conditions (at lower pH).

Analysis of the effluent supernatants showed that passage through active trypsin derivative liberated more protein, hexosamine, fucose and sialic acid than passage through inactivated preparation of trypsin (Table 1). No uronic acid was found in either sample.

Table 1

Substances found in the cell-free supernatants from IDT and IDT-TLCK columns

A suspension of about 6.6×10^8 ascites tumour cells was filtered through active and inactive trypsin columns. Mean values from six experiments ± S.D. are given.

Preparation	Protein	Hexosamine	Fucose	Sialic acid
	µg/column			
Undialysed supernatant				
from IDT column	2977±111	122.8±4.0	48.1±2.2	47.4±3.3
from IDT-TLCK column	1180±45	53.9±3.2	26.4±1.7	26.0±2.0
Δ (effect of trypsin)	1797±120	68.9±5.1	21.7±2.8	21.4±3.9
Dialysed supernatant				
from IDT column	2441±57	112.0±7.1	37.7±2.2	37.2±3.6
from IDT-TLCK column	1072±44	49,0±3.0	16.5±1.0	18.7±1.7
Δ (effect of trypsin)	1369±72	63.0±7.7	21.2±2.4	18.5±4.0
Non-diffusible material	76%	91%	98%	86%

When supernatants from IDT and IDT-TLCK columns were dialysed against water, in both cases a rather abundant white sediment was formed. It was dissolved by adding the saline solution and analysed together with other non-diffusible substances. It was found that a significant amount, about 25%, of the peptide material obtained from the IDT column was diffusible (Table 1). On the other hand, most of hexosamine- and fucose-containing substances remained bound to the non-diffusible material. Molar ratios of hexosamine: fucose:sialic acid were about 4:2:1 in the supernatants from both columns (Table 2).

Table 2

Amount and molar ratios of hexosamine, fucose and sialic acid in cell-free supernatant from IDT and IDT-TLCK columns

Mean values from six experiments are given.

Preparation	Hexosamine	Fucose	Sialic acid	Molar ratios
	nmol/mg protein			
Undialysed				
from IDT column	229.2	98.5	51.3	4.5:1.9:1.0
from IDT-TLCK column	253.8	136.4	71.1	3.6:1.9:1.0
Dialysed				
from IDT column	254.9	94.2	49.2	5.2:1.9:1.0
from IDT-TLCK column	253.9	93.9	56.3	4.5:1.7:1.0

The spot test performed with the non-diffusible material showed that the precipitate formed during dialysis as well as supernatant gave positive reactions for protein, carbohydrates and lipids.

DISCUSSION

Since the use of the insoluble derivative of trypsin assures that the enzyme does not penetrate into the cell but acts on its surface, the obtained solutes may be considered as derived from the outer surface of the cells. In this respect the IDT-solubilized substances may be compared with the materials solubilized by other procedures applied to whole cells. Langley & Ambrose (1967) applied soluble trypsin to Ehrlich ascites-tumour cells. In the cell-free supernatant deproteinized with trichloroacetic acid and dialysed, a mucopeptide containing galactosamine and sialic acid was found. Trypsin liberated about 74% of the amount of sialic acid released with neuraminidase. Condington *et al.* (1972) obtained a glycoprotein fraction containing sialic acid, from mouse TA_3 mammary adenocarcinoma ascites cells by treatment with a soluble preparation of *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin. In successive 20 min treatments they obtained material containing similar amounts of protein but decreasing amounts of sialic acid and increasing of aminosugars. The purified mucoproteid from first treatment contained galactose, aminosugars and sialic acid in molar ratio about 4:3:1.

Baranowski & Lisowska (1963) treated isolated blood group substances M and N with trypsin and observed liberation of sugar-rich peptides from hydrophobic core. From this and other experiments Morawiecki (1964) suggested the model for arrangement of glycoproteins in the erythrocyte cell membrane. According to the model, hydrophilic parts of polypeptides with asymmetrically distributed carbohydrate chains protrude above the surface of the membrane. They would be liberated by trypsin action, while hydrophobic part of polypeptide chains remain submerged in the lipid bilayer of the cell membrane. Similar arrangement of glycoproteins could be expected in the cell membrane of Ehrlich ascites tumour cells, and probably also for fucose-containing (Buck *et al.*, 1970) histocompatibility antigens in the other cells.

This work was supported in part by a grant from the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences. The Authors thank the Monsanto Co. for generous gift of maleic acid-ethylene copolymer.

REFERENCES

Baranowski T. & Lisowska E. (1963). *Arch. Immunol. Ther. Exp.* **11**, 631 - 640.

Bitter T. & Muir H. M. (1962). *Anal. Biochem.* **4**, 330 - 334.

Boas N. F. (1953). *J. Biol. Chem.* **204**, 553 - 563.

Breguła U. (1968). *Ph. D. Thesis*, Inst. Immunologii i Terapii Dośw. PAN, Wrocław.

Buck C. A., Glick M. C. & Warren L. (1970). *Biochemistry* **9**, 4567 - 4576.

Condington J. F., Sanford B. H. & Jeanloz R. W. (1972). *Biochemistry* **11**, 2559 - 2564.

Dishe Z. & Shettles L. B. (1948). *J. Biol. Chem.* **175**, 595 - 603.

Edidin M. (1966). *J. Embryol. Exp. Morphol.* **16**, 519 - 530.

Finean J. B., Coleman R. & Green W. A. (1966). *Ann. N. Y. Acad. Sci.* **137**, 414 - 420.

Hilgert I., Kandutsch A. A., Cherry M. & Snell G. D. (1969). *Transplantation* **8**, 451 - 461.

Hummel B. C. W. (1959). *Can. J. Biochem. Physiol.* **37**, 1393 - 1399.

Kawiak J., Moskalewski S. & Darżynkiewicz Z. (1965). *Exp. Cell Res.* **39**, 59 - 68.

Langley O. K. & Ambrose E. J. (1967). *Biochem. J.* **102**, 367 - 372.

Levin Y., Pecht M., Goldstein L. & Katchalski E. (1964). *Biochemistry* **3**, 1905 - 1913.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265 - 275.

Morawiecki A. (1964). *Biochim. Biophys. Acta* **83**, 339 - 347.

Nathenson S. G. & Davies D. A. L. (1966). *Ann. N. Y. Acad. Sci.* **129**, 6 - 13.

Northrop J. H., Kunitz M. & Herriott R. M. (1948). *Crystalline Enzymes*, 2nd ed. p. 125 - 128. Columbia University Press, New York.

Shaw E., Mares-Guia M. & Cohen W. (1965). *Biochemistry* **4**, 2219 - 2224.

Shimada A. & Nathenson S. G. (1969). *Biochemistry* **8**, 4048 - 4062.

Spiro R. G. (1966). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 8, p. 3 - 26. Academic Press, New York, London.

Uriel J., Grabar P. & Wunderly C. (1957). *Clin. Chim. Acta* **2**, 35 - 42.

Zacharius R. M. & Zell T. (1969). *Anal. Biochem.* **30**, 148 - 152.

UWOLNIENIE MATERIAŁU Z POWIERZCHNI KOMÓREK NIEROZPUSZCZALNĄ POCHODNĄ TRYPSYNY

Streszczenie

Użyto kolumny z nierozpuszczalnej pochodnej trypsyny i kopolimeru kwasu maleinowego i etylenu dla uwolnienia materiału z powierzchni komórek raka wysiękowego Ehrlicha. W bezkomórkowym supernatancie pochodzącym z kolumny aktywnej stwierdzono około 2 razy więcej białka, heksozamin, fukozy i kwasów sialowych w porównaniu z kontrolą. Uwolniony materiał zawierał dializujące peptydy oraz heksozaminę i fukozę związane z substancjami niedializującymi. Stosunek molarny heksozamin:fukozy:kwasów sialowych wynosił około 4:2:1.

Received 15 May, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 20 1973 No. 2

J. SŁAWIŃSKI, BARBARA SZCZODROWSKA and MARIA WŁODARCZYK-GRAETZER

STUDIES ON THE CHEMILUMINESCENCE OF THE SYSTEM: PURPUROGALLINE - PEROXIDASE - HYDROGEN PEROXIDE

Laboratory of Physics, Department of Agriculture Mechanization, College of Agriculture, ul. Janosika 8, 71-424 *Szczecin, Poland*

1. Chemiluminescence appearing on oxidation of purpurogalline by horse-radish peroxidase and H_2O_2 in acetate buffer, pH 5.6, saturated with oxygen, was examined. 2. The kinetics of the luminescence and dissociation constants of the active complexes were determined. 3. The absorption, fluorescence and chemiluminescence spectra, quantum yield of chemiluminescence, and fluorescence of the oxidation products were studied. 4. The obtained data indicate that the chemiluminescence accompanies oxidative scission of six-membered purpurogallinquinone ring, with formation of carbonyl derivatives of α-tropolone in their singlet excited states.

In studies on the chemiluminescence accompanying peroxidative oxidation, pyrogallol was commonly used as substrate (Dure & Cormier, 1964; Nilsson, 1964; Klipson & Mamedow, 1966; Grabiec, 1968). The main product of enzymic oxidation of pyrogallol is 2,3,4,6′-tetrahydroxy-5(H)-cycloheptabenzene-5-one (purpurogalline, PPG[1]), which undergoes further oxidation with light emission (Nilsson, 1964; Sławiński, 1969; Włodarczyk-Graetzer *et al.*, 1970; Sławiński, 1971). However, very little is known about the mechanism of chemiluminescence in the reaction of PPG with peroxidase and H_2O_2.

PPG is a purple pigment appearing in galls, i.e. sites of abnormal cell proliferation on leaves or twigs of some plants. There is a structural relationship between PPG and colchicine which is a mitotic poison. It is likely that PPG and its glucosides participate in gall metabolism (Potesilowa *et al.*, 1969). PPG is composed of two condensed, resonating rings, benzene and tropolone (see Ia in Scheme 1). The question arises which of the rings is subject to processes resulting in the appearance of chemiluminescence. Are those processes truly enzymic in nature, or are they non-enzymic side reactions? In the present study the chemiluminescence kinetics and mechanisms operative in the chemiluminescent system composed of PPG - horse-radish peroxidase - H_2O_2 - Na-acetate buffer, pH 5.6, were studied.

[1] Abbreviations used: PPG, purpurogalline; HRP, horse-radish peroxidase.

MATERIALS AND METHODS

Reagents. Special reagents were from the following sources: Horse-radish peroxidase (HRP, EC 1.11.1.7), mol. wt. 40000 (Koch-Light. Lab., Colnbrook, Bucks., England); purpurogalline (Fluka AG, Buchs, Switzerland) additionally purified by recrystallization from methanol; trimethyl-PPG was prepared according to Barlttrop & Nicholson (1948). Other reagents were analytical grade products of Polish origin. Solvents before use were purified by distillation. Water twice-distilled with $KMnO_4$ in all-glass still was used throughout.

Tissue extracts. The plants and animal tissues were homogenized in acetate buffer, pH 4.7, at 20°C for 30 min according to Mikhlin & Bronowickaja (1949) and the extract used for experiments.

Light measurements. The intensity of chemiluminescence was measured as described previously (Sławińska & Sławiński, 1969). To eliminate the influence of changes in spectral distribution of emission excited on the total intensity of chemiluminescence, a blue filter with maximum transmission at 440 nm was used.

Spectrophotometric determinations were made in a Unicam SP 700 recording spectrophotometer (Cambridge, England).

Emission spectra of fluorescence and chemiluminescence were measured with a Zeiss monochromator (Jena, G.D.R.), EMI 6262B and RCA 1P28 photomultipliers (Electronic Ltd, Hayes, Middlesex, England) and recording Zeiss potentiometer G1-B1. Spectral band width at 460 nm were 2 nm and 10 nm for fluorescence and chemiluminescence spectra, respectively. The wavelength of exciting light was 360 nm.

Total quantum yield was measured by comparison with the low-level light standard (Lee & Seliger, 1965) according to the modified formula:

$$\eta_{CL\,(PPG)} = \frac{\Sigma I_{PPG}\, A_{PPG}\, c_L \eta_L \chi}{\Sigma I_L\, C_{PPG}}$$

where η_{PPG} and η_L are quantum yield of PPG and that of luminol, respectively; c_L and c_{PPG}, concentrations of luminol and PPG, respectively; A_{PPG}, cologarithm of the optical density of solution $I_0/I \simeq 1.23$; ΣI, light sum, i.e. area under the kinetic curve $I=f(t)$, equal to $\int_{t=0}^{\infty} I dt$; $\chi = 1.015$, correction factor taking into account difference between λ_{max}'s in chemiluminescence spectrum of PPG and that of luminol and spectral sensitivity of the used phototube.

Reaction conditions. The reaction was started by rapid injection of 2 ml of 625 μM-PPG in methanolic solution, with a glass syringe, to a mixture of 2 ml of 2.5 mM-H_2O_2 and 2 ml of 5.0 μM-peroxidase and 4 ml of 25 mM-Na-acetate buffer, pH 5.6, at 303°K. The solution was vigorously stirred by passing a stream of O_2 (CO_2-free). All determinations were made in triplicate.

Isolation of the oxidation products. As soon as the maximum intensity of chemiluminescence (I_{max}) was reached, the reaction was stopped by adding H_2SO_4. For chromatographic analysis, the products were extracted from the acidic solution

with ethyl ether, and the extract was evaporated to a small volume. The chromatography was performed on Whatman no. 1 paper with *n*-butanol - acetic acid - water (4:1:2.2, by vol.) using the ascending technique. For spectrofluorometric and spectrophotometric analysis, the acidic solution was evaporated in N_2 atmosphere under reduced pressure and then the total residue was extracted three times successively with: cyclohexane, benzene, ethyl ether, chloroform, ethanol, water and 0.1 M-NaOH.

The values of electron densities and bond-orders were calculated using the Hückel's method.

RESULTS

Characteristics of the light-producing reaction. The general properties of the peroxidase-catalysed oxidation of PPG and accompanying chemiluminescence were presented in the previous paper (Włodarczyk-Graetzer *et al.*, 1970). In the temperature range 288 - 298°K, the temperature coefficient, Q_{10}, of the light-producing reaction rate was found to be 1.12 ± 0.19, and activation energy (E_{act}) 10.5 ± 1.8 kJ per mol. The maximum value of chemiluminescence intensity was observed when the substrate was added to the preformed active HRP-H_2O_2 complex.

In the course of oxidation blue-violet intermediates were formed (A_{max} at 560 - 600 nm within a pH range of 4.2 - 6.6). At low H_2O_2 concentrations (<10 μM) the intermediates were relatively stable (Fig. 1). The steady state of intermediates kinetics $\frac{d[A_{560-600}]}{dt} = 0$, corresponding to the maximum absorbance of the blue-violet compounds, was evident. The injection of H_2O_2, or H_2O_2 with HRP to the blue-violet solution at the steady state resulted in immediate activation of the chemiluminescence. The highest intensity of this activation was seen upon

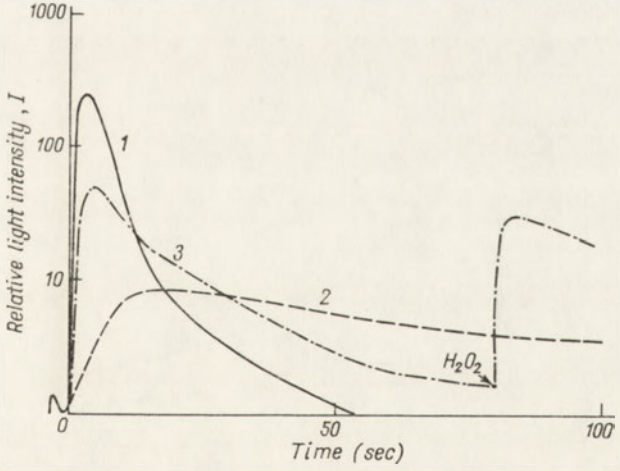


Fig 1. Course of the chemiluminescent reaction resulting from injection of purpurogalline (final concn. 125 μM) to a solution containing (in acetate buffer, pH 4.7): *1*, 4 mM-H_2O_2 and 1.0 μM-HRP; *2*, 4 mM-H_2O_2 and 0.08 μM-HRP; *3*, 0.5 mM-H_2O_2 and 1 μM-HRP, with 0.4 mM-H_2O_2 added 80 sec after start of the reaction (shown by arrow). Temperature 303°K. A small peak before zero time is due to injection of HRP to H_2O_2-buffer solution.

injecting H_2O_2+HRP solution. It should be noted that trimethyl-PPG used as substrate gave no chemiluminescence.

It is well known that in the course of peroxidative reaction free radicals are formed from hydrogen donor (Piette *et al.*, 1963; Nilsson, 1964). Therefore the effect of free-radical inhibitors and O_2 on the chemiluminescence was studied in the investigated system (Table 1). The obtained results indicated that typical radical inhibitors strongly quenched the luminescence. When the concentration of dissolved O_2 was lowered by passing a stream of argon through the solution, the intensity of chemiluminescence was reduced to 15%. Thus, the reaction leading to the generation of excited molecules proceeds *via* free-radical steps. This is in agreement with the low value of activation energy. The respiratory inhibitors, KCN, NaN_3 and

Table 1

Effect of free-radical inhibitors and O_2 *on chemiluminescence of the system PPG+HRP+*H_2O_2*+acetate buffer*, pH 5.6

I^0_{max}, inhibitor added to HRP before the addition of H_2O_2; I_{max}, inhibitor added to HRP-H_2O_2 mixture. The inhibition is expressed in relation to I_{max} without inhibitor taken as 100.

Inhibitor (200 μM)	I^0_{max}	I_{max}
	Inhibition (%)	
Ascorbic acid	81	89
1,4-Dihydroxybenzene	79	78
α-Naphthol	90	58
2,6-Ditertbutyl-*p*-cresol	77	51
KCN	66	85
NaF	—	1
NaN_3	25	16
CO	—	41
Argon	—	85

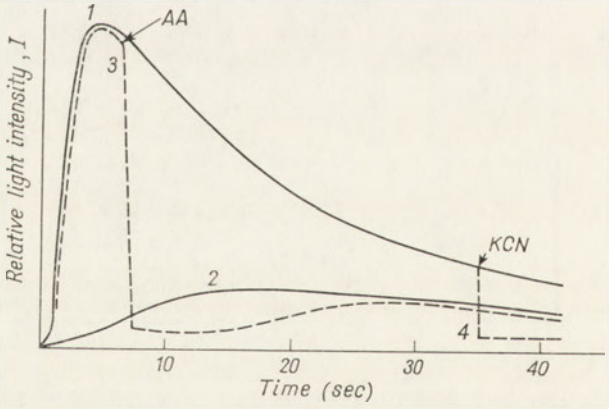


Fig. 2. Effect of inhibitors at 200 μM concentration on the course of the chemiluminescent reaction of 0.1 μM-HRP, 0.5 mM-H_2O_2, acetate buffer, pH 5.6, and 125 μM-PPG: *1*, without inhibitor; *2*, ascorbic acid or KCN added prior to PPG; *3*, ascorbic acid added 6 sec after start of the reaction; *4*, KCN added 35 sec after start of the reaction.

CO, quenched the chemiluminescence. NaF at the concentration of 200 μM was non-inhibitory.

Upon addition of the inhibitors to the whole system during the reaction, there was always a decrease in chemiluminescence intensity (Fig. 2).

Kinetic studies. The Michaelis constants, K_m, for PPG (Fig. 3) and H_2O_2 (Fig. 4) were calculated from the Lineweaver-Burk double reciprocal plot, from the Woolf-Hofstee v versus v/[S] plot, and from the [S]/v *versus* [S] plot. The K_m for PPG was on average 35 μM and V_{max} 1355 relative units, and K_m for H_2O_2 was 15 mM and V_{max} 3×10^4 relative units.

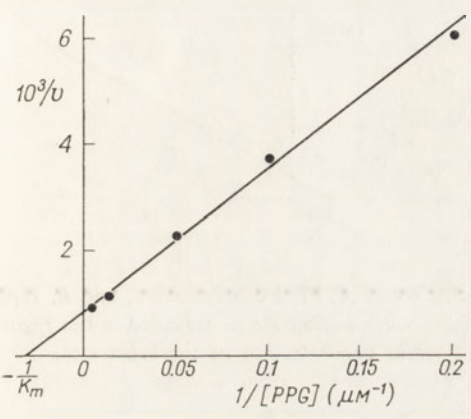


Fig. 3

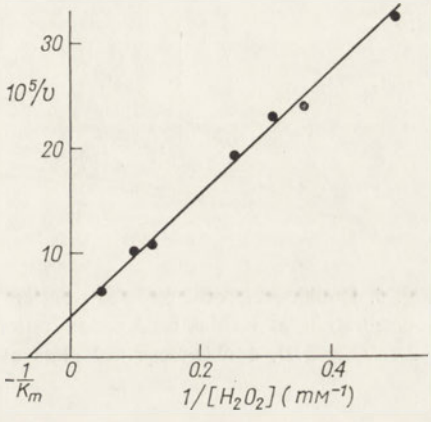


Fig. 4

Fig. 3. Double reciprocal plot of reaction velocity *versus* PPG concentration. The reaction mixture contained 1 μM-HRP, 0.5 mM-H_2O_2, acetate buffer, pH 5.6, and 5 - 100 μM-PPG. The light emission was measured with the use of a blue filter (λ_{max} 440 nm). v is expressed in relative units for I_{max}.

Fig. 4. Double reciprocal plot of reaction velocity *versus* H_2O_2 concentration (2 - 20 mM) at 125 μM-PPG. Other conditions as in Fig. 3.

In a more adequate treatment of the investigated system the combining of two substrates, namely H_2O_2 and PPG, with the enzyme should be taken into consideration. The appropriate dissociation constants of the enzyme-substrates complexes: K_a, K_b, K'_a, and K'_b (Dixon & Webb, 1958) were calculated graphically from two reciprocal quantities (Fig. 5): K_a for H_2O_2 = 10 mM; K_b for PPG = 11.2 μM; K'_b = 62 μM and K'_a = 125 mM. The values of K_a and K_b correspond within experimental error to those of K_m for PPG and H_2O_2, respectively.

Absorption and emission spectra. The absorption spectrum of PPG in methanol solution (Fig. 6) was found to be very close to that reported by Haworth *et al.* (1948) for PPG in ethanol. The methanol solution of PPG with H_2O_2 in acetate buffer, pH 5.6, exhibited almost the same absorption. When HRP was injected to this mixture, after 10 min the absorption in the range of 256 - 333 nm disappeared. The absorption at 230 - 245 nm and 305 - 370 nm characteristic of tropolone derivatives (Dyer, 1967) was relatively stable (up to 30 min).

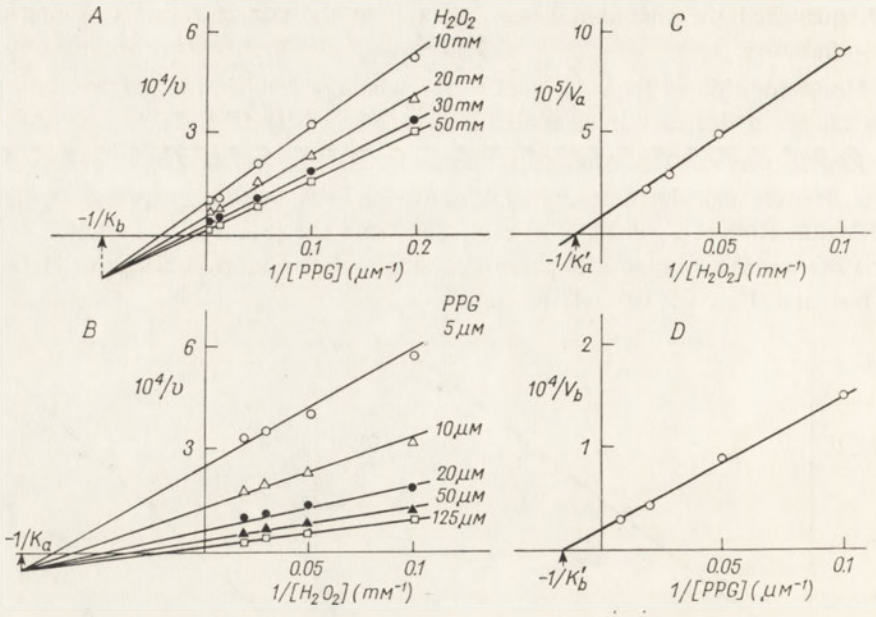


Fig. 5. Double-reciprocal plots of reaction velocity *versus* *A*, PPG concentration, and *B*, H_2O_2 concentration, at various fixed concentrations of the second substrate as indicated in the Figure. *C* and *D*, double-reciprocal plots of V_{max} *versus* concentration of the substrates.

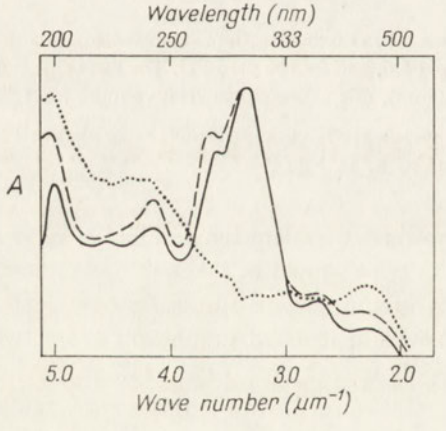


Fig. 6

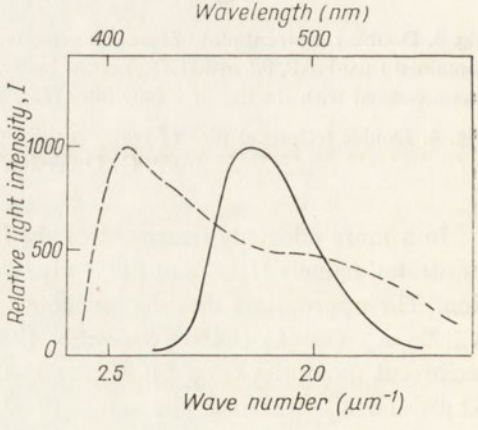


Fig. 7

Fig. 6. Absorption spectra of: ——, PPG in methanol; - - -, PPG+H_2O_2+acetate buffer, pH 5.6, in methanol - water (2:8) solution; •••, PPG+H_2O_2+acetate buffer+HRP, measured 10 min after start of the reaction. The concentration of the components was: 125 μM-PPG, 0.5 mM-H_2O_2 and 1 μM-HRP.

Fig. 7. Emission spectra of the reaction mixture: ——, chemiluminescence spectrum (average values obtained at 5 - 20 sec of the reaction); - - -, fluorescence spectrum after 10 min of the reaction (excitation at 360 nm). The reaction mixture contained 1 μM-HRP, 0.5 mM-H_2O_2 and 125 μM-PPG.

None of the components of the studied system was fluorescent but in the course of PPG oxidation the solution revealed a pale-blue fluorescence. Spectra of fluorescence after 10 min of the enzymic reaction and of chemiluminescence at 5-20 sec are presented in Fig. 7. The spectrum of chemiluminescence showed the maximum at 470 nm and a shoulder at 550 nm. A very broad spectrum of the fluorescence,

Table 2

The fluorescence colour of the spots on the chromatograms

The enzymic reaction mixture contained: 125 μM-PPG, 60 mM-H_2O_2, 10 μM-HRP, 4 mM-Na-acetate buffer, pH 5.6. The non-enzymic reaction mixture contained: 200 μM-PPG, 150 mM-H_2O_2, 125 mM-Na_2CO_3, pH 10.6. The temperature was 293°K. The reaction was stopped at I_{max}.

System			
Enzymic		Non-enzymic	
Colour	R_F	Colour	R_F
pale blue	0.29	pale blue	0.34
yellow	0.44	blue	0.65
yellow-brown	0.64	yellow	0.67
blue	0.84	azure	0.72
blue	0.94		

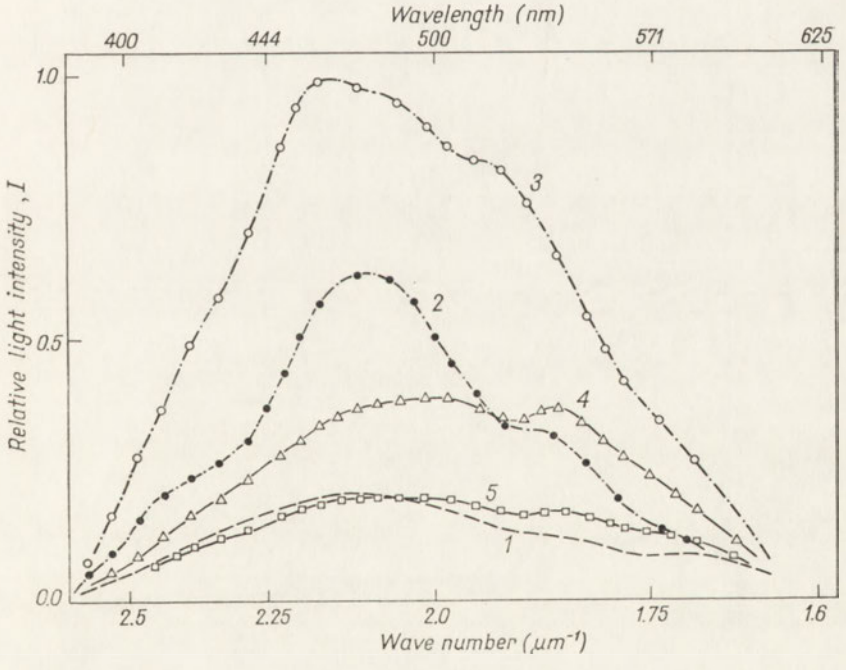


Fig. 8. Fluorescence spectra of enzymic reaction products extracted successively with: *1*, benzene; *2*, chloroform; *3*, ethyl ether; *4*, ethanol; *5*, water. The spectra were corrected for the spectral distribution of photomultiplier sensitivity and transmittance of monochromator prism.

ranging from red to UV, may be due to the presence of a number of fluorescers in the reaction mixture and/or to light scattering due to HRP colloid solution.

Fluorescence of the reaction products. The products of enzymic and non-enzymic oxidation of PPG were submitted to chromatography and visualized in UV light. Some of the fluorescent products were not distinctly separated on the chromatogram, forming streaks. The fluorescence colour of the distinctly separated spots and their R_F values are shown in Table 2. It is evident that the oxidation products obtained in the two systems were not the same. The reaction product which exhibited pale-blue fluorescence was not extractable with ethyl ether from the acidic water solution.

The products of PPG oxidation extracted with solvents differing in their dielectric constant, exhibited fluorescence in the range 380 - 620 nm with maxima

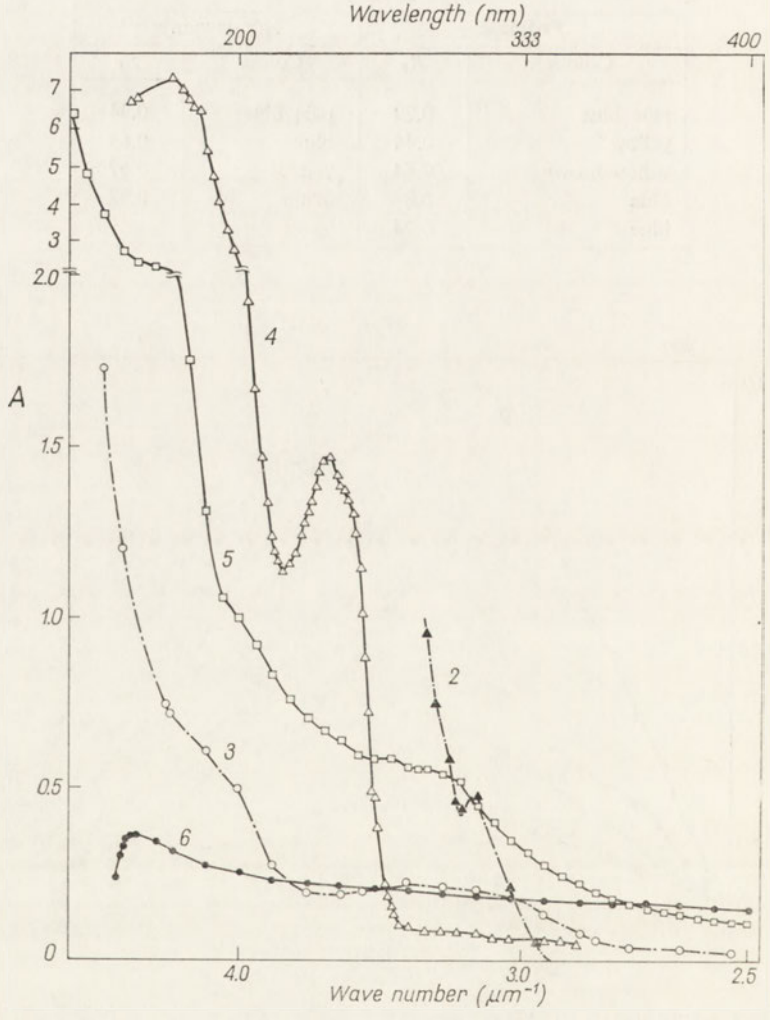


Fig. 9. Absorption spectra of enzymic reaction products extracted successively with different solvents: *2 - 5*, same as in Fig. 8; *6*, NaOH.

at about 480 nm (Fig. 8). The fluorescent products were insoluble in cyclohexane, sparingly soluble in benzene, and readily soluble in polar solvents such as chloroform, ethyl ether, ethanol and water.

The fluorescence spectra of the reaction products extracted with water, ethanol, or ethyl ether show a shoulder at 520 - 540 nm. In general, the fluorescence spectra are very close to the chemiluminescence spectrum of the whole reaction mixture (see Fig. 7) and differ from the fluorescence spectrum of the reaction mixture which evidently was distorted by light-scattering. The absorption spectra of the extracted products (Fig. 9) of PPG oxidation soluble in polar solvents, such as ether, ethanol and water, exhibited absorption maxima at 225, 275 and 310 nm, respectively, which is in agreement with the fluorescence spectra and suggests that these products might be considered as tropolone derivatives containing polar groups.

It should be noted that the colour of products formed from PPG in the reaction with H_2O_2 and HRP under oxygen atmosphere was bright yellow, whereas that in anaerobic conditions (N_2 and/or Ar) was brown.

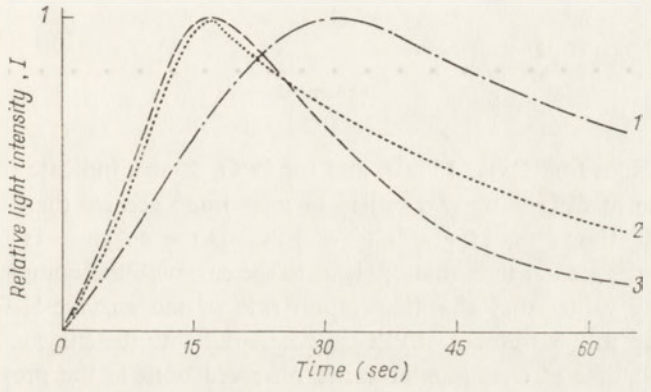


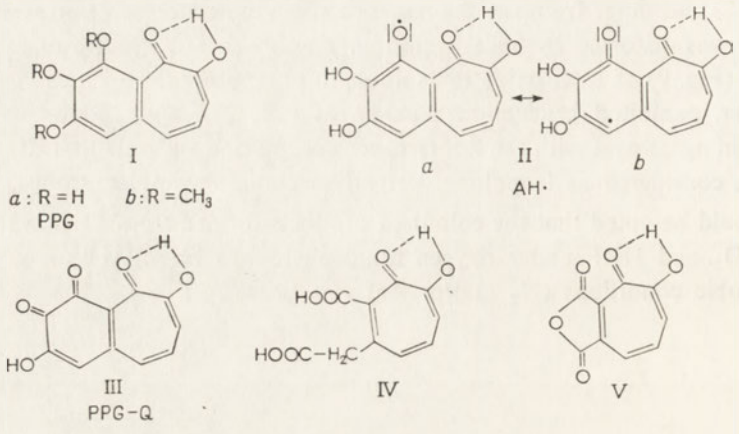
Fig. 10. Course of chemiluminescence resulting from injection of PPG (final concentration 125 μM) to a mixture containing 2 ml of extract (corresponding to 0.1 g of tissue) in acetate buffer, pH 4.7, and 4 mM-H_2O_2, at 303°K. *1*, Cabbage leaves; *2*, wheat roots; *3*, fish (*Abramis brama*) kidney. The ratio of actual I_{max} values for cabbage, wheat and fish kidney was 1:7.5:5.7.

Quantum yield of chemiluminescence (η_{CL}). The total η_{CL} of the system: 125 μM-PPG+1 μM-HRP+5 mM-H_2O_2+acetate buffer, pH 5.4, measured at 306°K was $(4.8 \pm 0.2) \times 10^{-6}$. η_{CL} was largely dependent on PPG concentration, e.g. for 0.1 mM-PPG it was 5.7×10^{-6}, and for 25 μM-PPG, 1.2×10^{-5}. The dependence of ΣI_{PPG} on PPG concentration under conditions when ΣI_{PPG} was proportional to η_{CL} has been discussed in a previous paper (Włodarczyk-Graetzer *et al.*, 1970).

Experiments with tissue extracts. Addition of PPG to crude extracts from wheat roots, cabbage leaves or fish kidney also gave marked chemiluminescence (Fig. 10). This indicates that PPG is a suitable substrate for the peroxidase present in various tissues.

DISCUSSION

The obtained results indicate that PPG is a suitable substrate for the reaction catalysed by peroxidase. The lack of chemiluminescence with trimethyl-PPG (Ib) as substrate indicates that hydrogens at 2, 3 and 4 positions of the phenolic ring are essential for the reaction.



Scheme 1

The K_m values for H_2O_2, 15 mM, and for PPG, 35 μM, indicate that the affinity of the enzyme to PPG is by two orders of magnitude greater than to H_2O_2. The ratio of K'_a/K'_b shows that the affinity of PPG to the enzyme - H_2O_2 complex is about 2000 times greater than that of H_2O_2 to the enzyme-PPG complex. It is evident from the V_{max} values that the dissociation rate of the enzyme-H_2O_2 complex is 22 times higher in the presence of PPG as compared with the enzyme-PPG complex.

The time-course of chemiluminescence observed both in the previous (Włodarczyk-Graetzer *et al.*, 1970) and in the present investigations reveals the following relationships:

$$\tau_{max} \sim \frac{1}{[HRP]} \qquad I_{max} \sim [HRP]$$

where τ_{max} is the time after which chemiluminescence intensity reaches a maximum value I_{max}.

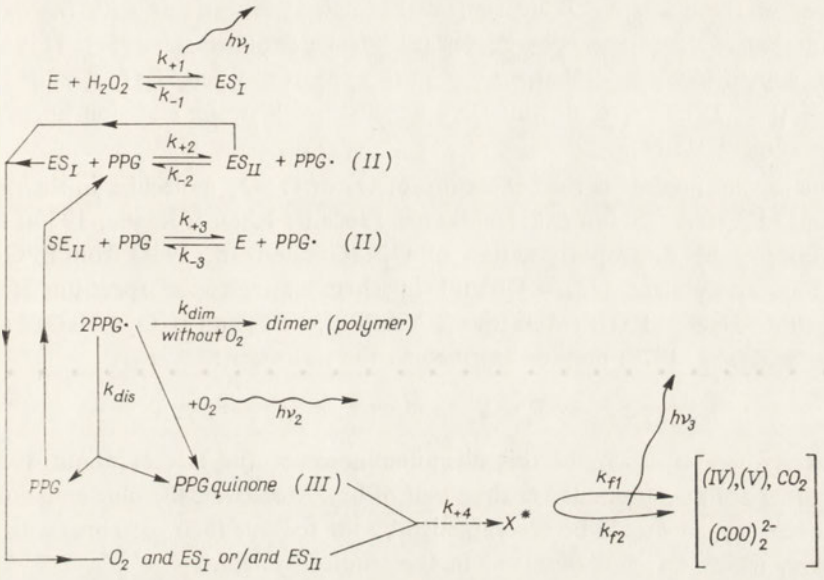
Kinetic curves $I = f(t)$ for $t > \tau_{max}$ could be presented in the form:

$$I \approx \exp(-k_{f_1} t) + \exp(-k_{f_2} t)$$

where k_{f_1} and k_{f_2} are the rate constants of the light-producing reactions. The values of k_{f_1} obtained both from the slope of $\ln I = f(t)$ curves and from the relation $k_{f_1} = \frac{0.693}{\tau_{1/2}}$ are $(0.9 - 1.2) \times 10^{-1}$ sec^{-1} (where $\tau_{1/2}$ is the half-time of luminescence drop). At 20 - 50 mM-H_2O_2, the k_{f_1} value was found to be independent of PPG concentration over the range 5 - 50 μM. The value of k_{f_2} calculated from

the slope of $\ln I = f(t)$ for $t > 60$ sec was $(1.0 - 3.8) \times 10^{-2} \text{sec}^{-1}$. Both values greatly depend on HRP-H_2O_2 concentration. The pH-dependence of I_{max} of the chemiluminescence was characteristic of peroxidase activity.

The kinetic data implied that in the enzymic oxidation of PPG the rates of production and disappearance of the intermediate directly involved in the luminescent reaction, were dependent upon the enzyme concentration. On the basis of the obtained data the course of reactions presented in Scheme 2 may be suggested. The



Scheme 2

three initial processes, regarded as virtually proper enzymic ones, are presented according to Haldane; since the oxidation of PPG is irreversible (k_{-1}, k_{-2} and k_{-3} are near 0) and $k_{+2} > k_{+3}$, the value of k_{+3} could be estimated from the relation (Dixon & Webb, 1958):

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}}$$

K_m would be $< 4.8 \times 10^2 \text{ mol}^{-1} \text{ sec}^{-1}$, which is a rather small value, in agreement with experimentally observed values of τ_{max} of the luminescence. This suggests that the brutto reaction velocity of the formation of a transient, blue-violet anion of PPG-quinone (III^{1-}; see II and III in Scheme 1) is limited by the rate of reaction with k_{+3}. In the reaction step with rate constant k_{+4}, leading to the generation of excited species X*, a blue-violet anion of PPG-quinone reacts with oxidation agent, probably O_2 or enzymically activated H_2O_2. The rate of this step is strongly accelerated by dissolved O_2 and HRP-H_2O_2 complex. Thus, both reactions (k_{+3} and k_{+4}) involved oxidation with participation of the enzyme - H_2O_2 complex.

The elimination of excited species X* proceeds by two or more steps with rate constants k_{f_1} and k_{f_2}. This seems to be confirmed by the fact that several types of fluorescers are formed in the course of PPG oxidation.

The proposed scheme has been analysed in terms of energetic and spectroscopic criteria of chemiluminescence. The results of this analysis indicate that the three processes generating excited, light-emitting species are possible.

The first one is an apparently true enzymic reaction between HRP and H_2O_2 ($h\nu_1$). Chemiluminescence is associated with the oxidation of cyclic amino acid residues of the enzyme (Likhtenstein & Purmal, 1966), and/or with the oxidative degradation of the iron-porphyrin ring (Sławińska & Sławiński, 1969). This luminescence is manifested as a sharp, small peak appearing when H_2O_2 is added to the enzyme (Fig. 1) and does not interfere seriously with strong emission $h\nu_3$ observed in the studied system.

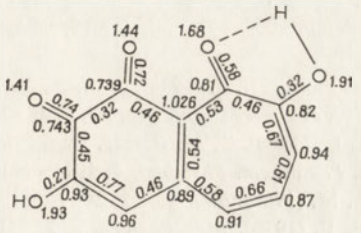
The second process is the generation of O_2 or O_2-O_2 molecules in their excited $^1\Delta g$ and $^1\Sigma_g^+$ states (Stauff & Schmidkunz, 1962a,b; Khan & Kasha, 1970), formed for example, by disproportionation of O_2^- (Gunderman, 1968) from PPG$\cdot + O_2$. The blue component ($\lambda_{max} = 470$ nm) in chemiluminescence spectrum for the pyrogallol$+H_2O_2+$KOH (Meluzova & Vassil'ev, 1970) and H_2O_2+NaOCl systems (Khan & Kasha, 1970) may be ascribed to the transition:

$$^1\Delta g\ ^1\Sigma_g^+ \rightarrow {}^3\Sigma_g^-\ {}^3\Sigma_g^- \text{ and/or } {}^1\Delta g\ {}^1\Delta g \rightarrow {}^3\Sigma_g^-\ {}^3\Sigma_g^-$$

However, quantum yield of this chemiluminescence ($h\nu_2$) is of about 10^{-9}, i.e. three orders of magnitude lower than that of $h\nu_3$. Moreover, the blue emission from the singlet oxygen should be accompanied by the red one (630 - 780 nm) with higher intensity, which was not observed in the studied system.

The third group of processes leading to the observed chemiluminescence is an oxidative ring-opening of ortho- and/or paraquinones of PPG (IV). An initial step is a nucleophilic attack of O_2, O_2^-, O_2H^- or polarized H_2O_2 molecule in the HRP-H_2O_2 complex on a positively polarized C-atom of the $\overset{-\delta}{\underline{O}}\!-\!\overset{+\delta}{C}<$ quinoid group of PPG-quinone. The quinoid arrangement undergoes destruction *via* a peroxy-intermediate and carbonyl derivatives of *α*-tropolone, IV and V, are formed. These reactions liberate 260 - 950 kJ/mole and the sum of $\Delta H + E_{act}$ is sufficient to promote the emission with wavelength longer than 440 nm. The above reactions in non-enzymic systems were described in detail by Collier (1966, 1969) and Sławiński (1971). This mechanism is supported by electron densities and bond-orders, calculated by Hückel's MO method. It is evident from the electronic diagram for PPG-quinone (Scheme 3) that, firstly, the carbonyl groups are in fact strongly polarized since electron densities are greatest at quinoid oxygens, and secondly $>C\!=\!\!-\!\!-C<$ bond between quinoid carbonyls has the smallest value of bond order, i.e. it is the weakest bond in the molecule. Since chemiluminescence, fluorescence and absorption spectra as well as the course of chemical reaction in both systems are very similar, it is most probable that carbonyl derivatives of *α*-tropolone, generated in excited states are emitters of luminescence. It was found that the majority of the oxidation

products exhibit fluorescence (Table 2, Fig. 8), the emission band of which overlaps the long-wave band of the absorption spectrum (Fig. 6). If it is assumed that the observed chemiluminescence is due to $^1S \rightarrow {}^0S$ transition, the luminescence quantum yield η_L would be in the range of $10^{-3} - 10^{-1}$. Thus, excitation quantum yield $\eta_{exc} = \frac{\eta_{CL}}{\eta_L}$ would be equal to $10^{-5} - 10^{-3}$ and in agreement with other chemiluminescent processes (Vassil'ev, 1967; Belyakov & Vassil'ev, 1970). It may be



Scheme 3

easily calculated that only $10^{-6} - 10^{-4}$ photons with an average energy 245 kJ/mole are emitted per one molecule of the enzyme per second. Unfortunately, the lack of the spectral characteristics of carbonyl derivatives of α-tropolone makes impossible more exact calculations.

The proposed scheme is in line with experimental data, and includes several radical steps: oxidation of PPG, dismutation of two AH·semiquinones of PPG (II) and dimerization. The latter yields a yellow-brown product ($\lambda_{max} = 450$ nm), observed as a brown spot on the chromatogram with a low R_F value. Since the yield of this process increases under anaerobic conditions under N_2 or Ar, it is clear that the dimerization of PPG-semiquinone radical is a competitive reaction with regard to chemiluminescence elimination, involving O_2 and/or H_2O_2 activated by HRP.

It is obvious that generation of excited species is coupled with oxidative destruction of quinoid ring and not with simple detachment of electron or hydrogen from donor. Although many questions concerning the reaction mechanisms remain to be elucidated, the described chemiluminescent system offers an extremely sensitive (even to 10^{-14} M-HRP) and rapid method for kinetic and analytical studies of peroxidative reactions.

The authors wish to express their gratitude to Professor Dr. K. L. Wierzchowski of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, for his criticism of the manuscript and stimulating discussion. Doc. Dr. D. Sławińska is acknowledged for her help in performing the fluorescence measurements and for valuable remarks.

REFERENCES

Barlttrop J. A. & Nicholson J. S. (1948). *J. Chem. Soc.* 116 - 120.
Belyakov V. A. & Vassil'ev R. F. (1970). *Photochem. Photobiol.* **11**, 179 - 192.
Collier P. D. (1966). *J. Chem. Soc.* (*C*) 2255 - 2261.
Collier P. D. (1969). *J. Chem. Soc.* (*C*) 612 - 621.
Dixon M. & Webb E. (1958). *Enzymes*, p. 26 - 33, 63 - 162, 354 - 360. Longmans, Green and Co., London.
Dure L. S. & Cormier M. J. (1964). *J. Biol. Chem.* **239**, 2351 - 2359.
Dyer J. R. (1967). *Spektroskopia absorpcyjna w chemii organicznej*, p. 27, P.W.N., Warszawa.
Grabiec S. (1968). *Bull. Acad. Pol. Sci., Ser. Sci. Biol.* **16**, 249 - 251.
Gunderman K. D. (1968). *Chemilumineszenz Organischer Verbindungen*, p. 15, 16. Springer-Verlag, Berlin.
Haworth R. D., Moore B. P. & Pauson B. P. (1948). *J. Chem. Soc.* 1045 - 1051.
Khan A. U. & Kasha M. (1970). *J. Amer. Chem. Soc.* **92**, 3293 - 3300.
Klipson N. A. & Mamedow T. G. (1966). *T. M. Obszcz. Insp. Prirody* **16**, 22 - 24.
Lee J. & Seliger H. H. (1965). *Photochem. Photobiol.* **4**, 1015 - 1018.
Likhtenstein G. I. & Purmal A. M. (1966). *Biofizika* **11**, 245 - 248.
Meluzova G. B. & Vassil'ev R. F. (1970). *Mol. Photochem.* **2**, 251 - 257.
Mikhlin D. M. & Bronowickaja Z. S. (1949). *Biochimia* **14**, 379 - 381.
Nilsson R. (1964). *Acta Chem. Scand.* **18**, 389 - 401.
Piette L. H., Yamazaki I. & Mason H. S. (1963). *Swobodnyje radikały w biologiczeskich systemach.* Izd. Nauka, Moskwa.
Potesilowa H., Alcaraz C. & Santavy F. (1969). *Coll. Czech. Chem. Commun.* **34**, 2128 - 2133.
Sławińska D. & Sławiński J. (1968). *Wiad. Chem.* **22**, 165 - 185 and 267 - 296.
Sławińska D. & Sławiński J. (1969). *Acta Biochim. Polon.* **16**, 347 - 354.
Sławiński J. (1969). *Zesz. Nauk. W.S.R.* Szczecin, Monografia 18.
Sławiński J. (1971). *Photochem. Photobiol.* **13**, 489 - 497.
Stauff J. & Schmidkunz H. Z. (1962a). *Z. Physik. Chem.* **33**, 273 - 297.
Stauff J. & Schmidkunz H. Z. (1962b). *Z. Physik. Chem.* **35**, 295 - 308.
Vassil'ev R. F. (1967). In *Progress in Reaction Kinetics* **4**, 305 - 387.
Włodarczyk-Graetzer M., Szczodrowska B. & Sławiński J. (1970). *Zesz. Nauk. W.S.R.* Szczecin 395 - 410.

BADANIA CHEMILUMINESCENCJI W UKŁADZIE PURPUROGALINA - PEROKSYDAZA - NADTLENEK WODORU

Streszczenie

1. Badano chemiluminescencję towarzyszącą utlenianiu purpurogaliny przez H_2O_2 i peroksydazę chrzanu w buforze octanowym o pH 5,6, nasyconym tlenem w temperaturze 30°C.

2. Oznaczano kinetykę luminescencji i stałe dysocjacji aktywnych kompleksów.

3. Zbadano widma absorbcji, fluorescencji i chemiluminescencji, wydajność kwantową chemiluminescencji oraz fluorescencję produktów utleniania.

4. Uzyskane wyniki wskazują, że chemiluminescencja towarzyszy utleniającemu rozerwaniu sześcio-członowego pierścienia chinonu purpurogaliny, w wyniku czego tworzą się karbonylowe pochodne α tropolonu we wzbudzonych stanach singletowych.

Received 19 May, 1972.

ACTA BIOCHIMICA POLONICA

Vol. 20 1973 No. 2

ZOFIA LASSOTA, BOŻENNA OLSZAŃSKA and EWA GRĄBCZEWSKA

HETEROGENEITY AND LABELLING PATTERNS OF RNA FROM CHICKEN EMBRYOS

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, 02-532 *Warszawa; and Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzębiec, Poland*

The polyacrylamide-gel electrophoresis patterns of total RNA from 5- and 6-day-old chick embryos were found to be similar to those of total RNA from embryonic as well as adult liver. Two minor fractions located between two major ones in the 28S - 18S region of total embryonic RNA correspond to ribosomal RNA and may be explained by the lability of 28S rRNA. Two fractions less mobile than 28S RNA are synthesized in the nucleus and represent the heavy rapidly-labelled RNA, probably the precursor rRNA.

Comparative studies seem to indicate that evolution of organisms was accompanied by changes in the size of the transcription unit of rRNA and of the intermediate of its conversion (Perry *et al.*, 1970), as well as in the size of the cytoplasmic rRNA, especially of its heavier component (Loening, 1968). However, considerable lability of this component, differing from one animal species to another (Loening, 1968), makes difficult comparative evaluations.

The data obtained so far concern some species of invertebrates, and among vertebrates mainly the amphibians and mammals (Attardi & Amaldi, 1970), whereas there are but scarce data on avian RNA. Characterization of avian nuclear and cytoplasmic RNA could throw some light on phylogenetic changes in the synthesis and conversion of these particles. Therefore we have undertaken studies aiming at investigating the main steps in the process of conversion of nuclear RNA into cytoplasmic RNA in chick embryo.

MATERIAL

Reagents. [2-^{14}C]- and [U-^{14}C]uridine were products of UVVVR (Praha, Czechoslovakia). DNase I from bovine pancreas, free of RNase, was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Actinomycin D and 4S *E. coli* RNA were products of Calbiochem A.G. (Luzern, Switzerland). Triton X-100, tris and *N,N,N',N'*-

tetramethylenediamine (TEMED) were from B.D.H. Chemicals Ltd (Poole, Dorset, England). PPO and POPOP were from Reanal (Budapest, Hungary). Acrylamide and *N,N'*-methylene-bis-acrylamide from Eastman Kodak Co. (Rochester, N. Y., U.S.A.) were recrystallized from chloroform and acetone, resp. Commercial sucrose was purified according to Perzyński & Szafrański (1971).

27S RNA of *E. coli* phage F_2 was a gift from the Department of Protein Biosynthesis of the Institute of Biochemistry and Biophysics (Warszawa, Poland).

Animals. The experiments were carried out on 5- and 6-day-old whole chick embryos and livers of 10-day-old or adult chicken of the Leghorn breed. In some experiments, where indicated in the text, hybrids (♀ Leghorn × Red Island × ♂ Polish breed żółtonóżka kuropatwiana) were used. The embryos were taken out from eggs and their eyes were removed. Livers were immediately isolated from the obtained embryos or from decapitated chicken.

METHODS

Labelling with [^{14}C]*uridine.* The incubation medium prepared according to Wilt (1967) consisted of: 0.7% NaCl, 0.017% $CaCl_2$, 0.037% KCl, 0.02 M-tris, pH 7.5. The freshly obtained embryos were washed with the medium and incubated at 38°C in the same medium supplemented with [2-^{14}C]- or [U-^{14}C]uridine to a specific activity of 10 μCi/ml. Where indicated in the text, the embryos after incubation were washed, transferred to another portion of the medium containing actinomycin D (20 μg/ml) and the incubation continued at the same temperature.

After incubation the embryos were washed several times with the medium or water at 4°C and used directly or frozen in solid CO_2 and stored at -40°C not longer than for 60 days.

When 20 - 30 Leghorn embryos were incubated for 30 min in 10 ml of the medium containing 0.5 mg of [2-^{14}C]uridine (100 μCi), the total RNA isolated from 1 g contained $2 - 2.5 \times 10^{-6}$ mg of labelled uridine in the case of 5-day-old embryos and twice as much in the case of 6-day-old ones. After 60 min, the amount of uridine incorporated into RNA was doubled. Under the same conditions, embryos of the hybrids showed several-fold lower incorporation of radioactivity.

Subcellular fractions. The fractionation was carried out on an ice-bath. The ribosomal fraction from livers or whole embryos was obtained according to Petermann & Pavlovec (1963a) by differential centrifugation of homogenates prepared in 0.3 M-sucrose in the presence of Mg-bentonite. The final 105 000 *g* sediment was suspended in 0.1 M-sodium acetate buffer of pH 5 containing 0.1 M-NaCl and 1 mM-EDTA.

The nuclear fraction was isolated either from the 20 000 *g* sediment obtained in the above procedure of ribosome isolation or from whole embryos. The tissue, 2 g (or a corresponding amount of the sediment) was gently homogenized in the Potter-Elvehjem homogenizer with 5 ml of 0.25 M-sucrose solution containing 1% of sodium citrate, and centrifuged at 1000 *g* for 5 min. The obtained sediment was homogenized for 2 min in 0.25 M-sucrose containing 3 mM-$CaCl_2$ and 0.1%

of Triton X-100 (10 ml/g of tissue) in a Virtis (The Vir-Tis Co. Inc., Gardiner, New York, U.S.A.) homogenizer. The homogenate was filtered through 16 layers of gauze and centrifuged at 800 *g* for 5 min. This procedure, combining the elements of Dounce (1955) and Hymer & Kuff (1964) methods permitted to obtain from chick embryos a preparation of nuclei which, after staining with the Pappenheim-Unna solution, was microscopically reasonably pure.

The crude cytoplasmic fraction was collected in the course of isolation of nuclei from whole embryos by pooling the supernatants of the first and second centrifugation in 0.25 M-sucrose. These supernatants were supplemented with polyvinyl sulphate to a concentration of 10 μg/ml, and made 0.1 M with respect to NaCl, and 1 mM with respect to EDTA.

Isolation of RNA. RNA was isolated by the phenol-cresol method (Hastings & Kirby, 1966) at pH 5. The whole tissue or the subcellular fractions were homogenized in an ice-bath in a glass Potter-Elvehjem homogenizer in a mixture composed (per 1 g of tissue) of: 10 ml of the NaCl-EDTA-acetate buffer, pH 5 (see above), 10 ml of phenol saturated with the same buffer and containing 0.1% of 8-hydroxyquinoline, and 2 ml of 10% sodium dodecyl sulphate. After 20 min of shaking at room temp. and centrifuging at 17 000 *g* for 15 min, the aqueous phase was collected, added with NaCl to a concentration of 0.5 M, shaken for 20 min with an equal volume of a phenol-cresol - 0.1% 8-hydroxyquinoline mixture saturated with the NaCl-EDTA-acetate buffer, pH 5, and centrifuged as above. RNA was precipitated from the aqueous phase with 2 vol. of cold absolute ethanol, usually overnight, at 4°C. From the crude RNA preparation DNA was removed by incubation with RNase-free DNase in 0.15 M-NaCl - 0.01 M-$MgCl_2$ - 0.01 M-tris buffer, pH 7.2, for 20 min at 38°C. The enzyme was removed by repeating the extraction with the phenol-cresol - 0.5 M-NaCl mixture. RNA precipitated with ethanol from the aqueous phase was dissolved in the NaCl-EDTA-acetate buffer, pH 5, and reprecipitated with ethanol at -20°C.

The amount of total RNA obtained by this procedure, calculated per 1 g of fresh tissue, was: from whole embryos about 2 mg, from embryonic nuclear fraction about 0.2 mg, and from adult chicken liver, 4 - 5 mg.

Density-gradient centrifugation. A 5 - 20% linear sucrose gradient was applied according to Britten & Roberts (1960), the gradient being prepared with 0.01 M-sodium acetate buffer, pH 5, containing 0.1 mM-EDTA. About 1 mg of RNA dissolved in 0.2 ml of the same buffer was layered over 4 ml of the gradient and centrifuged in the Spinco-Beckman L2 39 SW rotor at 35 000 rev./min at 4°C for 6 h. Fractions of about 0.1 ml were collected from the bottom of the tube, and freeze-dried.

Disc-gel electrophoresis was performed by the method of Bishop *et al.* (1967) in bisacrylamide cross-linked 2.4 and 4.8% polyacrylamide, at pH 7.2. After 30 - 45 min of prerun, samples of about 50 μg of RNA (0.05 ml) were layered on gels (7.7×0.9 cm) and the electrophoresis was carried out at 10 mA per gel for 90 min. The gels were fixed with 1 M-acetic acid and stained with 1% solution of

methylene blue in 1% acetic acid, then destained by several changes of 1% acetic acid.

The gels were photographed and the negatives scanned in visible light in a double-beam recording microdensitometer MK III (C.S. Joyce-Loebl, Gateshead-on-Tyne, England).

Determination of radioactivity. Radioactivity was measured in a Tri-Carb Packard scintillation counter in vials containing 50 µl of the sample tested, 5 ml of the scintillation fluid (3 g of PPO and 0.1 g of POPOP in 1000 ml of toluene) and 1 ml of absolute ethanol. The recovery of ^{14}C counts was 47%.

Determination of RNA was carried out at 260 nm in Unicam SP 500 spectrophotometer, using the coefficient $E^{0.1\%}_{1\ cm} = 23$.

RESULTS

Total RNA. The total RNA of adult chicken liver separated in 2.4% polyacrylamide gel into seven fractions (Fig. 1a, Plate 1A); the sum of fractions *3* through *6* corresponded to 60% of the whole preparation, and 35% was found in fraction *7*. The 27S RNA of *E. coli* phage F_2 applied as a marker of the heavy fractions was located between the fractions *3* and *4*, and the 4S *E. coli* RNA applied as a marker for the light fractions, overlapped fraction *7*.

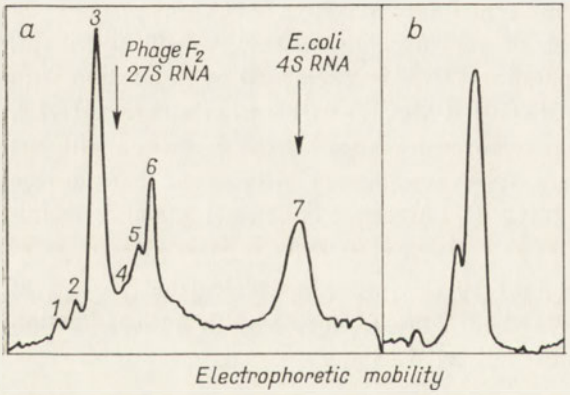


Fig. 1

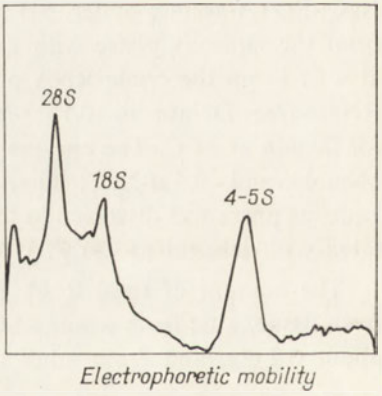


Fig. 2

Fig. 1. Densitograms of electrophoretograms of: *a*, total RNA from adult chicken liver (see Plate 1A) separated on 2.4% gel; the electrophoretic mobility of markers is shown by arrows. *b*, Light fractions (<18S) collected from sucrose gradient, separated in 4.8% gel.

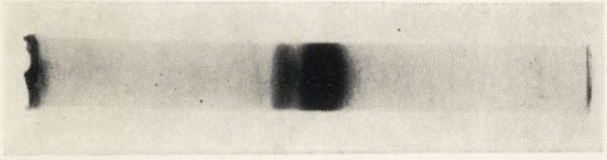
Fig. 2. Densitogram of electrophoretogram of whole RNA from liver of 10-day-old embryos, separated in 2.4% gel.

Taking into account the electrophoretic mobility in relation to that of the markers, and the electrophoretic pattern of RNA from rat liver obtained under similar conditions by Dingman & Peacock (1968), we have arbitrarily designated the major RNA fractions as follows: fraction *3*, 28S; fraction *6*, 18S, and fraction *7*, which appeared to be inhomogeneous, 4 - 5S. When total RNA was fractionated in sucrose-

Plate 1

Polyacrylamide gel electrophoresis of:

A, total RNA from adult chicken liver (2.4% gel);



B, light fractions (< 18S) of total RNA from liver of 10-day-old embryos (4.8% gel);

C, total RNA of 5-day-old embryos (2.4% gel);

D, nuclear RNA from 5-day-old embryos* (2.4% gel);

E, crude cytoplasmic fraction from 5-day-old embryos (2.4% gel).

* Hybrids.

density gradient, the isolated light fractions separated on electrophoresis in 4.8% polyacrylamide into two bands, corresponding presumably to 4S and 5S RNA (Fig. 1b).

Total RNA from liver of 10-day-old embryo (Fig. 2) gave a pattern similar to that of RNA of adult chicken liver; the most mobile 4 - 5S fraction separated also into two components (Plate 1B).

In RNA isolated from whole 5- and 6-day-old embryos (Plate 1C) the major fractions found in the liver RNA were also present. RNA of 6-day-old embryos separated by sucrose-density-gradient centrifugation was collected in three fractions (*I*, heavy; *II*, intermediate; *III*, light — Fig. 3a) and submitted to electrophoresis. The heavy fraction *I* contained two components less mobile than 28S RNA. In fraction *II*, two major bands of 28S and 18S RNA were accompanied by two minor ones situated between them. In the region of the light fractions (*III*) two bands appeared in 4.8% gel, similar to those observed in liver RNA.

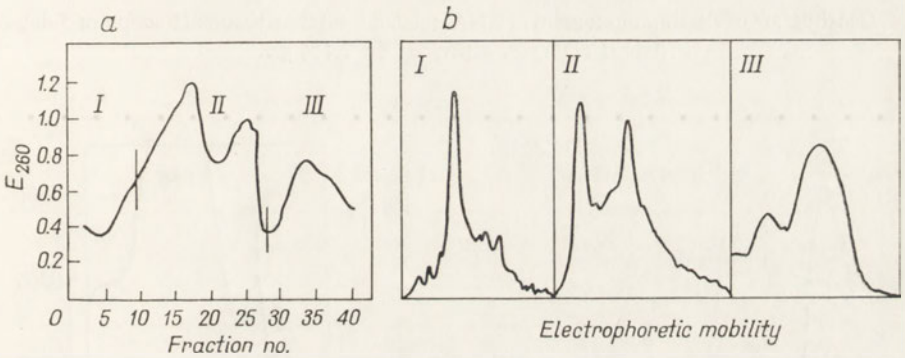


Fig. 3. *a*, Fractionation of total RNA from 6-day-old embryos by sucrose-gradient centrifugation. The fractions were collected as shown in the diagram and submitted to electrophoresis, fractions *I* and *II* in 2.4% gel, fraction *III* in 4.8% gel, and their densitograms are presented.

Ribosomal RNA from embryos. The rRNA isolated from 5-day-old embryos, gave on gel electrophoresis (Fig. 4) two major fractions, 28S and 18S, two bands located between 28S and 18S, and two bands in the region of the light fractions, 4 - 5S. The 28S:18S ratio was close to 2, which is a value characteristic of ribosomes. In the region between the start and 28S only traces of heavier fractions were visible.

Nuclear RNA from embryos. In the RNA isolated from the nuclear fraction, beside the two major fractions corresponding to 28S and 18S rRNA (the ratio of which was shifted, compared with total RNA, in favour of 28S RNA), two distinct bands migrating slower than 28S RNA were visible (Plate 1D).

In the crude cytoplasmic fraction obtained after removal of nuclei, the content of RNA components slower than 28S RNA was very low (Plate 1E).

Incorporation of [^{14}C]*uridine into embryos* in vitro. Total RNA isolated from 5-day-old embryos incubated for 30 min with [2-^{14}C]uridine, gave in sucrose gradient the pattern presented in Fig. 5. In the region of heavy RNA fractions, which

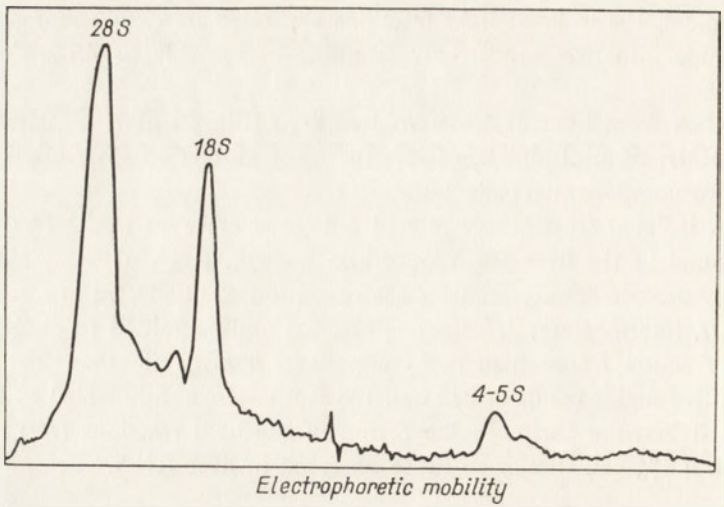


Fig. 4. Densitogram of electrophoretogram of RNA isolated from the ribosomal fraction of 5-day-old hybrid embryos, separated on 2.4% gel.

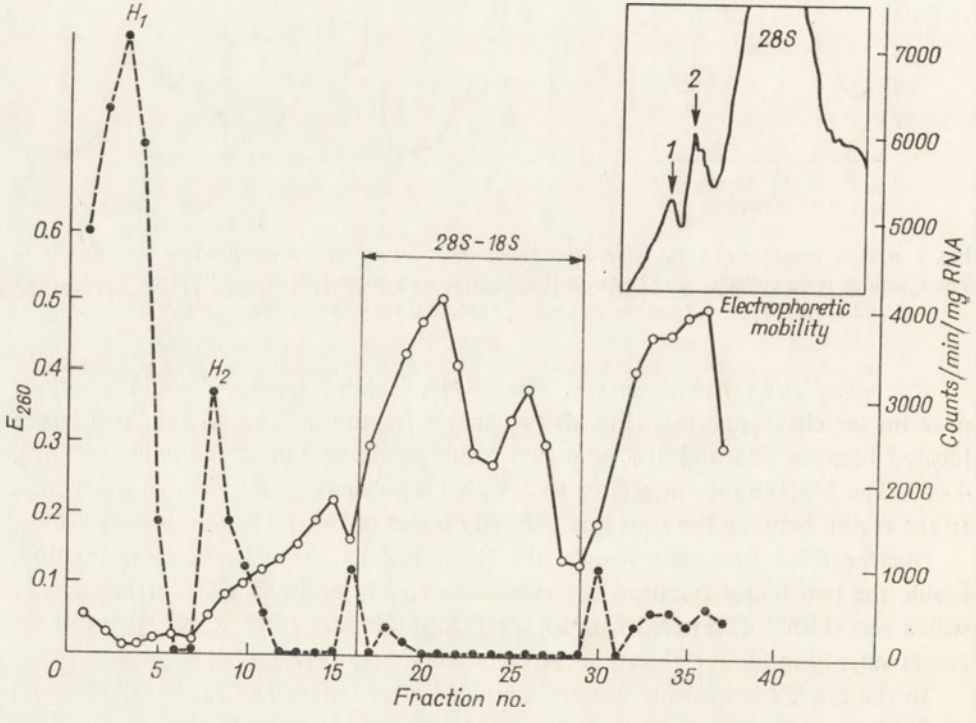


Fig. 5. Sucrose-density-gradient centrifugation and labelling patterns of total RNA isolated from forty 5-day-old embryos, incubated for 30 min in 10 ml of medium containing [2-^{14}C]uridine (10 μCi, 0.056 mg/ml). Insert, densitogram of the heavy fractions, separated in 2.4% gel. ○, E_{260}; ●, radioactivity; H_1 and H_2, main peaks of radioactivity.

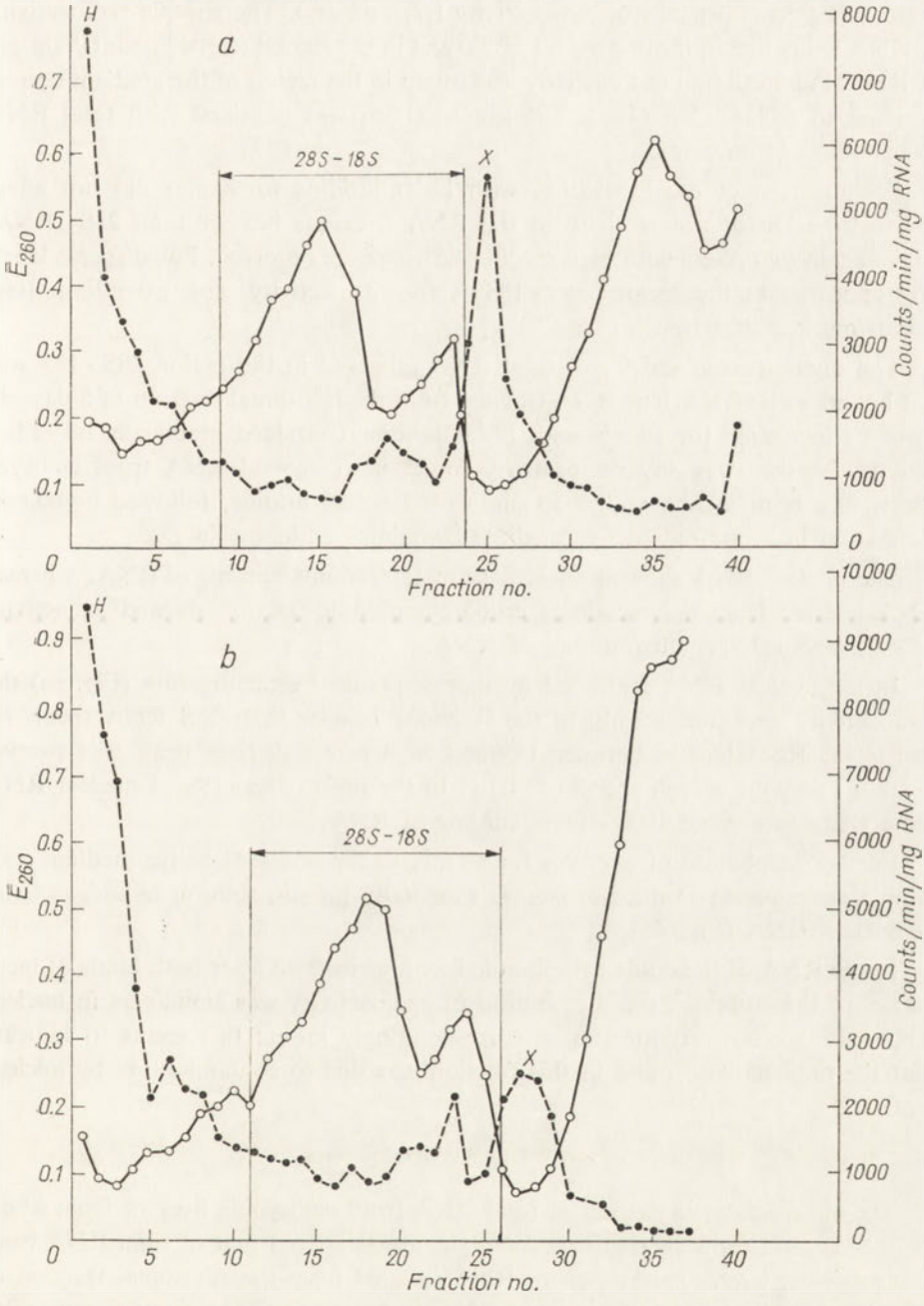


Fig. 6. Sucrose-density-gradient centrifugation and labelling pattern of nuclear RNA isolated from thirty 5-day-old embryos incubated: *a*, for 30 min in 10 ml of the medium containing [U-^{14}C]uridine (10 μCi, 0.0112 mg/ml); *b*, incubated as in *a*, then for 60 min in non-radioactive medium supplemented with actinomycin D (20 μg/ml). *H* and *X*, main peaks of radioactivity. ○, E_{260}; ●, radioactivity.

on electrophoresis correspond to the two fractions less mobile than 28S RNA, there appeared two peaks of radioactivity (H_1 and H_2). The specific radioactivity of RNA amounted in the first peak to 7000 and in the second to 3000 counts/min/mg of RNA. Practically no radioactivity was found in the region of the gradient corresponding to rRNA (28S - 18S). The same pattern was obtained with total RNA from 6-day-old embryos.

Prolongation of the incubation with [2-^{14}C]uridine to 60 min did not affect the distribution of radioactivity in the RNA fractions heavier than 28S rRNA, where again two peaks with high specific activity were observed. Polydisperse labelling appeared in the region 28S - 18S RNA, its activity not exceeding 1000 counts/min/mg of RNA.

The slight specific activity of total RNA observed in the region 28S - 18S was not bound with rRNA. The RNA isolated from the ribosomal fraction of 5-day-old embryos incubated for 30 min with [2-^{14}C]uridine contained practically no radioactivity. Neither was any radioactivity found in ribosomal RNA from embryos which had been incubated for 30 min with labelled uridine, followed by 60 min incubation in a non-radioactive medium containing actinomycin D.

The nuclear RNA showed an activity of 1500 counts/min/mg of RNA, whereas RNA isolated from the remaining crude cytoplasmic fraction showed an activity of only about 100 counts/min/mg of RNA.

In the nuclear RNA separated by sucrose-gradient centrifugation (Fig. 6a) the radioactivity appeared mainly in the fractions heavier than 28S RNA (peak *H*) and in the RNA located between 18S and 5S, where a distinct peak was formed (peak *X*) showing a high specific activity. In the region 28S - 18S of nuclear RNA the activity was about 1000 counts/min/mg of RNA.

Further incubation of embryos for 60 min in the non-radioactive medium containing actinomycin D did not change essentially the distribution of radioactivity in nuclear RNA (Fig. 6b).

In the RNA of the crude cytoplasmic fractions isolated after both kinds of incubation of the embryos, the distribution of radioactivity was similar as in nuclear RNA, the specific activities being correspondingly lower; this seems to indicate that the radioactivity found in this fraction was due to contamination by nuclear material.

DISCUSSION

The electrophoretic pattern of total RNA from embryonic liver or from whole 5- or 6-day-old embryos does not differ significantly from that of total RNA from adult chicken liver. Comparison of RNA isolated from the ribosomal fraction of embryos permits to identify in total RNA three major rRNA components, arbitrarily designated 28S, 18S and 5S. These designations, however, do not imply the molecular weight of avian rRNAs. The fastest-moving component, separable from 5S rRNA on 4.8% gel, could be tRNA, similarly as observed by Dingman & Peacock (1968) in the cytoplasm of rat liver.

Electrophoretic heterogeneity of total RNA in the region 28S - 18S is related to rRNA, as it was observed also in the RNA preparations obtained from the ribosomal fraction. Petermann & Pavlovec (1963b) have reported that ribosomal RNA of rat liver contains a stable 22S fraction, amounting to about 10% of rRNA. The gel electrophoresis of rat liver rRNA prepared in our laboratory according to Petermann & Pavlovec (1963a) revealed, however, not one but two minor fractions located between the major components (unpublished results). This picture was consistent with the electrophoretic pattern of cytoplasmic RNA of rat liver presented by Dingman & Peacock (1968). Thus it seems that heterogeneity of heavy rRNA fractions from rat liver and chick embryo is similar.

Heterogeneity in the 28S - 18S region of rRNA from chicken embryos may be due to the lability of 28S RNA. Degradation of this component is usually observed under conditions favouring splitting of hydrogen bonds, and is ascribed to hidden breaks preexisting in the chain and masked by secondary bonds (Applebaum *et al.*, 1966). Depending on conditions and origin of ribosomes, the course of 28S rRNA degradation and the products formed may be different. Insect 28S rRNA within 10 min at 37°C breaks partially into two fragments, one being less, and another more mobile than 18S (Rubinstein & Clever, 1971). At higher temperature, insect 28S rRNA is split completely into two fragments of similar size, whereas rat liver 28S rRNA gives polydisperse products (Applebaum *et al.*, 1966). The thermal splitting of 28S rRNA of duck erythroblasts results in release of a small 7S fragment but the electrophoretic mobility of the remaining molecule decreases, owing to a change in conformation (Pene *et al.*, 1968); HeLa cells 28S rRNA behaves like duck 28S rRNA in contrast to mouse 27S rRNA, preserving under these conditions the original mobility (Eliceiri & Green, 1969).

So it may be assumed that under the rather mild conditions of our experiments, the chicken 28S rRNA underwent partially a specific degradation into products less mobile than 18S rRNA.

Heterogeneity of chicken embryo rRNA may be also due to the heterogeneity of ribosome population, which has been observed in bacteria (Schaup *et al.*, 1969). Recently Dahlberg & Peacock (1971) reported on electrophoretic heterogeneity of both 23S and 16S *E. coli* rRNA, but they ascribed it to differences in primary structure (16S) and conformation (23S) related to maturation.

In total embryonic RNA the two fractions less mobile than 28S are of nuclear origin; they do not occur at all in the RNA isolated from ribosomal fraction and appear in small amounts in the RNA of crude cytoplasm.

Scherrer *et al.* (1966) found that nuclear RNA of duck erythroblasts contained, along with 45S and 35S rRNA precursors, rapidly-labelled 70S and 60S RNA, considered as a polycistronic transcription products, containing mRNA. In their experiments RNA was isolated with hot phenol, whereas we have carried out the phenol step at room temperature; this explains why in chicken RNA we have not observed any 60 - 70S fractions which on electrophoresis in 2.4% gel would have remained at the start.

Thus we may consider the two slower components of chicken embryo RNA as precursors of rRNA; this is also supported by the labelling experiments. After 30 min of incubation of embryos *in vitro* with [^{14}C]uridine, when the bulk of radioactivity is in the nucleus and ribosomal RNA still contains no label, there are in total RNA two highly radioactive fractions, corresponding to the two electrophoretically slowest components. The analysis of nuclear RNA from embryos also confirmed the preferential high labelling of the RNA heavier than the 28S component. Apart from this, there appeared also a rapidly-labelled nuclear fraction moving faster than 18S RNA but we can not interpret its character.

Maturation of the rRNA precursor in the chicken nucleus seems to be rather slow, as 60-min chase in the presence of actinomycin D did not result in shifting of the label to the 28S - 18S region of nuclear or cytoplasmic RNA. In HeLa cells on 60-min chase with actinomycin D, the radioactivity in the 45S component disappeared, and in the 35S component considerably decreased, although even after two hours no label was found in the cytoplasmic 28S rRNA (Penman, 1966).

This work was supported by the Polish Academy of Sciences within the project 09.3.1.

REFERENCES

Applebaum S. W., Ebstein R. P. & Wyatt G. R. (1966). *J. Mol. Biol.* **21**, 29 - 41.
Attardi G. & Amaldi F. (1970). *Annu. Rev. Biochem.* **39**, 183 - 226.
Bishop D. H. L., Claybrook J. R. & Spiegelman S. (1967). *J. Mol. Biol.* **26**, 373 - 387.
Britten R. J. & Roberts R. B. (1960). *Science* **131**, 32 - 33.
Brown D. D. (1967). In *Methods in Developmental Biology* (F. H. Wilt & N. U. Wessels, eds.) p. 691. Thomas Y. Crowell Co., New York.
Dahlberg A. E. & Peacock A. C. (1971). *J. Mol. Biol.* **55**, 61 - 74.
Dingman C. W. & Peacock C. A. (1968). *Biochemistry* **7**, 659 - 668.
Dounce A. L. (1955). In *The Nucleic Acids, Chemistry and Biology* (E. Chargaff & J. N. Davidson, eds.) vol. 2, pp. 104 - 108. Academic Press, New York.
Eliceiri G. J. & Green H. (1969). *J. Mol. Biol.* **41**, 253 - 260.
Hastings J. R. M. & Kirby K. S. (1966). *Biochem. J.* **100**, 532 - 539.
Hymer W. C. & Kuff E. L. (1964). *J. Histochem. Cytochem.* **12**, 359 - 363.
Loening U. (1968). *J. Mol. Biol.* **38**, 355 - 365.
Peacock A. C. & Dingman C. W. (1967). *Biochemistry* **6**, 1818 - 1827.
Pene J., Knight E. & Darnell J. (1968). *J. Mol. Biol.* **33**, 609 - 623.
Penman S. (1966). *J. Mol. Biol.* **17**, 117 - 130.
Perry R. P., Cheng T.-Y., Freed J. J., Greenberg J. R., Kelly D. E. & Tartof K. D. (1970). *Proc. Nat. Acad. Sci. U.S.* **65**, 609 - 616.
Perzyński S. & Szafrański P. (1971). *Acta Biochim. Polon.* **18**, 373 - 385.
Petermann M. L. & Pavlovec A. (1963a). *J. Biol. Chem.* **238**, 318 - 323.
Petermann M. L. & Pavlovec A. (1963b). *J. Biol. Chem.* **238**, 3717 - 3724.
Rubinstein L. & Clever U. (1971). *Biochim. Biophys. Acta* **246**, 517 - 529.
Schaup H. W., Best J. B. & Goodman A. B. (1969). *Nature* **221**, 864 - 865.
Scherrer K., Marcaud L., Zajdela F., London J. M. & Gros F. (1966). *Proc. Nat. Acad. Sci. U. S.* **56**, 1571 - 1578.
Tiollais P., Galibert F. & Boiron M. (1971). *Proc. Nat. Acad. Sci. U.S.* **68**, 1117 - 1120.
Wilt F. H. (1967). In *Methods in Developmental Biology* (F. H. Wilt & N. K. Wessels, eds.) p. 667. Thomas Y. Crowell Co., New York.

HETEROGENNOŚĆ I ZNAKOWANIE SIĘ RNA EMBRIONÓW KURZYCH

Streszczenie

Całkowity RNA 5- i 6-dniowych embrionów kurzych dzieli się podczas elektroforezy w żelu poliakrylamidowym w analogiczny sposób jak całkowity RNA z embrionalnej i dorosłej wątroby kurzej. Dwie mniejsze frakcje występujące w obszarze 28S - 18S całkowitego RNA embrionów są pochodzenia rybosomalnego, a ich obecność można przypisać labilności 28S rRNA. Dwie frakcje mniej ruchliwe niż 28S powstają w jądrze i reprezentują ciężki szybkoznakujący się RNA. Są to zapewne prekursory rRNA.

Received 9 June, 1972.

ACTA BIOCHIMICA POLONICA

Vol. 20 1973 No. 2

WANDA DOBRYSZYCKA and J. OSADA

EFFECT OF MODIFICATION ON PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF HAPTOGLOBIN

REACTION WITH SUCCINIC ANHYDRIDE

Department of Clinical Investigation, Biological-Pharmaceutical Institute, Medical School, ul. Szewska 38/39, 50-139 *Wrocław, Poland*

Direct succinylation of haptoglobin (Hp) resulted in about 33% decrease of peroxidase activity of the complex of Hp with haemoglobin. Succinylation of reduced-alkylated Hp preparation resulted in 76% decrease of activity; succinylation of Hp preparation reduced-alkylated in the presence of 8 M-urea led to complete inactivation of the complex and induced dissociation of Hp into α and β chains, as shown by starch-gel and polyacrylamide-gel electrophoresis. In double gel diffusion experiments, rabbit antiserum against native Hp gave precipitin lines with reduced-alkylated-succinylated Hp as well as with succinylated β chain.

The chemical and biological properties of haptoglobin (Hp)[1] have been studied in our laboratory by degradation of the molecule (Dobryszycka & Lisowska, 1966; Lisowska & Dobryszycka, 1967; Dobryszycka & Kukral, 1970), or by blocking of tyrosine or tryptophan residues with specific reagents (Dobryszycka *et al.*, 1969; Dobryszycka & Bec, 1971).

Habeeb *et al.* (1958) demonstrated that chemical modification of proteins with succinic anhydride results in an unfolding of the compact conformation of several proteins accompanied by a large increase of net negative charge produced by the replacement of the positively charged ε-amino groups of lysine residues by negatively charged $-NH-CO-CH_2-CH_2-COO^-$ groups.

In the present work haptoglobin was submitted to succinylation, and the effect of this modification on the ability of Hp to form an active complex with haemoglobin was studied; moreover, the role of Hp amino groups in its antigenic activity was investigated.

[1] Abbreviations used: Hp, haptoglobin; Hb, haemoglobin; TNBS, 2,4,6-trinitrobenzenesulphonic acid; URA-Hp, haptoglobin reduced-alkylated in the presence of 8 M-urea; RA-Hp, haptoglobin reduced-alkylated in the absence of urea.

MATERIALS AND METHODS

Preparations used. Succinic anhydride was a product of B.D.H. (Poole, England), 2-mercaptoethanol of Koch-Light (Colnbrook, Bucks, England), iodoacetamide of Carl Roth (Karlsruhe, G.F.R.), Sephadex G-200 of Pharmacia (Uppsala, Sweden). Haemoglobin was prepared from horse erythrocytes by the method of McQuarrie & Beniams (1954). Human haptoglobin, type 2-1, was prepared from ascitic fluid as described by Dobryszycka & Lisowska (1966). Preparations that showed purity of over 90% as determined by the peroxidase method of Jayle (1951) were freeze-dried and used for succinylation.

Haptoglobin modification. Reduction and alkylation were carried out by two procedures: A, according to Shim & Bearn (1964) with some modifications: Hp, 100 mg, was dissolved in 5 ml of 0.55 M-tris buffer, pH 8.2. Urea and mercaptoethanol were added to final concentrations of 8 M and 0.5 M, respectively. The mixture was left overnight in the refrigerator, and iodoacetamide in 0.55 M-tris buffer was added to a final concentration of 1 M. Alkylation proceeded for 3 h at 4°C in darkness, then the solution was dialysed against 0.2 M-KCl at 4°C. This preparation is further referred to as URA-Hp.

B, By the method of Fleischmann *et al.* as described by Lenard & Singer (1966): to 100 mg of the protein dissolved in 5 ml of 0.55 M-tris buffer, pH 8.2, 0.325 ml of 2-mercaptoethanol was added. After standing at room temperature for 1 h, the solution was cooled in an ice-bath, then was added with 1 g of iodoacetamide dissolved in 5 ml of 0.55 M-tris buffer. After 1 h at 0°C, the solution was dialysed as above. This preparation is further referred to as RA-Hp.

Succinylation of native and reduced-alkylated preparations of Hp was performed according to Lenard & Singer (1966) using 30 mg of succinic anhydride per 100 mg of protein (the concentration of protein was 10 mg per 1 ml of the solution). An automatic titration device was used to maintain the pH 8 by addition of 5 M-KOH. The reaction was continued for about 2 h. The succinylated protein was dialysed against water and freeze-dried.

The URA-succinylated Hp preparation was separated into α and β chains on a column of Sephadex G-200 equilibrated with 0.01 M-tris buffer, pH 8.2.

Analytical procedures. Titration of acid groups was carried out as follows: 10 mg of the protein was dissolved in 5 ml of 0.1 M-KOH and left for 24 h at room temperature. The excess of KOH was titrated with 1 M-HCl using an automatic "Titrator" (Radiometer, Copenhagen, Denmark).

Determination of free amino groups was carried out with TNBS as described by Habeeb (1966).

Protein was determined by the method of Lowry *et al.* (1951).

Electrophoresis. Starch-gel electrophoresis was carried out for 18 h in borate buffer, pH 9.6, according to Smithies (1955), and disc electrophoresis in polyacrylamide gel by the method of Davis (1964) in tris-glycine buffer, pH 8.3, at 4 mA per tube.

Immunochemical assays. Antibodies directed against Hp were obtained in rabbits, each rabbit being given about 10 mg of the protein in the foot-pad, intravenously and intramuscularly. Antiserum was obtained approximately 4 weeks following the initial injection. Horse antihuman antiserum was a product of Wytwórnia Surowic i Szczepionek (Warszawa, Poland), series no. 20672.

Double diffusion in 1% Difco agar in 0.05 M-veronal buffer, pH 8.2, was performed by the method of Ouchterlony (1949). Quantitative reaction was carried out by the method described by Dobryszycka & Bec (1971).

RESULTS

The increase in peroxidase activity of Hb observed on its binding with Hp was used to estimate the capacity of the modified Hp to form an active complex with Hb. The binding ability of the directly succinylated Hp decreased by 33% as compared with native Hp. RA-succinylated Hp retained about 24% of the binding activity of native Hp, whereas URA-succinylated Hp was inactive. Neither α nor β chains showed any binding activity (Table 1).

Table 1

Effect of succinylation of haptoglobin on peroxidase activity of the complex with haemoglobin

For details see Methods.

Preparation	Reaction with Hb (%)
Native HP	100
Directly succinylated Hp	67
Reduced-alkylated-succinylated Hp	24
Reduced-alkylated in the presence of 8 M-urea and succinylated Hp	0
Succinylated α chain	0
Succinylated β chain	0

Titration curves of native and succinylated URA-Hp preparations were different over the pH range 5 - 12 (Fig. 1), the difference corresponding to a loss of the positively charged amino groups replaced by negatively charged acidic groups.

The estimation of free amino groups showed 42 amino groups per mol in native Hp, 47 in 8 M-urea treated Hp, and 9.7 groups in URA-succinylated Hp (Fig. 2).

On starch-gel electrophoresis URA-succinylated Hp migrated faster than native Hp (Fig. 3), and was separated into three protein bands. The α and β chains separated from this preparation on Sephadex G-200 gave in starch gel two and one bands, respectively. A similar pattern was obtained in polyacrylamide-gel electrophoresis (Fig. 4). When electrophoresis of succinylated Hp was carried out with the addition

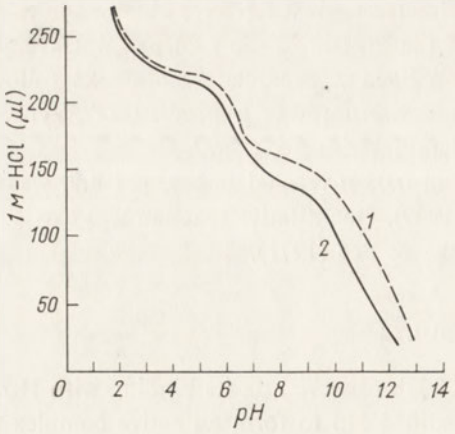


Fig. 1

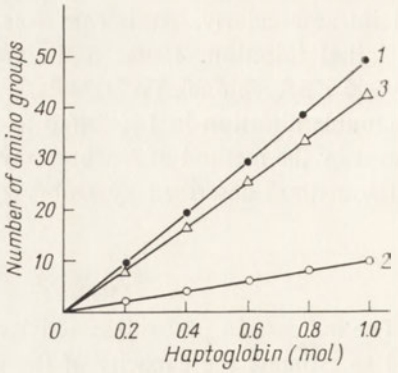


Fig. 2

Fig. 1. Titration curves of *1*, native and *2*, URA-succinylated haptoglobin. The protein, 10 mg, was dissolved in 5 ml of 0.1 M-KOH and left for 24 h at room temperature; excess of KOH was titrated with 1 M-HCl.

Fig. 2. Determination of amino groups in: *1*, native haptoglobin; *2*, URA-succinylated Hp; *3*, native Hp treated with 8 M-urea. The TNBS method of Habeeb (1966) was used; for calculation, the molecular weight of Hp was accepted as 85 000.

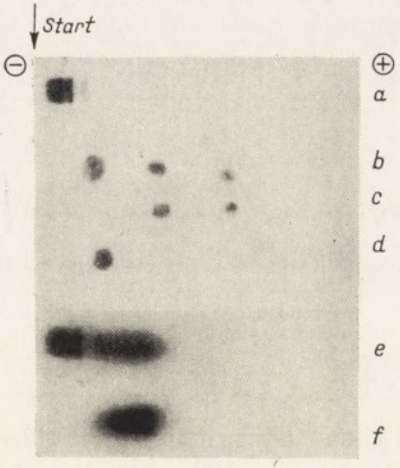


Fig.3

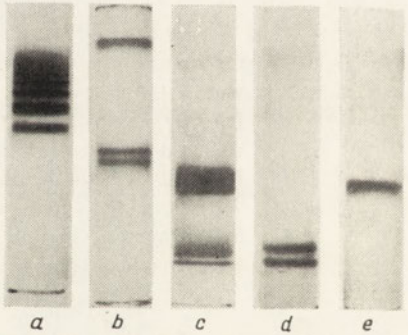


Fig.4

Fig. 3. Starch-gel electrophoretic patterns of: *a*, native haptoglobin; *b*, URA-succinylated Hp; *c*, succinylated *α* chain; *d*, succinylated *β* chain; *e*, native Hp+Hb; *f*, URA-succinylated Hp+Hb. Electrophoresis was carried out at pH 8.6 according to the standard procedure of Smithies (1955). Strips *a* - *d* were stained with Amido Black 10 B in methanol - acetic acid solution, strips *e* and *f* were treated with benzidine - hydrogen peroxide solution.

Fig. 4. Polyacrylamide-gel electrophoretic patterns of: *a*, native haptoglobin; *b*, RA-Hp; *c*, URA-succinylated Hp; *d*, succinylated *α* chain; *e*, succinylated *β* chain. Electrophoresis was carried out at pH 8.3 according to Davis (1964). Staining was done with Amido Black 10 B.

of Hb and stained with benzidine and hydrogen peroxide, one spot corresponding to free haemoglobin was observed (Fig. 3f).

The effect of succinylation on immunochemical properties of Hp was studied by the double gel diffusion technique and by quantitative precipitin reaction. In double gel diffusion, antiserum against Hp_{2-1} or antihuman antiserum in the center wells were used against the preparations of succinylated Hp as well as succinylated α and β chains in outer wells (Fig. 5).

Native Hp gave a distinct precipitin line with anti-Hp antiserum; URA-succinylated Hp and β chain gave less distinct but visible lines, which seem to indicate incomplete antigenic identity. No precipitation was observed with succinylated α chain.

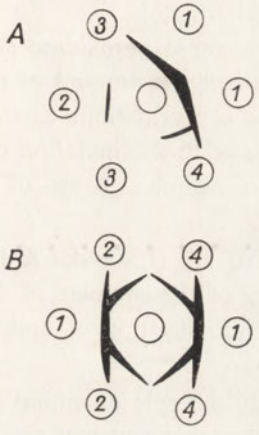


Fig. 5

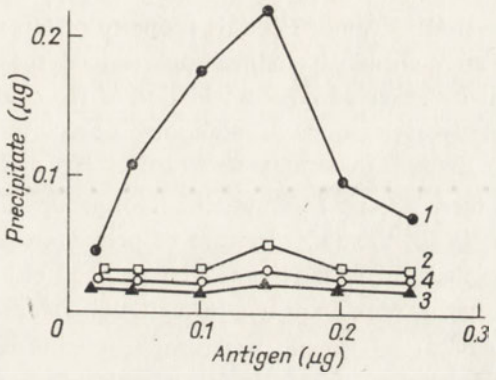


Fig. 6

Fig. 5. Double gel diffusion analysis of succinylated haptoglobin. In the center wells: *A*, rabbit anti-native Hp_{2-1} antiserum, and *B*, horse antihuman antiserum; in the outer wells: *1*, native Hp; *2*, URA-succinylated Hp; *3*, succinylated α chain; *4*, succinylated β chain. Antigen concentration was 0.05%, except for *2* and *4* in *B*, which was 0.2%.

Fig. 6. Precipitin curves of native and succinylated haptoglobin with anti-native Hp_{2-1} antiserum. Antigens used: *1*, native Hp; *2*, URA-succinylated Hp: *3*, succinylated α chain; *4*, succinylated β chain.

Quantitative precipitin curves obtained with anti-Hp antiserum and Hp preparations are shown in Fig. 6. Under the optimum conditions for native Hp (Dobryszycka & Bec, 1971), URA-succinylated Hp and succinylated α chain showed practically no precipitating capacity. At 0.15 µg of β chain a slight maximum of precipitate (0.05 µg) was obtained.

DISCUSSION

Succinylation of protein leads to large conformational changes due presumably to the electrostatic repulsion stemming from the large increase in the net charge (Klotz & Keresztes-Nagy, 1963). Succinic anhydride at pH near neutral reacts with protein primarily at the free amino groups. However, *O*-succinylation of

hydroxy amino acids and *S*-succinylation of cysteine can occur at least in pepsinogen (Gounaris & Perlmann, 1967) and aldolase (Hass, 1964), respectively. With oligomeric proteins extensive succinylation is accompanied by dissociation of the protein into subunits (Meighen & Schachman, 1970a,b). In contrast, limited succinylation of proteins often may be achieved without disruption of tertiary or quarternary structures (Karesztes-Nagy *et al.*, 1965). Scanu *et al.* (1968) have shown that 90% of the ε-amino groups of lysine residues of high-density lipoprotein can be succinylated without the protein dissociating into subunits.

A molecule of Hp consists of four polypeptide chains held together by disulphide bonds (Shim & Bearn, 1964). Each molecule comprises a pair of similar or identical α chains having an approximate mol. wt. of 9000 and another pair of β chains of mol. wt. 36 000.

Haemoglobin, when complexed with Hp, shows enhanced peroxidase activity, and we took advantage of this property of Hp to estimate the amount of protein which after chemical modification retained the native conformation. Changes in the activity observed on succinylation of Hp could be due to succinylation of thiol and OH-tyrosyl groups, in addition to succinylation of ε-amino groups of lysine, and to changes in tertiary structure of Hp molecule.

As there are no free cysteine thiol groups in native Hp (Lisowska & Dobryszycka, 1967), the 33% decrease of peroxidase activity of the complex of directly succinylated Hp with Hb may be ascribed either to modification of amino and OH-tyrosyl groups or to conformational changes.

Shinoda (1965) applied trinitrophenylation to study the role of amino groups in the formation of the Hp-Hb complex and concluded that some of the amino groups are essential for active complex formation. Different groups introduced on the residue of a protein may exert various effects on its biological properties. It has been shown by Dobryszycka & Bec (1972) that the effect of modification of tyrosyl residues on peroxidase activity of the Hp-Hb complex differed depending on the type of modification applied, viz. acetylation, nitration or iodination.

Urea-treated Hp was found to contain 47 free amino groups, which is in agreement with the lysine content as calculated from the data of Lombart *et al.* (1965). In the URA-succinylated Hp preparation there were only 10 free amino groups. Thus it seems likely that the relatively small loss of biological activity of directly succinylated Hp is due to the combined effect of the modification of ε-amino groups and changes in the conformation of Hp molecule. The reduced-alkylated Hp retained 25% of its ability to form an active complex with Hb. But when URA-Hp was submitted to succinylation, the activity was abolished and the modified protein could be separated into polypeptide chains.

Bezkorovainy *et al.* (1969) reported that practically no disulphide bridges remained in transferrin, conalbumin and orosomucoid after the reduction-alkylation treatment. Electrophoresis of succinylated URA-Hp in starch gel as well as in the polyacrylamide gel gave three bands, one of them comprising twice as much protein as the two others. It is known (Shim *et al.*, 1965) that while Hp_{1-1} and Hp_{2-2} are

composed of equal amounts of α and β chains, in Hp_{2-1}, which was used in our work, there exist two types of α chains, slightly differing in molecular weight.

Zschocke & Bezkorovainy (1970) reported that fully succinylated transferrin, immune globulins and reduced-alkylated orosomucoid failed completely to give precipitin lines with antisera to the native proteins. In our experiments, succinylated URA-Hp and even separated succinylated β chain, both inactive in the interaction with Hb, gave precipitin lines when tested with rabbit antiserum against native Hp or with horse antihuman serum. Thus the modified β chain still remained in some way related to the native protein, whereas α chain was found to be completely unrelated.

REFERENCES

Bezkorovainy A., Zschocke R. & Grohlich D. (1969). *Biochim. Biophys. Acta* **181**, 295 - 304.

Davis B. J. (1964). *Ann. N. Y. Acad. Sci.* **121**, 404 - 427.

Dobryszycka W. & Bec I. (1971). *Biochim. Biophys. Acta* **243**, 178 - 186.

Dobryszycka W. & Bec I. (1972). *Abstr. Commun. 8th Meet. Eur. Biochem. Soc.* no. 460.

Dobryszycka W. & Kukral J. C. (1970). *Arch. Immun. Ther. Exp.* **18**, 527 - 536.

Dobryszycka W. & Lisowska E. (1966). *Biochim. Biophys. Acta* **121**, 42 - 50.

Dobryszycka W., Pusztai A. & Kukral J. C. (1969). *Biochim. Biophys. Acta* **175**, 271 - 281.

Gounaris A. D. & Perlmann G. E. (1967). *J. Biol. Chem.* **242**, 2739 - 2745.

Habeeb A. F. S. A. (1966). *Anal. Biochem.* **14**, 328 - 336.

Habeeb A. F. S. A., Cassidy H. G. & Singer S. J. (1958). *Biochim. Biophys. Acta* **29**, 587 - 593.

Hass L. F. (1964). *Biochemistry* **3**, 535 - 541.

Jayle M. F. (1951). *Bull. Soc. Chim. Biol.* **33**, 876 - 880.

Keresztes-Nagy S., Lazer L., Klapper M. H. & Klotz I. M. (1965). *Science* 357 - 359.

Klotz I. M. & Keresztes-Nagy S. (1963). *Biochemistry* **2**, 445 - 452.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265 - 275.

Lenard J. & Singer S. J. (1966). *Nature (London)* **210**, 536 - 537.

Lisowska E. & Dobryszycka W. (1967). *Biochim. Biophys. Acta* **133**, 338 - 345.

Lombart C., Dautrevaux M. & Moretti J. (1965). *Biochim. Biophys. Acta* **97**, 270 - 274.

McQuarrie M. B. & Beniams H. N. (1954). *Proc. Soc. Exp. Biol. Med.* **86**, 627 - 632.

Meighen E. A. & Schachman H. K. (1970a). *Biochemistry* **9**, 1163 - 1176.

Meighen E. A. & Schachman H. K. (1970b). *Biochemistry* **9**, 1177 - 1184.

Ouchterlony O. (1949). *Acta Pathol. Microbiol. Scand.* **26**, 507 - 515.

Scanu A., Reader W. & Edelstein C. (1968). *Biochim. Biophys. Acta* **160**, 32 - 45.

Shim B. S. & Bearn A. G. (1964). *J. Exp. Med.* **120**, 611 - 628.

Shim B. S., Lee T. H. & Kang Y. S. (1965). *Nature (London)* **207**, 1264 - 1267.

Shinoda T. (1965). *J. Biochem. (Tokyo)* **57**, 100 - 102.

Smithies O. (1955). *Biochem. J.* **61**, 629 - 641.

Zschocke R. H. & Bezkorovainy A. (1970). *Biochim. Biophys. Acta* **200**, 241 - 246.

WPŁYW MODYFIKACJI NA FIZYKO-CHEMICZNE I BIOLOGICZNE WŁAŚCIWOŚCI HAPTOGLOBINY

REAKCJA Z BEZWODNIKIEM BURSZTYNOWYM

Streszczenie

Bezpośrednia sukcynylacja haptoglobiny (Hp) powodowała 33% spadek aktywności peroksydazowej kompleksu Hp z hemoglobiną. Sukcynylacja Hp po redukcji i alkilowaniu powodowała 76% spadek aktywności peroksydazowej kompleksu lub w przypadku użycia mocznika — całkowity zanik aktywności. Zastosowanie tego ostatniego postępowania powodowało dysocjację Hp na łańcuchy α i β, co wykazano przy pomocy elektroforezy w żelu skrobiowym i poliakryloamidowym. Badania przy pomocy podwójnej dyfuzji w żelu wykazały, że królicza surowica odpornościowa przeciwko natywnej Hp dawała łuki precypitacyjne z redukowaną-alkilowaną-sukcynylowaną Hp oraz z sukcynylowanym łańcuchem β.

Received 1 July, 1972.

ACTA BIOCHIMICA POLONICA

Vol. 20 1973 No. 2

M. CHARĘZIŃSKI and T. BORKOWSKI

STUDIES ON BINDING OF AMINO ACIDS BY MITOCHONDRIAL AND CYTOPLASMIC tRNA FROM CALF BRAIN

Department of Physiological Chemistry, Medical School, ul. Lubartowska 85, 20-123 *Lublin, Poland*

Aminoacylation of tRNA isolated from brain mitochondria and cytoplasm was studied using ^{14}C-labelled arginine, glutamic acid, glycine, leucine, phenylalanine and serine. The acceptor activity of tRNA was always higher in the presence of aminoacyl-tRNA synthetases isolated from the homologous subcellular fraction. Both in the mitochondrial and the cytoplasmic systems, formation of glutamyl-tRNA was the highest.

The presence of both tRNA and aminoacyl-tRNA synthetases in mitochondria of *Neurospora crassa* was demonstrated by Barnett & Brown (1967) and Barnett *et al.* (1967), and in mitochondria of mammalian liver by Buck & Nass (1968).

A high concentration of sRNA in brain mitochondria, four times that found in liver mitochondria, has been reported by Borkowski *et al.* (1967, 1968). As demonstrated by Borkowski & Charęziński (1971) the low-molecular-weight RNA from brain mitochondria binds amino acids in the presence of mitochondrial or cytoplasmic aminoacyl-tRNA synthetases. Moreover, differences were observed in the activity of these synthetases toward tRNA's isolated from mitochondria or cytoplasm, on incubation with ^{14}C-labelled amino acids of the protein hydrolysate from algae. Buck & Nass (1968) demonstrated significant differences in the enzyme specificity and acceptor activity of tRNA's from liver cytoplasm and mitochondria.

The present paper reports the results of experiments in which binding of particular amino acids to specific tRNA's has been compared both in homologous systems (enzyme and tRNA from mitochondria or cytoplasm) and in heterologous ones (mitochondrial enzyme and cytoplasmic tRNA, or *vice versa*).

MATERIALS AND METHODS

Material. Fresh calf brains, obtained from the municipal slaughterhouse in Lublin, were used for experiments. The brain was isolated immediately after killing of the animal and cooled in ice. All further manipulations were performed at 0 - 4°C. The brain, after removal of blood vessels, was homogenized in a Potter-Elvehjem

homogenizer with 10 vol. of 0.44 M-sucrose - 1 mM-EDTA solution, adjusted to pH 7.2 with 1 M-KOH. Mitochondria were isolated by the method of Løvtrup & Zelander (1962), and their purity was checked by electron microscopy.

Isolation of mitochondrial and cytoplasmic tRNA's. Nucleic acids were isolated from mitochondria by phenol extraction (Borkowski *et al.*, 1968) and fractionated by methylated albumin column chromatography (Mandell & Hershey, 1960). The tRNA eluted from the column with 0.4 M-NaCl, was precipitated with 2.5 vol. of ethanol and stored at -20°C.

From the post-mitochondrial fraction microsomes were removed by centrifugation at 100 000 *g* for 70 min in a WAC (Janetzki, G.D.R.) ultracentrifuge, to the supernatant sodium acetate was added to a concentration of 2%, and RNA was precipitated with 2.5 vol. of ethanol and left overnight at -20°C. After centrifugation, the sediment was dissolved in 0.14 M-NaCl - 0.01 M-tris·HCl buffer, pH 7.2, RNA was extracted by the phenol method and tRNA isolated as described for the mitochondrial tRNA. The endogenous amino acids bound to tRNA were removed by incubation in NH_4OH solution of pH 10 at 37°C for 30 min; then the mixture was neutralized and tRNA precipitated with 2.5 vol. of ethanol.

The E_{280}/E_{260} ratios for the mitochondrial and cytoplasmic tRNA preparations were within the range 0.48 - 0.50. The concentration of tRNA was calculated from the extinction at 260 nm; 1 mg of tRNA corresponded to 20 extinction units (Rubin *et al.*, 1967).

Isolation of mitochondrial and cytoplasmic aminoacyl-tRNA synthetases. Mitochondria (corresponding to 32 g of brain) were suspended in 30 ml of 0.15 M-KCl - 1 mM-EDTA - 1 mM-2-mercaptoethanol - 0.05 M-tris·HCl buffer, pH 7.5, disintegrated by ultrasonic treatment in an MSE (100 W, no. 7100) instrument at the maximum amplitude, 4×1 min, with intermittent cooling to 0°C, and centrifuged at 100 000 *g* for 70 min. The sediment was discarded and from the supernatant the enzymic protein was precipitated at 0.6 ammonium sulphate saturation. After 2 h the sediment was collected by centrifugation at 100 000 *g* for 30 min and dissolved in 4 ml of 0.2 M-potassium-phosphate buffer, pH 7.5, containing 0.01 M-2-mercaptoethanol. To remove endogenous tRNA, it was passed over a DEAE-cellulose column (0.9×12 cm) which was equilibrated and eluted with the same buffer according to Barnett *et al.* (1967). The first protein fraction contained the enzymic activity; it was dialysed against 0.01 M-2-mercaptoethanol - 0.05 M-tris·HCl buffer, pH 7.5, for 20 h to remove phosphates, then the protein solution was concentrated using polyethyleneglycol.

From the post-microsomal supernatant (see above) the protein fraction precipitating at 0.25 - 0.6 ammonium sulphate saturation was collected, dissolved in 4 ml of 0.01 M-2-mercaptoethanol - 0.2 M-potassium-phosphate buffer, pH 7.5, and the aminoacyl-tRNA synthetases isolated as described for the mitochondrial enzymes.

The concentration of enzymic protein was determined by the method of Lowry *et al.* (1951).

Determination of activity of aminoacyl-tRNA synthetases. The standard incubation mixture contained in a volume of 0.3 - 0.5 ml: tris·HCl buffer, pH 7.5, 50

μmol; $MgCl_2$, 10 μmol; KCl, 2.5 μmol; NH_4Cl, 2.5 μmol; reduced glutathione, 5 μmol; ATP, 2 μmol; [^{14}C]amino acid, 1 μCi, or a mixture of six [^{14}C]amino acids, 1 μCi each; tRNA, 10 - 30 μg, and enzyme protein, 0.5 mg. The control sample contained no tRNA. The time of incubation was 20 min at 37°C.

In experiments with one amino acid, 50 μl portions of the incubation mixture were applied directly to discs of Whatman 3 *MM* paper, dried, treated with cold 10% trichloroacetic acid to precipitate the formed [^{14}C]aminoacyl-tRNA complex, and washed as described by Rubin *et al.* (1967).

In the experiments with six amino acids incubated simultaneously, the following procedure was used. After incubation the mixture was cooled rapidly to 0°C, carrier RNA (8 extinction units) was added, diluted to 3 ml with cold water, and extraction with an equal volume of 90% phenol in water was carried out for 1 h at 0°C. After centrifugation at 10 000 *g* for 30 min, from the aqueous phase the RNA and [^{14}C]aminoacyl-tRNA's were precipitated with 2.5 vol. of ethanol and left at -20°C for 12 h. The sediment was centrifuged and washed three times with cold 70% ethanol to remove free radioactive amino acids. The sediment was hydrolysed with 2 M-NH_4OH for 1 h at 37°C. The ammonia was removed by evaporation, to the residue 2 ml of water was added, RNA was precipitated with 2.5 vol. of ethanol, centrifuged off, and the supernatant containing the amino acids released from the complex was freeze-dried. The preparation was dissolved in 0.1 ml of water, carrier

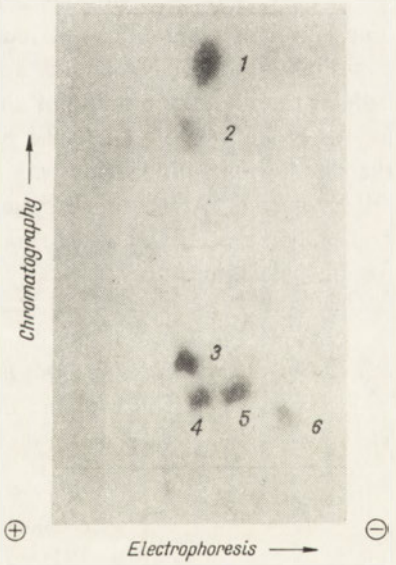


Fig. 1. Electrochromatogram of amino acids obtained from the aminoacyl-tRNA complex formed during incubation with the mixture of six amino acids. *1*, Leucine; *2*, phenylalanine; *3*, glutamic acid; *4*, serine; *5*, glycine; *6*, arginine.

amino acids (2 mg each) were added, and the solution submitted to electrochromatography according to Fischl & Segal (1963) on Whatman 3 *MM* paper. In the first dimension the electrophoresis was carried out in 7.8% acetic acid - 2.5% formic acid mixture (1:1, v/v), pH 2.2 at 20 V/cm for 1 h; in the second dimension chromatography in *n*-butanol - acetic acid - water (4:1:1, by vol.) was applied. The amino

acids were detected with ninhydrin (Fig. 1), the particular spots were cut out and radioactivity measured.

The acceptor activity of tRNA was expressed as pmol of [^{14}C]amino acid, calculated per 100 μg of tRNA per 1 mg of enzyme protein.

Radioactivity measurements. Counting in the liquid scintillator was carried out as described by Rubin *et al.* (1967) in USB-2 type counter (Polon, Warszawa, Poland). The liquid scintillator system contained per sample 20 mg of PPO and 0.5 mg of POPOP in 5 ml of toluene. For each amino acid a separate calibration curve was constructed by determining the dependence of concentration on the number of impulses in the scintillation counter. The amount of particular amino acids bound to tRNA was calculated from the measured number of impulses/min.

Reagents. The following uniformly ^{14}C-labelled amino acids were used: L-glutamic acid (125 mCi/mmol), L-arginine (143 mCi/mmol), L-glycine (52 mCi/mmol), L-leucine (20.2 mCi/mmol), L-phenylalanine (115 mCi/mmol) and L-serine (20 mCi/mmol), all from UVVR (Praha, Czechoslovakia); non-labelled amino acids were from B.D.H. Lab. (Poole, Dorset, England). ATP-disodium salt, reduced glutathione, 2-mercaptoethanol and yeast RNA were from Koch-Light Lab. (Colnbrook, Bucks, England). DEAE-cellulose was from Whatman Biochem. (Maidstone, Kent, England).

RESULTS

In the experiments with six amino acids, the incubation mixture contained arginine, glycine, glutamic acid, leucine, phenylalanine and serine. In the systems containing both tRNA's and aminoacyl-tRNA synthetases from the same subcellular fraction, the binding of all amino acids tested was higher than in the heterologous systems (Table 1). In the system: mitochondrial enzyme - cytoplasmic tRNA the binding of amino acids was higher than in the system: cytoplasmic enzyme - mitochondrial tRNA. In all systems the binding of glutamic acid was several-fold higher than that of any other amino acid. The lowest acceptor activity was observed for phenylalanine.

Table 1

Binding of amino acids by brain mitochondrial and cytoplasmic tRNA on incubation with the mixture of six amino acids

The activity is expressed as pmol of the bound amino acid/100 μg of tRNA/mg of enzyme protein.

Amino acid	Mitochondrial enzyme		Cytoplasmic enzyme	
	mitochondrial tRNA	cytoplasmic tRNA	cytoplasmic tRNA	mitochondrial tRNA
Arginine	13.6	4.2	7.6	1.8
Glutamic acid	358.0	260.0	281.0	121.4
Glycine	20.2	8.4	15.8	10.6
Leucine	14.6	9.8	22.4	7.2
Phenylalanine	3.6	1.8	4.0	0.8
Serine	19.4	8.2	19.6	6.2

Table 2

Binding of amino acids by brain mitochondrial and cytoplasmic tRNA on incubation with one amino acid

The activity is expressed as pmol of the bound amino acid/100 μg of tRNA/mg of enzyme protein.

Amino acid	Mitochondrial enzyme		Cytoplasmic enzyme	
	mitochondrial tRNA	cytoplasmic tRNA	cytoplasmic tRNA	mitochondrial tRNA
Glutamic acid	390.0	236.0	380.0	138.0
Leucine	79.2	50.0	66.4	27.2
Phenylalanine	68.8	47.4	65.0	26.0
Serine	25.2	10.8	23.8	6.6

When the incubation system contained a single amino acid, the results obtained were similar (Table 2). The highest aminoacylation of tRNA was observed in homologous systems, and the lowest in the system composed of cytoplasmic enzyme and mitochondrial tRNA. Of the four amino acids tested, the highest binding was observed with glutamic acid and the lowest with serine, whereas the binding of leucine and phenylalanine was several-fold higher than on incubation with the mixture of six amino acids.

DISCUSSION

The presented analytical procedure permits to study the acceptor activity of tRNA simultaneously for several amino acids. It is especially suitable for preliminary assay of mixtures of different tRNA's. In our experiments, unresolved mixtures of tRNA's and of aminoacyl-tRNA synthetases isolated from calf brain were used.

The observed highest binding of glutamic acid is in agreement with the earlier results of Szafrański & Sułkowski (1959) who, using the hydroxamic method, studied the activation of amino acids in various guinea pig tissues and found very high formation of glutamyl-AMP in brain tissue. The high requirement of glutamic acid in the biosynthesis of brain protein suggested by these facts is supported by the occurrence of acidic protein fractions specific for the brain, reported by Moore (1965).

Formation of aminoacyl-tRNA complexes in brain mitochondrial and cytoplasmic homologous or heterologous systems is similar to that reported by Barnett *et al.* (1967) for *N. crassa* and by Burkard *et al.* (1970) for the chloroplast and cytoplasmic systems from *Phaseolus vulgaris*.

The higher acceptor activity observed in our experiments in homologous systems as compared with the heterologous ones, could be due to different amounts of tRNA and specific aminoacyl-tRNA synthetases in the mitochondrial and cytoplasmic preparations, or to the structurally and functionally different tRNA's and enzymes being present in brain mitochondria and cytoplasm.

The results of Barnett *et al.* (1967) who in *N. crassa* mitochondria found specific systems of tRNA and aminoacyl-tRNA synthetases for aspartic acid, phenylalanine and leucine, seems to point to this last possibility. However, the occurrence of different tRNA species specific for the particular amino acids should also be considered, as distinct differences in intracellular location of leucyl-tRNA's in tobacco leaves were found by Guderian *et al.* (1972).

This work was supported by the Polish Academy of Sciences within the Project 0.9.3.1.

REFERENCES

Barnett W. E. & Brown D. H. (1967). *Proc. Nat. Acad. Sci. U.S.* **57**, 452 - 458.
Barnett W. E., Brown D. H. & Epler J. R. (1967). *Proc. Nat. Acad. Sci. U.S.* **57**, 1775 - 1781.
Borkowski T., Borkowska I., Sikorska K. & Kulesza S. (1967). *Bull. Acad. Pol. Sci., Ser. Sci. Biol.* **15**, 511 - 516.
Borkowski T., Sikorska K. & Borkowska I. (1968). In *Macromolecules and Function of the Neuron* (Z. Lodin & S. P. R. Rose, eds.) p. 187 - 192. Excerpta Medica Foundation, Amsterdam.
Borkowski T. & Charęziński M. (1971). *J. Neurochem.* **18**, 851 - 857.
Buck C. A. & Nass M. M. K. (1968). *Proc. Nat. Acad. Sci. U. S.* **60**, 1045 - 1052.
Burkard G., Guillemaut P. & Weil J. H. (1970). *Biochim. Biophys. Acta* **224**, 184 - 198.
Fischl J. & Segal S. (1963). *Clin. Chim. Acta* **8**, 399 - 405.
Guderian R. H., Pulliam L. R. & Gordon M. P. (1972). *Biochim. Biophys. Acta* **262**, 50 - 65.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265 - 275.
Løvtrup S. & Zelander T. (1962). *Exp. Cell. Res.* **27**, 468 - 473.
Mandell J. & Hershey A. D. (1960). *Anal. Biochem.* **1**, 66 - 67.
Moore B. W. (1965). *Biochem. Biophys. Res. Commun.* **19**, 739 - 744.
Rubin I. B., Kelmers A. D. & Goldstein G. (1967). *Anal. Biochem.* **20**, 533 - 544.
Szafrański P. & Sułkowski E. (1959). *Acta Biochim. Polon.* **6**, 133 - 141.

BADANIA NAD WIĄZANIEM AMINOKWASÓW PRZEZ tRNA MITOCHONDRIALNY I CYTOZOLOWY MÓZGU CIELĘCEGO

Streszczenie

Aminoacylację tRNA izolowanych z mitochondriów i cytozolu komórek mózgowych badano przy zastosowaniu argininy, kwasu glutaminowego, glicyny, leucyny, fenyloalaniny i seryny znakowanych ^{14}C. Aktywność akceptorowa tRNA była zawsze wyższa w obecności aminoacylo-tRNA syntetaz izolowanych z homologicznej frakcji podkomórkowej. Zarówno w układzie mitochondrialnym jak i cytozolowym tworzenie się glutamylo-tRNA było znacznie większe w stosunku do innych aminokwasów.

Received 5 August, 1972.

ACTA BIOCHIMICA POLONICA

Vol. 20 1973 No. 2

A. PASZEWSKI and J. GRABSKI

STUDIES ON β-CYSTATHIONASE AND O-ACETYLHOMOSERINE SULFHYDRYLASE AS THE ENZYMES OF ALTERNATIVE METHIONINE BIOSYNTHETIC PATHWAYS IN *ASPERGILLUS NIDULANS*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, 02-532 *Warszawa, Poland*

Two enzymes directly involved in homocysteine synthesis, i.e. β-cystathionase and *O*-acetylhomoserine sulfhydrylase, were identified and characterized in the fungus *Aspergillus nidulans*. Synthesis of these enzymes is partially repressed in the presence of methionine or homocysteine in the growth medium. *In vitro*, neither enzyme is inhibited by methionine. *O*-Acetylhomoserine sulfhydrylase is partially inhibited by *O*-acetyl-L-serine.

In fungi two alternative pathways of biosynthesis of homocysteine, the immediate precursor of methionine, are known:

O-Acetyl-L-homoserine + L-cysteine → L-cystathionine + acetic acid (1a)

L-Cystathionine + H_2O → L-homocysteine + pyruvic acid + NH_3 (1b)

and

O-Acetyl-L-homoserine + H_2S → L-homocysteine + acetic acid (2)

The reaction (1a) is catalysed by cystathionine γ-synthase, the reaction (1b) by β-cystathionase and the reaction (2) by *O*-acetyl-L-homoserine sulfhydrylase (OAHS)[1]. All three reactions have been studied in *Neurospora crassa* (Flavin & Slaughter, 1964; Kerr & Flavin, 1968, 1970; Wiebers & Garner, 1967a,b; Kerr, 1971). Biochemical data showed that the enzymes of both pathways have appreciable activities but genetic evidence suggests that the pathway involving cystathionine is the main one in this organism. Auxotrophic mutants lacking cystathionine γ-synthase or β-cystathionase but with normal activity of OAHS have been isolated (Kerr & Flavin, 1970; Kerr, 1971). On the other hand, in *Saccharomyces cerevisiae* a methionine-requiring mutant has been found which hardly shows any activity

[1] Abbreviations: OAHS, *O*-acetyl-L-homoserine sulfhydrylase; OASS, *O*-acetyl-L-serine sulfhydrylase.

of OAHS (Cherest *et al.*, 1969). This points to the sulfhydrylase reaction as the main path of homocysteine synthesis in yeast.

The existence of two alternative pathways of homocysteine biosynthesis makes them a very interesting model for studying the regulation of enzyme synthesis in fungi. This report concerns some biochemical and regulatory aspects of β-cystathionase and OAHS in *Aspergillus nidulans*.

MATERIAL AND METHODS

Chemicals. *O*-Acetyl-DL-homoserine was synthesized according to Wiebers & Garner (1967b), *O*-acetyl-L-serine was synthesized by the method of Greenstein & Winitz (1961). DL-allo-Cystathionine was purchased from Calbiochem Inc. (Los Angeles, Calif., U.S.A.), DL-homocysteine thiolactone·HCl and 1-naphthylethylenediamine diHCl from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), L-cysteine from Loba-Chemie (Wien, Austria), L-methionine and pyridoxal-5′-phosphate from Merck (Darmstadt, West Germany). Human γ-globulin (mol. wt. 150 000) was a product of Warsaw Serum and Vaccine Laboratories (Warszawa, Poland) and egg white lysozyme (mol. wt. 17 500) of Reanal (Budapest, Hungary). Cysteic acid and homocysteic acid were prepared from cysteine and homocysteine according to Bessman *et al.* (1967). All inorganic compounds were reagent grade.

Biological material. The biotine-requiring strain of *A. nidulans* (*biI*) used in this work was obtained from the Department of Genetics, University of Glasgow. It behaves like wild type with respect to methionine biosynthesis.

Media and culture conditions. Liquid minimal medium contained mineral salts as described by Cove (1966) and microelements corresponding to the Difco Yeast Nitrogen Base without amino acids. Glucose was the sole carbon source. Erlenmeyer flasks (250 ml) containing 150 ml of minimal medium, supplemented, if necessary, with appropriate amino acids at final concentration of 2 mM (calculated for L-form), were inoculated with 10 ml of a heavy conidial suspension in the same medium and incubated at 30 - 31°C in a rotary shaker for 18 - 20 h. Mycelium was then filtered on surgical gauze and washed with water. Blotted mycelial pad was mixed with glass powder and ground in a chilled mortar with 5 - 7 volumes of 0.1 M-potassium phosphate buffer, pH 7.5, which was added in small portions during grinding. The resulting slurry was centrifuged at 15 000 *g* for 15 min at 4°C and the supernatant (3 - 6 mg protein/ml) was used as the enzyme source. Freshly prepared extracts were used throughout this work.

Enzyme assays. *O*-Acetylhomoserine sulfhydrylase (OAHS) activity was estimated in a reaction mixture similar to that described by Wiebers & Garner (1967a). It contained in the final volume of 0.5 ml: *O*-acetyl-DL-homoserine, 40 μmol; sodium sulfide, 5 μmol; pyridoxal-5′-phosphate, 0.2 μmol; potassium phosphate buffer, pH 7.5, 100 μmol, and mycelium extract corresponding to 0.3 - 0.7 mg protein. Two blanks were prepared: one without enzyme and one without *O*-acetylhomoserine. The reaction was started by the addition of sodium sulfide, carried out at 37°C for 15 min and terminated by immersing the tubes in ice-bath.

The amount of homocysteine was estimated in 50-µl portions of reaction mixtures exactly as described by Kredich & Tomkins (1966). A standard curve was prepared with pure homocysteine thiolactone in alkaline solution as described by Abrahamson & Shapiro (1965).

For *O*-acetylserine sulfhydrylase (OASS) assay the reaction mixture in a volume of 0.5 ml contained: *O*-acetyl-L-serine, 15 µmol; pyridoxal-5′-phosphate, 0.2 µmol; potassium phosphate buffer, pH 7.2, 100 µmol; sodium sulfide dissolved in the same buffer, 5 µmol and mycelium extract corresponding to 0.3 - 0.7 mg protein. The control was without *O*-acetylserine. The reaction was started by the addition of sodium sulfide, carried out at 37°C for 15 min, and terminated by immersing the tubes in ice-bath. Cysteine formed was estimated by the method of Gaitonde (1967) as modified by Pieniążek (personal communication): 0.2 ml of reaction mixture was treated with 1 ml of ninhydrin-acid solution (250 mg of ninhydrin dissolved in 16 ml of glacial acetic acid and 4 ml of concentrated hydrochloric acid) and kept at 100°C for 5 min. Then the mixture was cooled and 2 ml of 96% ethanol was added. Extinction was read at 560 nm with the control as reference. For calculation the standard curve for cysteine was used.

For *β*-cystathionase assay the reaction mixture contained in a final volume of 0.5 ml: DL-allo-cystathionine, 2 µmol; pyridoxal-5′-phosphate, 0.2 µmol; potassium phosphate buffer, pH 7.8, 100 µmol, and mycelium extract (0.3 - 0.6 mg protein). Cystathionine was omitted in the control. Incubation was carried out at 37°C for 45 min and the reaction was terminated by immersing the tubes in ice-bath. Since a part of cystathionine is cleaved by another enzyme, *γ*-cystathionase, to cysteine which gives the same colour reaction as homocysteine in the method of Kredich & Tomkins (1966), to estimate the amount of homocysteine formed two procedures were used.

In the first cysteine and homocysteine were oxidized to cysteic and homocysteic acids, respectively, and separated electrophoretically. After incubation, to the reaction mixture 1 ml of water was added followed by 0.15 ml of 1.5 M-perchloric acid. The protein precipitate was removed by centrifugation and amino acids were isolated from the supernatant on Dowex 50 (H^+) column as described by Delivier-Klutchko & Flavin (1965). The eluent containing amino acids was evaporated. Cysteine (and cystine) and homocysteine (and homocystine) in the residue were oxidized by treatment with 1.5 ml of mixture of 85% formic acid and 30% H_2O_2 as described by Bessman *et al.* (1967). After 1 hour the mixture was evaporated and the dried residue was dissolved in 0.2 ml of water. This solution (50 µl) was applied on Whatman no. 3 paper in a 4 cm band. The proper sample, its control, and cysteic and homocysteic acid standards (20 µg of each) were applied on the same sheet. Electrophoresis was done as described by Bessman *et al.* (1967). The dried paper was immersed in 0.4% ninhydrin solution in acetone, dried again and kept overnight in the dark at room temperature. The colour spots were fixed with a mixture of 2 ml of saturated copper nitrate solution, 0.5 ml of 10% nitric acid and 200 ml of acetone, similar to that described by Fischer & Dorfel (1953). The spots corresponding to cysteic and homocysteic acids were eluted with 4 ml of

ethanol during 15 min. Extinction at 504 nm was read and the amount of the two acids was calculated from standard curves.

In the second method, the sum of homocysteine and cysteine was estimated according to Kredich & Tomkins (1966) as in the assay for OAHS, cysteine according to Pieniążek as in the assay for OASS, and the amount of homocysteine formed was calculated.

The second procedure is much simpler, and in fact it was normally used once the reaction products had been identified electrophoretically. Usually the specific activity for β-cystathionase was slightly higher when estimated by the second method. It should be noted that during oxidation in the first method and during treatment with acid-ninhydrin reagent in cysteine estimation in the second procedure, a part of cystathionine is chemically decomposed giving cysteine and homocysteine. To account for the chemical decomposition of cystathionine this substance was added to the control reaction mixtures at the end of incubation.

The protein content in the extracts was determined by the method of Lowry *et al.* (1951).

RESULTS

Extracts from the *bi1* strain of *A. nidulans* showed activity of both β-cystathionase and OAHS. Gel filtration of the extracts through a Sephadex G-25 column (0.5× 25 cm) did not change the specific activities of the enzymes. In the mycelium extract the activity of OAHS was linear with protein concentration up to about 3.3 mg/ml, and with time to about 20 min; the respective values for β-cystathionase were 1.2 mg protein and 50 min. The specific activity of OAHS was about 20 times higher than that of β-cystathionase. The K_m of β-cystathionase was 0.33 mM (Fig. 1), and that of OAHS for *O*-acetyl-L-homoserine 22 mM (Fig. 2). When the dependence of OAHS activity on sulfide concentration was studied, a sigmoidal curve (Fig. 3),

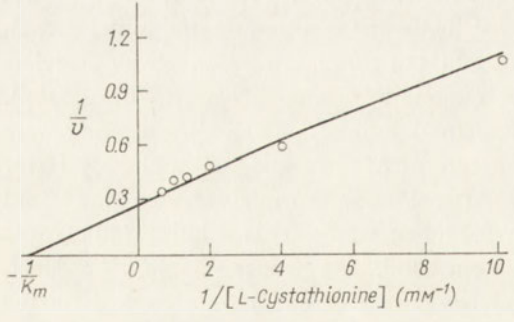


Fig. 1

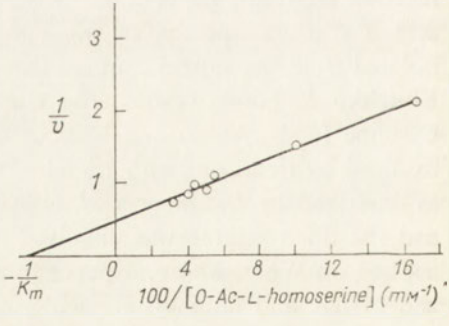


Fig. 2

Fig. 1. Effect of L-cystathionine concentration on β-cystathionase activity. The assays were performed by the procedures of Kredich & Tomkins (1966) and Gaitonde (1967). The velocity, v, is expressed in terms of E_{540}.

Fig. 2. Effect of *O*-acetyl-L-homoserine concentration on *O*-acetylhomoserine sulfhydrylase activity. Sulfide concentration was 10 mM. The velocity, v, is expressed in terms of E_{540}.

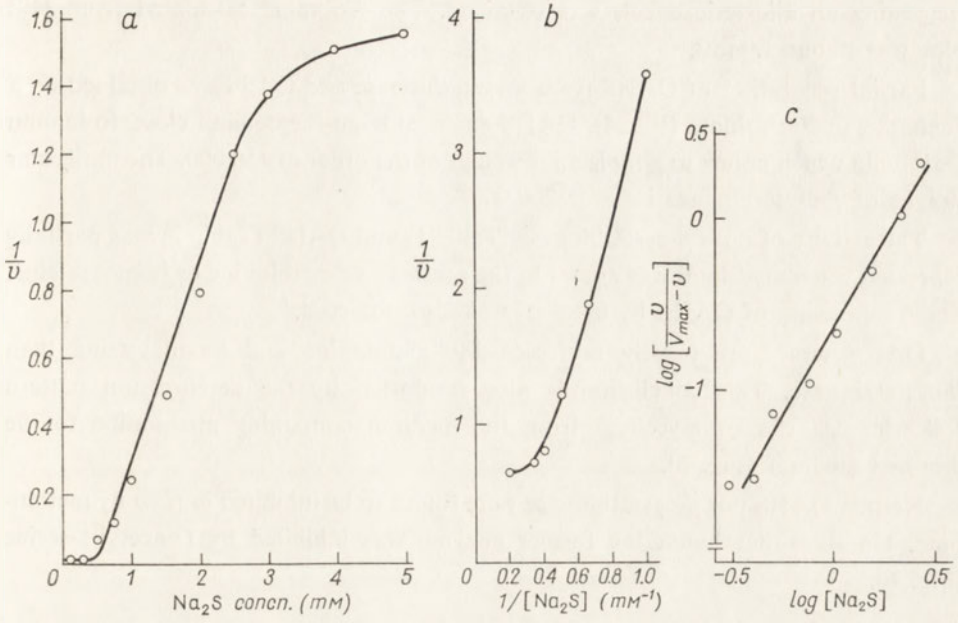


Fig. 3. Effect of sulfide concentration on *O*-acetylhomoserine sulfhydrylase activity; *a*, [S] - v plot; *b*, double-reciprocal plot; *c*, Hill plot. Concentration of *O*-acetyl-L-homoserine was 45 mM. The velocity, v, is expressed in terms of E_{540}.

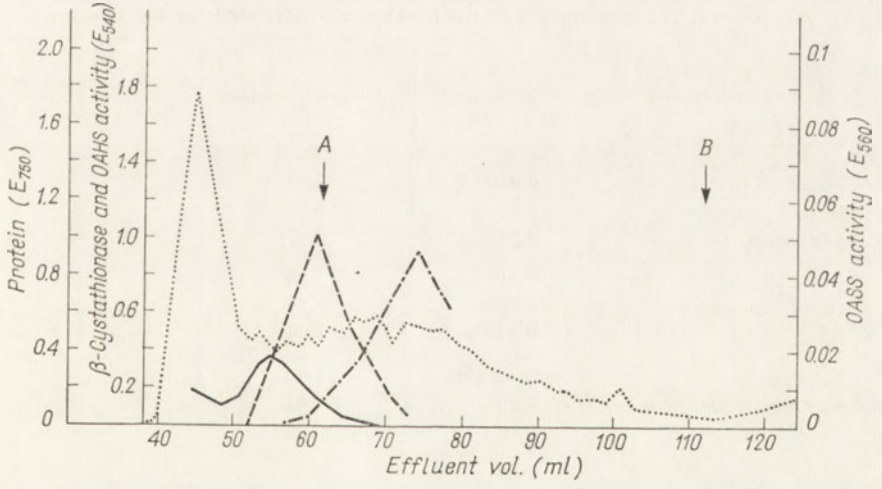


Fig. 4. Separation of β-cystathionase, *O*-acetylhomoserine sulfhydrylase (OAHS) and *O*-acetylserine sulfhydrylase (OASS) of *Aspergillus nidulans* by gel filtration on Sephadex G-200 column (1.5 × 82 cm). The 105 000 *g* supernatant of mycelial homogenate (1.5 ml) was applied to the column equilibrated with 0.1 M-potassium phosphate buffer, pH 7.5, and eluted with the same buffer (0.25 ml/min). Fractions of 1.5 ml were collected and in 0.1 ml samples enzyme activities and protein were estimated as described in the Methods. The arrows mark the elution volume of *A*, human γ-globulin and *B*, lysozyme. ——, β-Cystathionase (E_{540}); - - -, OAHS (E_{540}); ·····, OASS (E_{560}); ···, protein (E_{750}).

suggesting an allosteric effect, was obtained. The K_m value calculated from Hill plot was about 1.9 mM.

Partial separation of OAHS from β-cystathionase and OASS was obtained on a Sephadex G-200 column (Fig. 4). OAHS emerged from the column close to human γ-globulin which points to a molecular weight of the order of 150 000. The molecular weight of β-cystathionase in over 200 000.

The activity of both β-cystathionase (Table 1) and OAHS (Table 2) was partially repressed when mycelium was grown in the presence of methionine or homocysteine. Some repression of OAHS by cysteine was also observed.

OAHS was more readily repressed by methionine and homocysteine than β-cystathionase. This conclusion is also supported by the derepression pattern following transfer of mycelium from the medium containing methionine to the minimal medium (Fig. 5).

Neither OAHS nor β-cystathionase were found to be inhibited *in vitro* by methionine. On the other hand, the former enzyme was inhibited by *O*-acetyl-L-serine (Fig. 6).

Table 1

Regulation of cystathionine-cleaving enzymes in Aspergillus nidulans

Culture conditions and enzyme assays were as described in Material and Methods; in expts. 5 and 6 the results of the electrophoretic assay are given. The enzyme activity is expressed as nmol/min/mg protein. The supplements to the medium are calculated for the L-form.

Expt.	Supplement to minimal medium (2 mM)	γ-Cystathionase	β-Cystathionase	Repression of β-cystathionase (%)
1	None	0.016	1.67	—
	Homocysteine	0.19	0.68	60
	Methionine	0.24	0.97	42
2	None	0.21	2.06	—
	Homocysteine	0.25	1.53	26
	Methionine	0.29	1.44	31
3	None	non-measurable	1.44	—
	Homocysteine	0.10	1.01	29
	Methionine	0.10	0.91	36
4	None	0.26	2.34	—
	Methionine	0.79	1.14	52
5	None	0.17	1.50	—
	Methionine	0.48	0.48	68
6	None	—	1.33	—
	Methionine	—	0.55	59
Average repression by homocysteine				38
	methionine			48

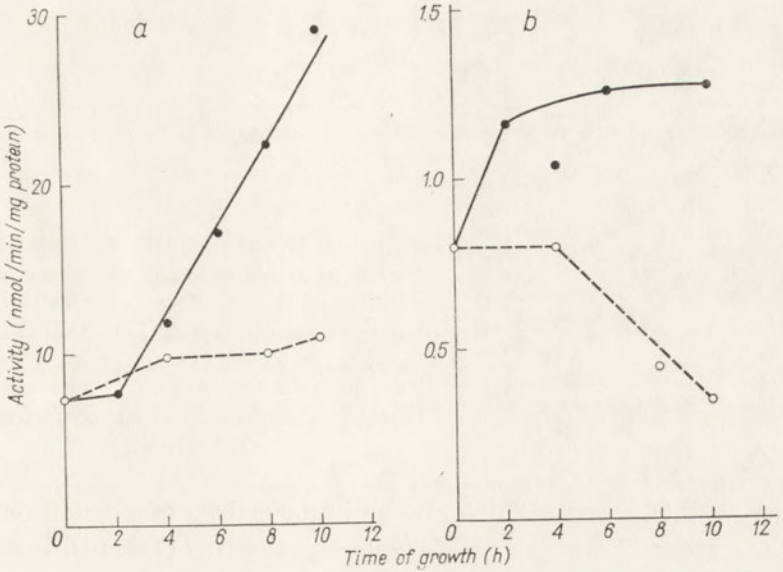


Fig. 5. Derepression of *a*, *O*-acetylhomoserine sulfhydrylase and *b*, β-cystathionase activity after transfer of mycelium to minimal medium. The mycelium grown for 14 h in methionine-containing medium was divided at zero time into two parts. One was transferred to minimal medium (solid line), the other, control, was kept in the methionine-containing medium (dotted line).

Table 2

Regulation of O-*acetylhomoserine sulfhydrylase in* Aspergillus nidulans

Culture conditions and enzyme assays were as described in Material and Methods.

Expt.	Supplement to minimal medium (2 mM)	Specific activity (nmol/min/mg protein)	Repression (%)
1	None	36.2	
	Homocysteine	2.7	93
	Methionine	6.7	71
2	None	21.3	
	Methionine	4.6	79
3	None	46.0	
	Homocysteine	8.9	81
	Methionine	18.7	60
	Cysteine	37.4	20
4	None	29.3	
	Cysteine	11.2	62
5	None	40.7	
	Cysteine	20.0	51
6	None	48.0	
	Cysteine	19.7	69
	Average repression by homocysteine		87
	methionine		70
	cysteine		50

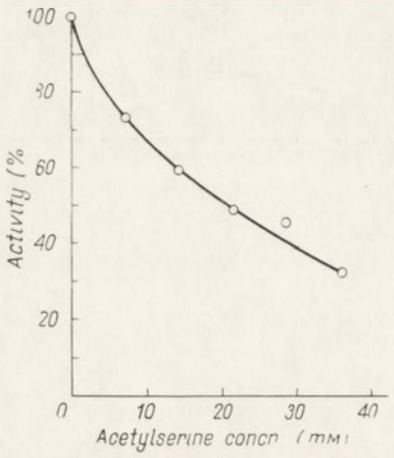


Fig. 6. Inhibition of OAHS by *O*-acetyl-L-serine. Formation of homocysteine was measured like in β-cystathionase assay by combination of the Kredich-Tomkins (1966) and Gaitonde (1967) methods, as in the reaction mixture cysteine is simultaneously formed from *O*-acetyl-L-serine and sulfide. *O*-Acetyl-L-homoserine concentration was 30 mM.

In the conditions of β-cystathionase assay the activity of γ-cystathionase was found to be about 10 - 15% of the β-cystathionase activity (Table 1). It should be noted, however, that the activity of the γ-enzyme was usually higher when mycelium was grown in the presence of methionine than in its absence.

When *O*-acetylhomoserine was substituted in the reaction mixture by homoserine, homocysteine was synthesized at a rate equal to 5 - 10% of normal activity. This suggests that homoserine is not a natural substrate for OAHS in *A. nidulans*.

DISCUSSION

The occurrence in *A. nidulans* of both β-cystathionase and OAHS may suggest that two alternative pathways of homocysteine synthesis are operative in this organism. The activity of β-cystathionase is similar to that observed in *S. cerevisiae* (H. Cherest, personal communication) and *Neurospora* (Kerr & Flavin, 1970). In *A. nidulans* this enzyme is partially repressed by methionine or homocysteine. To our knowledge no data concerning the regulation of this enzyme in fungi have been reported previously.

OAHS is repressed by methionine in all three above-mentioned fungi. Methionine is also an inhibitor of this enzyme *in vitro* in *Neurospora* and *S. cerevisiae* but not in *A. nidulans*. Kerr & Flavin (1970) suggested that sulfhydrylase reaction might provide an alternative pathway for methionine biosynthesis in *Neurospora*, while the main pathway involves cystathionine. This conclusion is supported by the existence of specific mutants (see introduction). On the other hand, the biochemical and genetic data available strongly suggest that in *S. cerevisiae* the sulfhydrylase reaction is the main path of homocysteine synthesis.

It seems likely that in *A. nidulans* both pathways lead to homocysteine formation. This conclusion is supported by the fact that both β-cystathionase and OAHS proved to be repressible by homocysteine. More than 60 methionine mutants have been isolated by Gajewski & Litwińska (1968) and mapped in six different loci. The majority of these mutants grow on methionine or homocysteine but none on

DL-allo-cystathionine. Since cystathionine supports growth of several mutants with impaired sulfate reduction pathway, it seems unlikely that in the case of these methionine-requiring mutants there is a cystathionine uptake problem. Preliminary enzymic examination of these mutants (Paszewski, unpublished) revealed that they have normal activities of both β-cystathionase and OAHS. Thus it seems that in these mutants some methionine-requiring processes may be impaired rather than methionine biosynthesis. In spite of many efforts we have not succeeded in obtaining "true" mutants blocked before homocysteine. The difficulty in getting such mutants could be accounted for if two biosynthetic pathways leading to homocysteine were operating, and so a block in one of them would not cause auxotrophy.

Unfortunately we have not been able so far to demonstrate cystathionine γ-synthase activity in *A. nidulans* so it is difficult to make an evaluation of the contribution of either pathway in methionine biosynthesis in this organism.

We are grateful to Drs. H. de Robichon-Szulmajster and H. Cherest (Laboratoire d'Enzymologie; CNRS, Gif-sur-Yvette, France) for advice and helpful discussions. This work was supported by the Polish Academy of Sciences within the Project 09.3.1.

REFERENCES

Abrahamson L. & Shapiro S. K. (1965). *Arch. Biochem. Biophys.* **109**, 376 - 382.

Bessman S. P., Koppanyi Z. H. & Wapnir R. A. (1967). *Anal. Biochem.* **18**, 213 - 219.

Cherest H., Eichler F. & de Robichon-Szulmajster H. (1969). *J. Bacteriol.* **97**, 328 - 336.

Cove D. J. (1966). *Biochim. Biophys. Acta* **113**, 51 - 56.

Delivier-Klutchko C. & Flavin M. (1965). *J. Biol. Chem.* **240**, 2537 - 2549.

Fischer F. G. & Dorfel H. (1953). *Biochem. Z.* **324**, 544 - 566.

Flavin M. & Slaughter C. (1964). *J. Biol. Chem.* **239**, 2212 - 2219.

Gaitonde M. K. (1967). *Biochem. J.* **104**, 627 - 633.

Gajewski W. & Litwińska J. (1968). *Mol. Gen. Genetics* **102**, 210 - 220.

Greenstein J. P. & Winitz M. (1961). *Chemistry of the amino acids*, vol. 2. John Wiley & Sons Inc., New York.

Kerr D. S. (1971). *J. Biol. Chem.* **246**, 95 - 102.

Kerr D. S. & Flavin M. (1968). *Biochem. Biophys. Res. Commun.* **31**, 124 - 130.

Kerr D. S. & Flavin M. (1970). *J. Biol. Chem.* **245**, 1842 - 1855.

Kredich N. M. & Tomkins G. M. (1966). *J. Biol. Chem.* **241**, 4955 - 4965.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265 - 275.

Wiebers J. L. & Garner H. R. (1967a). *J. Biol. Chem.* **242**, 12 - 23.

Wiebers J. L. & Garner H. R. (1967b). *J. Biol. Chem.* **242**, 5644 - 5649.

BADANIA β-CYSTATIONAZY I SULFHYDRYLAZY *O*-ACETYLOHOMOSERYNOWEJ JAKO ENZYMÓW ALTERNATYWNYCH DRÓG BIOSYNTEZY METIONINY U *ASPERGILLUS NIDULANS*

Streszczenie

Zidentyfikowano u grzyba *Aspergillus nidulans* dwa enzymy bezpośrednio związane z biosyntezą homocysteiny — prekursora metioniny — tj. β-cystationazę i sulfhydrylazę *O*-acetylohomoserynową. Synteza obu enzymów ulega częściowej represji, jeśli w pożywce znajduje się metionina lub homocysteina. *In vitro* metionina nie jest inhibitorem żadnego z tych enzymów. Sulfhydrylaza *O*-acetylohomoseryny jest częściowo hamowana przez *O*-acetylo-L-serynę.

Received 5 October, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 20 1973 No. 2

Z. KAZIMIERCZUK, J. GIZIEWICZ and D. SHUGAR

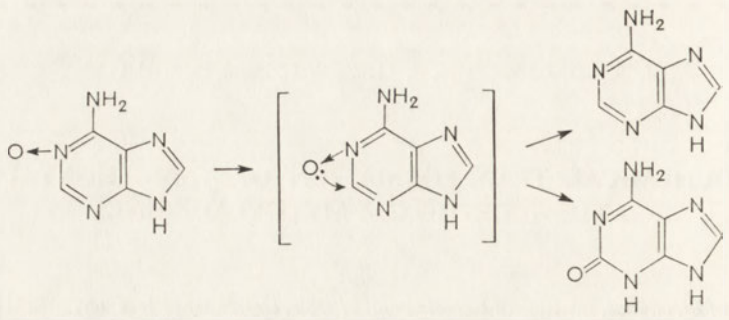
PHOTOCHEMICAL TRANSFORMATION OF N_1-METHOXYADENINE AND N_1-METHOXY-9-METHYLADENINE

Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Żwirki i Wigury 93, 02-089 *Warszawa; and Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka* 36, 02-532 *Warszawa, Poland*

1. Ultraviolet irradiation (254 nm) of the neutral form of 1-methoxyadenine in anhydrous methanol leads essentially to quantitative transformation to adenine (64%) and 2-methoxyadenine (30%). First-order kinetics for disappearance of 1-methoxyadenine prevail over the entire course of the reaction. Irradiation of the neutral and cationic forms in aqueous medium led to formation of the same two major products, but in somewhat different proportions. 2. The quantum yields for these reactions were quite high, from 0.16 to 0.20 M/E, and were independent of concentration or the presence of oxygen. 3. Irradiation of 1-methoxy-9-methyladenine under analogous conditions led to formation of 9-methyladenine as the sole UV-absorbing product, with even higher quantum yields, from 0.28 to 0.46. 4. Structures for the neutral and ionic forms of 1-methoxyadenine and 1-methoxy-9-methyladenine are proposed in connection with the difference in photochemical behaviour of 1-methoxyadenine and 1-methoxy-9-methyladenine. 5. The mechanism of formation of 2-methoxyadenine from 1-methoxyadenine is probably similar to that formulated for photochemical transformation of adenine-1-oxide, i.e. *via* an oxaziridine intermediate.

Of the natural bases found in nucleic acids, both adenine and guanine, and their nucleosides and nucleotides, are relatively resistant photochemically, either as monomers in aqueous medium, or when incorporated in polynucleotide chains. It is consequently of interest that some of the *N*-oxides of purines exhibit unusual photochemical sensitivity (Brown *et al.*, 1964, 1965; Cramer & Schlingloff, 1964), with quantum yields (Levin *et al.*, 1964) an order of magnitude greater than those for photohydration or photodimerization of pyrimidines (Shugar, 1960). Brown *et al.* (1964) first demonstrated that irradiation of adenine-1-oxide (or adenosine-1-oxide) at 254 nm led to formation of a mixture of adenine (or adenosine) and isoguanine (or isoguanosine, crotonoside). It was proposed by Brown *et al.* (1964) that the formation of isoguanine from adenine-1-oxide could be reasonably interpreted as a rearrangement *via* an unstable oxaziridine intermediate, as shown in

Scheme 1. This is in line with the well-known fact that most rearrangements of purine *N*-oxides involve loss of the *N*-oxide function with the transfer of the oxo group to a vicinal carbon atom (Lister, 1971).



Scheme 1

The yield of reaction products is markedly dependent on the pH of the medium (Cramer & Schlingloff, 1964), i.e. on the ionic forms of the reactant and/or intermediate(s). In slightly alkaline medium, one of the intermediates in the photolysis of adenine-1-oxide undergoes photocyclization to isoguanine, leading to an increased yield of the latter.

We have found it possible to scale up the photochemical transformation of adenosine-1-oxide to the point where it has become a suitable procedure for the preparative synthesis of isoguanosine, in fact superior to normal chemical syntheses. It has also proven feasible to extend this photochemical synthesis to the preparation, from the N_1-oxides of adenosine nucleotides, of the corresponding isoguanosine nucleotides (Kazimierczuk & Shugar, in preparation).

Several observations suggest that the foregoing type of photochemical transformation may be more general in scope. For example, irradiation of 6-methylpurine-1-oxide at 254 nm yielded largely 2-hydroxy-6-methylpurine, together with some 6-methylpurine (Brown *et al.*, 1964). On the other hand, 6-thiopurine-3-oxide is transformed largely to 6-thiopurine, together with some 2-hydroxy-6-thiopurine as a minor product (Brown *et al.*, 1965).

The present study was designed to investigate the possibility of extending these photochemical reactions to related derivatives of purine-*N*-oxides. The compound selected for this purpose was 1-methoxyadenine, on the assumption that this could conceivably undergo rearrangement to 2-methoxyadenine, the enol analogue of isoguanine. This expectation was, in fact, realized. It was hoped also to prepare in this way 9-substituted 2-methoxyadenine, i.e. the enol form of 9-substituted isoguanine, and, eventually, the enol form of isoguanosine, a compound isolated some years ago from the marine sponge *Cryptotethia crypta* by Bergmann & Burke (1956). This latter aim was not attained, for reasons described below.

MATERIALS AND METHODS

Authentic 1-methoxyadenine and 1-methoxy-9-methyladenine were synthetized according to the procedures of Fujii & Itaya (1971), and 2-methoxyadenine by the method of Bergmann & Burke (1956). Adenine was a product of Waldhof Pharma (Mannheim, German Federal Republic). All four compounds were checked by melting points, ultraviolet spectrophotometry, and chromatography.

Irradiation products were isolated on a preparative scale by chromatography on Whatman no. 3 *MM* paper with water-saturated *n*-butanol, or on PF_{254} silica gel with chloroform - methanol (85:15, v/v). For analytical purposes, Whatman no. 1 paper and three different solvent systems (cf. Table 1) were used.

Buffers utilized for pH measurements were 0.01 M-acetate and ammonia-ammonium chloride, and the measurements were carried out with a Radiometer PHM22 instrument, using semi-micro glass electrodes. Extremes of pH were obtained with appropriate concentrations of HCl and NaOH.

Two systems of irradiation were employed. For analytical purposes the source was a British Thermal Syndicate mercury resonance lamp, of which over 95% of the emitted radiation was at 254 nm. The radiation from this source was passed through a 5-mm layer of 30% acetic acid to remove wavelengths below 230 nm. The intensity of this source at the exit side of the filter (which was contained in a 5-mm path length spectro cuvette) was 7.5×10^{16} quanta cm^{-2} min^{-1}. With this system, used to follow the course of a reaction, the solutions were irradiated in 1-mm or 10-mm path length spectro cuvettes. Light intensities were measured actinometrically, using the photohydration reaction of a $\sim 10^{-4}$ M neutral aqueous solution of uridine (Shugar, 1960), for which the quantum yield is 0.021, and is virtually independent of irradiation wavelength. Actinometry was carried out in the same cuvette employed for irradiation of solutions.

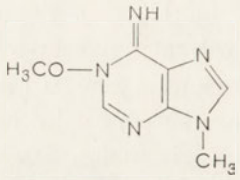
Another source, used for preparatory work, was a photochemical reactor constructed from a Phillips 40-watt germicidal lamp (Fikus *et al.*, 1965), making possible irradiation of larger volumes of solution. Following completion of a photochemical reaction, the solutions were concentrated to small volume under reduced pressure and then subjected to chromatography, on paper for analytical purposes, and on silica gel for isolation of products.

RESULTS

For purposes of simplicity, it is advisable to consider first some data regarding the ground-state structures of the neutral and ionic forms of 1-methoxyadenine and 1-methoxy-9-methyladenine.

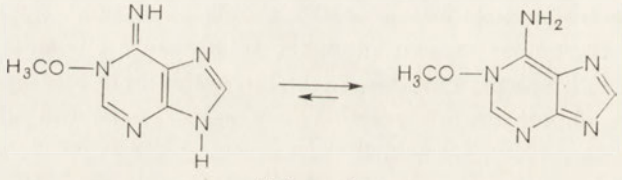
It was shown by Fujii & Itaya (1971) that 1-methoxyadenine exhibits two p*K* values, viz. 6.66 and 11.45. By contrast, 1-methoxy-9-methyladenine possesses only one p*K*, 8.55. Furthermore, the spectra of the neutral forms for both compounds (initial curves in Figs. 2 and 7) exhibit characteristic differences. The neutral form of 1-methoxy-9-methyladenine (initial curve, Fig. 7) shows a broad band in the

region of 290 nm, which is absent in the neutral form of 1-methoxyadenine, but is strikingly similar to that for the neutral form of 1-methyladenosine (Hall, 1971), a compound fixed in the imino form, as well as to that for 1-methylinosine (Psoda & Shugar, 1971), which is fixed in the keto form. It follows that the neutral form of 1-methoxy-9-methyladenine is, as might have been anticipated from purely formal considerations, in the imino form, as shown in Scheme 2. This is further supported by the pK value, 8.55, which is close to that for 1-methyladenosine, 8.8 (Martin & Reese, 1968).



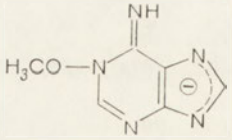
Scheme 2

From the foregoing, it appears reasonable to assume that the neutral form of 1-methoxyadenine, the spectrum of which differs appreciably from that for 1-methoxy-9-methyladenine, consists of a mixture of two mesomeric forms (Scheme 3),



Scheme 3

with the equilibrium in favour of the amino form. This would explain the ease with which this compound undergoes methylation at N_9 (Fujii & Itaya, 1971). Furthermore the spectrum of the anionic form of 1-methoxyadenine (Fig. 4) is strikingly similar to the spectrum of the neutral form of 1-methoxy-9-methyladenine, so that its structure is probably that shown in Scheme 4.



Scheme 4

In addition the absorption spectrum of 1-methoxyadenine in anhydrous methanol (initial curve in Fig. 1) is identical with that in aqueous medium at pH 9.2, so that it is the neutral form which prevails in methanol.

Unfortunately the data at our disposal are insufficient to establish the positions of protonation of the cationic forms of the foregoing compounds. As will be seen

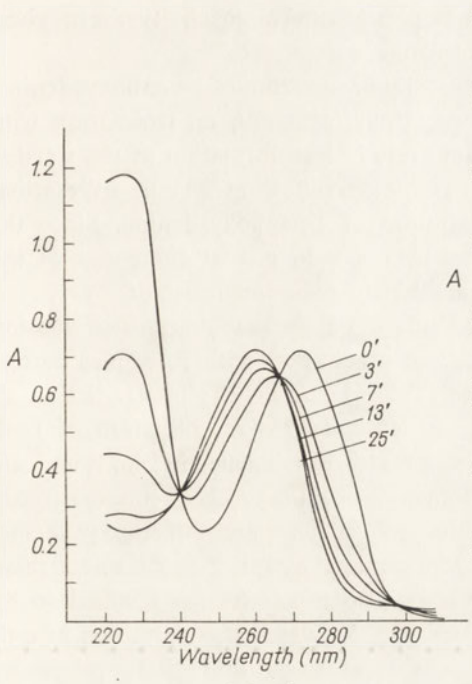


Fig. 1

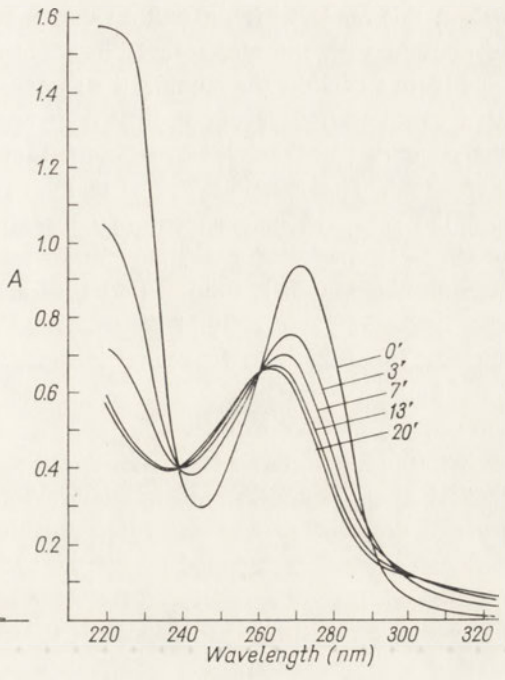


Fig. 2

Fig. 1. Change of absorption spectrum of 1-methoxyadenine (about 10^{-3} M in anhydrous methanol) on irradiation with mercury resonance lamp. Figures beside each curve indicate irradiation times in minutes.

Fig. 2. Photochemical transformation at 254 nm of 10^{-4} M-1-methoxyadenine in 0.01 M buffer, pH 9.2 (neutral form), followed by changes in absorption spectrum with time of irradiation (indicated by figures beside each curve) with mercury resonance lamp.

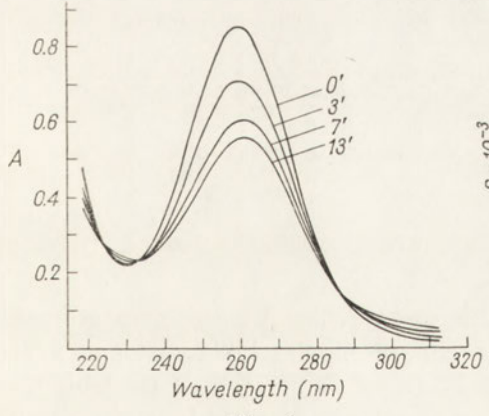


Fig. 3

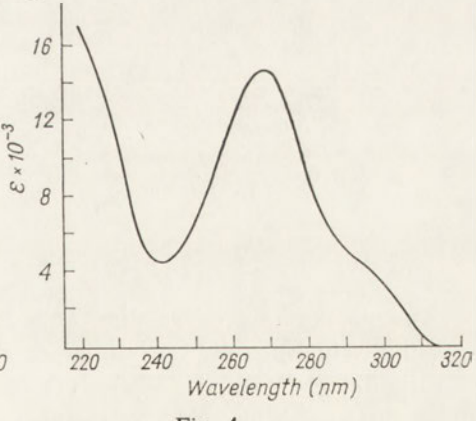


Fig. 4

Fig. 3. Course of photochemical transformation at 254 nm of 10^{-4} M-1-methoxyadenine in 0.01 M-acetate buffer, pH 4.1 (protonated form). Figures beside each curve indicate irradiation times (in minutes) with mercury resonance lamp.

Fig. 4. Absorption spectrum of anionic form of 1-methoxyadenine (pK=11.45) at pH 13.

below, this rendered it difficult to correlate photochemical reactivity for a given compound with the structure of its ionic form.

Figure 1 exhibits the modifications in absorption spectrum of 1-methoxyadenine, at a concentration of about 10^{-4} M in anhydrous methanol, on irradiation with the mercury resonance lamp. Note the rapid rate of transformation of the starting compound. The reaction was virtually terminated following 20 min irradiation, and the rate was independent of concentration of 1-methoxyadenine. Since the products themselves possess absorption at 254 nm even greater than that of the starting compound, it follows that they are highly radiation resistant.

Paper chromatography of the irradiated solution at various times of irradiation showed, in addition to 1-methoxyadenine, two other spots with R_F values corresponding to adenine and 2-methoxyadenine (Table 1). After 20 min irradiation all the starting product had disappeared. The resistance to 254 nm radiation of both the photoproducts, as well as the isosbestic points clearly delineated in Fig. 1, indicate that the photochemical transformation of 1-methoxyadenine proceeds simultaneously *via* two different pathways to give directly both adenine and 2-methoxyadenine, and that secondary reactions are involved to a minimal extent, if at all, under these conditions. The authenticity of the 2-methoxyadenine was further testified to by its absorption spectrum at various pH values (Fig. 5), which also permitted of estimation of its p*K* values, 3.6 and 10.2, as compared to 3.7 and 10.0 reported by Bergmann & Burke (1956).

Table 1

Ascending paper chromatography of 1-methoxyadenine, 1-methoxy-9-methyladenine and their photoproducts

Whatman paper no. 1 and the following solvent systems were used: (*A*) water - saturated *n*-butanol; (*B*) *n*-butanol - conc. NH_4OH - water, 4:1:1 (by vol); (*C*) *n*-butanol - glacial acetic acid - water, 5:2:3 (by vol.).

Compound	*A*	*B*	*C*
Adenine	0.38	0.24	0.63
1-Methoxyadenine	0.30	0.35	0.56
2-Methoxyadenine	0.54	0.42	0.71
1-Methoxy-9-methyladenine	0.17	0.47	0.55
9-Methyladenine	0.40	0.46	0.66

The proportions of adenine and 2-methoxyadenine formed were estimated quantitatively by elution from paper or PF_{254} silica gel chromatograms and spectrophotometry of the eluates (Table 2). It will be noted from the table that, under these conditions, 94% of the photochemically transformed 1-methoxyadenine was accounted for by the two products.

Figure 2 presents the spectral modifications resulting from irradiation of a 10^{-4} M solution of 1-methoxyadenine at pH 9.2, hence of the neutral form. It will be noted that the rate of photochemical transformation is similar to that in methanol,

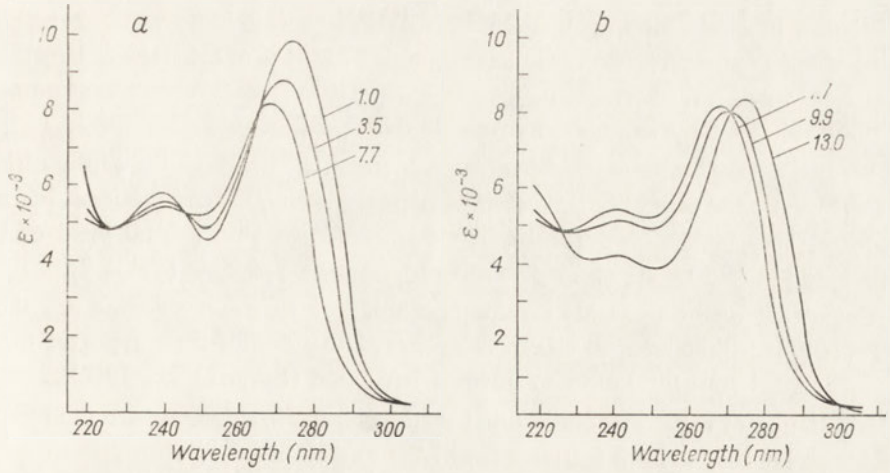


Fig. 5. Absorption spectrum of 2-methoxyadenine at different pH values, showing the presence of two ionic equilibria: (*a*) for protonation, probably on the ring N_1, with pK_1 3.6; (*b*) for dissociation of the imidazole hydrogen, with pK_2 10.2.

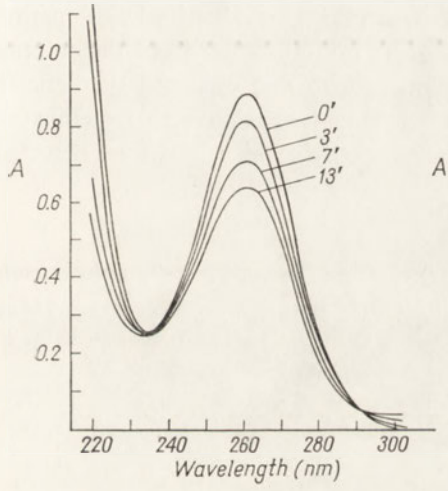


Fig. 6

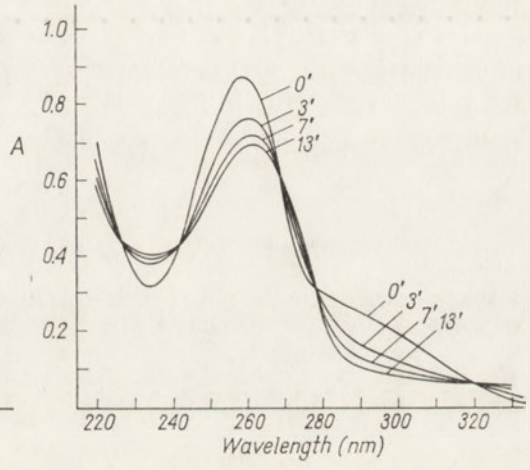


Fig. 7

Fig. 6. Course of photochemical transformation of a 10^{-4} M solution of 1-methoxy-9-methyladenine at pH 4.2 (cationic form), followed by changes in absorption spectrum with time of irradiation (indicated by figures beside each curve) with mercury resonance lamp.

Fig. 7. Course of photochemical transformation of a 10^{-4} M solution of 1-methoxy-9-methyladenine at pH 10.4 (neutral form), followed by changes in absorption spectrum with time of irradiation (indicated by figures beside each curve) with mercury resonance lamp.

but the absence of a well-defined isosbestic point in the neighbourhood of 290 nm suggests that the reaction is somewhat more complex than in methanol. However, the stable UV-absorbing photoproducts formed are in this instance equally radiation resistant since prolongation of the irradiation time was without effect. Paper chromatography of the irradiated solution demonstrated, as in the case of

irradiation in methanolic medium, only two UV-absorbing products, 2-methoxyadenine and adenine. The two products accounted for 81% of the starting 1-methoxyadenine (Table 2); furthermore their relative proportions were reversed as compared to these obtained by irradiation in methanol.

When 1-methoxyadenine was irradiated at pH 4.1, corresponding to the cationic form (Fig. 3), the same two UV-absorbing photoproducts were obtained, but with a considerably higher proportion of 2-methoxyadenine, and a total yield of UV-absorbing photoproducts of 71% relative to 1-methoxyadenine (Table 2).

Calculated quantum yields for disappearance of 1-methoxyadenine are shown in Table 2. It will be seen that these values are almost double those reported for the photochemical transformation of adenine-1-*N*-oxide (Levin *et al.*, 1964).

Irradiation of a 10^{-4} M solution of 1-methoxy-9-methyladenine with the mercury resonance lamp at pH 4.2 (cationic form) led to a decrease in absorption over the entire wavelength range (Fig. 6), which attained a limit following about 12 min irradiation. It is clear that the UV-absorbing photoproduct(s) formed in this case are highly radiation resistant. When the reaction was carried out with the Phillips 40-watt lamp on a preparative scale, and the irradiated solution concentrated and chromatographed, it exhibited only one UV-absorbing spot, identified as 9-methyladenine (Table 1). This was further confirmed by crystallization from the solution of 9-methyladenine, which proved to be identical with that obtained synthetically by Fujii & Itaya (1971). The yield of 9-methyladenine, relative to 1-methoxy-9-methyladenine, was 46%, and no 2-methoxy-9-methyladenine could be detected.

Table 2

Yield of photoproducts from 1-methoxyadenine and 1-methoxy-9-methyladenine

Results are expressed in terms of % conversion of the starting substance on irradiation at 254 nm with mercury resonance lamp under various conditions. Quantum yields (Ø) are calculated for disappearance of 1-methoxyadenine and 1-methoxy-9-methyladenine.

Compound	Conditions under which irradiated	Photoproducts (%)		Ø (M/E)
		adenine	2-methoxy-adenine	
1-Methoxyadenine	neutral form in anhydrous methanol	64	30	0.18
	pH=4.10 cationic form	27	44	0.20
	pH=9.23 neutral form	23	58	0.16
		9-methyladenine		
1-Methoxy-9-methyladenine	pH=4.20 cationic form	46		0.28
	pH 10.46 neutral form	58		0.46

When irradiation was conducted at pH 10.4, i.e. for the neutral form, with the mercury resonance lamp, the accompanying spectral modifications (Fig. 7) pointed to the disappearance of the imino structure (disappearance of the broad band at about 295 nm). Following about 13 min irradiation, no further modifications occurred, again pointing to the formation of radiation-resistant photoproduct(s). When the reaction was carried out on a larger scale with the Phillips 40-watt lamp, and the irradiated solution concentrated to small volume, paper chromatography demonstrated one major UV-absorbing spot and one minor spot. The former was identified as 9-methyladenine; the latter proved to be 6-methoxyamino-9-methylpurine, and was also found in a control non-irradiated solution. Its formation is readily explained as due to the alkaline lability of 1-methoxy-9-methyladenine, which is transformed *via* a Dimroth rearrangement in alkaline medium largely to 6-methoxyamino-9-methylpurine (Itaya *et al.*, 1972). No traces of 2-methoxy-9-methyladenine could be detected. When this dark reaction was taken into account, the yield of 9-methyladenine, as a result of photochemical transformation of 1-methoxy-9-methyladenine, was about 75%.

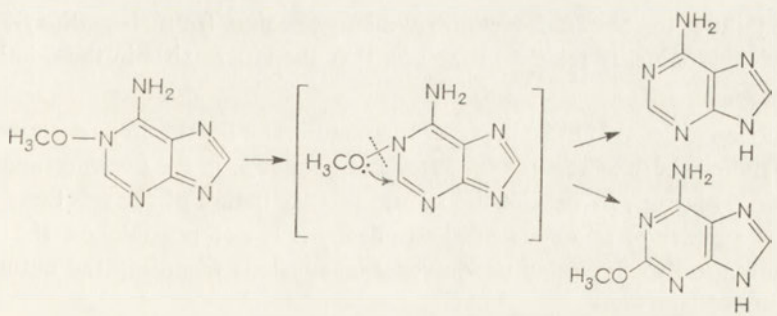
The quantum yield for disappearance of 1-methoxy-9-methyladenine was found to be 0.28 for the cationic form and about 0.43 for the neutral molecule, values which are remarkably high, in fact the highest hitherto reported for the photochemical transformation of purine or pyrimidine derivatives.

Finally, it is of some interest that all the foregoing photochemical transformations were unaffected with regard to rates of photoproduct formation when irradiations were conducted in the presence of oxygen.

DISCUSSION

The mechanism of formation of adenine and 2-methoxyadenine from 1-methoxyadenine is most likely similar to that proposed by Brown *et al.* (1964) for the formation of adenine and 2-hydroxyadenine from the N_1-oxide of adenine *via* an oxaziridine intermediate (Scheme 1), and is illustrated in Scheme 5.

Brown *et al.* (1964) irradiated unbuffered aqueous solutions of adenine-1-oxide. Up to about 50% photolysis, the ratio of 2-hydroxyadenine to adenine formed was approximately 1:1, further irradiation led to a more complex mixture of additional



Scheme 5

unidentified products. Our own results show that, both for the neutral and cationic forms of 1-methoxyadenine, there is a considerable preponderance of the 2-methoxy product. In fact, for the neutral form of 1-methoxyadenine, the ratio of 2-methoxyadenine to adenine is 2.5:1. An approximately inverse ratio was observed for irradiation of the neutral form in non-aqueous medium (see Table 2). Furthermore, formation of the two major photoproducts in methanol was almost quantitative (94%) and was also quite high in aqueous medium (over 70 - 80%). The results in Table 2 point to the possibility of selective formation of one of the two photoproducts by a suitable choice of solvent.

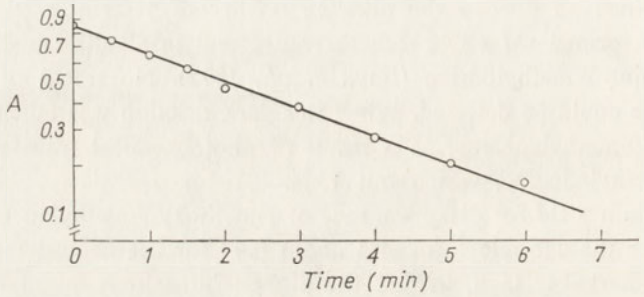


Fig. 8. Kinetics of disappearance of N_1-methoxyadenine, about 10^{-4} M in anhydrous methanol, on irradiation with mercury resonance lamp at 254 nm. Ordinate scale presents optical densities of 1-methoxyadenine, following successive periods of irradiation, at λ_{max} (272 nm), each corrected for absorption of photoproducts at this wavelength (cf. Levin *et al.*, 1964).

As in the case of adenine-1-oxide (Levin *et al.*, 1964), photolysis of 1-methoxyadenine followed a first-order reaction. This is illustrated for irradiation in methanol, where 1st-order kinetics prevailed up to the point where the starting product had practically disappeared (Fig. 8), in agreement with the absence of any concentration dependence of the reaction rate (see above). Taken in conjunction with the well-defined isosbestic points observed during the entire course of the reaction (Fig. 1), it appears reasonable to conclude that the two competing reactions for formation of 2-methoxyadenine and adenine (Scheme 5) are independent of each other, and that their relative rate constants are dependent only on the ionic form of the starting compound and the solvent medium.

The failure to obtain 2-methoxy-9-methyladenine from 1-methoxy-9-methyladenine is undoubtedly related to the fact that the latter exists in the 6-imino form (Scheme 2).

Finally mention should be made of the fact that all the reactions described above were unaltered, both as regards the rates and products, in the presence and absence of oxygen. However, in view of the intramolecular nature of the reactions, testified to by the absence of any concentration effects, it is not possible on the basis of this information to draw any unequivocal conclusions regarding the nature of the excited state(s) involved.

This investigation was supported by the Polish Academy of Sciences (Project 09.3.1); and profited also from partial support by The Wellcome Trust, the World Health Organization, and the Agricultural Research Service, U.S. Department of Agriculture.

REFERENCES

Bergmann W. & Burke D. C. (1956). *J. Org. Chem.* **21**, 226 - 228.
Brown G. B., Levin G. & Murphy S. (1964). *Biochemistry* **3**, 880 - 883.
Brown G. B., Levin G., Murphy S., Sele A., Reilly H. C., Tarnowski G. S., Schmid F. A., Teller M. N. & Stock C. C. (1965). *J. Med. Chem.* **8**, 190 - 195.
Cramer F. & Schlingloff G. (1964). *Tetrahedron Lett.* 3201 - 3204.
Fikus M., Wierzchowski K. L. & Shugar D. (1965). *Photochem. Photobiol.* **4**, 521 - 536.
Fujii T. & Itaya T. (1971). *Tetrahedron* **27**, 351 - 361.
Hall R. H. (1971). *Modified Nucleosides in Nucleic Acids.* Columbia University Press, New York.
Itaya T., Tanaka F. & Fujii T. (1972). *Tetrahedron* **28**, 535 - 547.
Levin G., Setlow R. B. & Brown G. B. (1964). *Biochemistry* **3**, 883 - 884.
Lister J. H. (1971). *Fused Pyrimidines.* John Wiley & Sons, New York.
Martin O. M. G. & Reese C. B. (1968). *J. Chem. Soc.* 1731 - 1738.
Psoda A. & Shugar D. (1971). *Biochim. Biophys. Acta* **247**, 507 - 513.
Shugar D. (1960). In *The Nucleic Acids* (E. Chargaff & J. N. Davidson, eds.) vol. 3, Academic Press, New York.

FOTOCHEMICZNA PRZEMIANA N_1-METOKSYADENINY I N_1-METOKSY-9-METYLOADENINY

Streszczenie

1. Naświetlanie promieniowaniem ultrafioletowym (254 nm) 1-metoksyadeniny w formie obojętnej w roztworze metanolowym prowadzi do prawie ilościowej przemiany w adeninę (64%) i 2-metoksyadeninę (30%). Zanik 1-metoksyadeniny w czasie reakcji zachodzi zgodnie z kinetyką reakcji pierwszego rzędu. Naświetlanie 1-metoksyadeniny w formie obojętnej i monokationu w środowisku wodnym prowadzi do tych samych produktów, ale w innym stosunku.

2. Wydajność kwantowa tych reakcji jest stosunkowo wysoka (0.16 - 0.20 M/E) i niezależna od stężenia i obecności tlenu.

3. Naświetlanie 1-metoksy-9-metyloadeniny w analogicznych warunkach jak 1-metoksyadeniny prowadzi do utworzenia tylko 9-metyloadeniny jako produktu absorbującego w ultrafiolecie. Reakcja fotochemiczna zachodzi również z wysoką wydajnością kwantową wynoszącą 0.28 - 0.46.

4. Na podstawie różnego zachowania w reakcji fotochemicznej 1-metoksyadeniny i 1-metoksy-9-metyloadeniny zaproponowano struktury form obojętnych i jonowych tych związków.

5. Mechanizm tworzenia 2-metoksyadeniny z 1-metoksyadeniny jest prawdopodobnie analogiczny jak w reakcji fotochemicznego przekształcenia *N*-tlenku adeniny, t.zn. produktem przejściowym jest związek typu oksazyrydyny.

Received 14 November, 1972.

ACTA BIOCHIMICA POLONICA

Vol. 20 1973 No. 2

W. DRABIKOWSKI, RENATA DĄBROWSKA and BARBARA BARYŁKO

PROPERTIES OF TROPONIN AND ITS CONSTITUENTS

Department of Biochemistry of Nervous System and Muscle, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warszawa, Poland

1. Three major components of troponin were obtained in a highly purified state on fractionation of troponin on DEAE-Sephadex A-50 in the presence of urea, EDTA with KCl gradient. The components were characterized in respect to electrophoretic mobility on polyacrylamide gel in the presence and absence of Ca^{2+} and in the presence of sodium dodecyl sulphate. 2. Two components: 23 500 daltons protein (TN-I) and 18 300 daltons protein (TN-C) are sufficient to confer Ca^{2+}-sensitivity to actomyosin. TN-C is the only one which has a high affinity for Ca^{2+}. The third component of mol. wt. 40 200 daltons (TN-B) interacts with tropomyosin and F-actin. 3. TN-C is distinguished by a high phenylalanine content and extremely high negative charge (isoelectric point at pH 3.8). All components differ in respect to thiol group content. 4. The components of mol. wt. 30 000 and 13 000 daltons, usually present in troponin preparations, are the products of proteolytic degradation of TN-B and TN-I, respectively. For minimizing this degradation the procedure of Ebashi *et al.* (1971) for preparation of troponin is recommended.

Ebashi & Kodama (1965) were the first who showed that the factor responsible for calcium sensitivity of myofibrils is a complex of tropomyosin and troponin. Subsequent studies led to the generally accepted view that troponin strongly interacts with tropomyosin, has a high affinity for Ca^{2+} and inhibits Mg^{2+}-stimulated actomyosin ATPase[1] in the presence of tropomyosin and in the absence of Ca^{2+} ions (for review see Ebashi & Endo, 1968). Until recently the multi-component structure of troponin (Greaser & Gergely, 1971; Drabikowski *et al.*, 1971a,b) was a matter of controversy. At present, however, there seems to be no more doubt that the active unit of troponin consists of three components, each responsible for a different function (Murray & Kay, 1971; Hartshorne, 1972; Wilkinson *et al.*, 1972; Schaub *et al.*, 1972; Ebashi & Ohtsuki, 1972). One of the components inhibits ATPase activity independently of the concentration of free Ca^{2+}. The inhibition

[1] Abbreviations: ATPase, adenosine triphosphatase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(β-aminoethyl-ether)-*N,N*-tetraacetic acid; HEDD, β-hydroxyl-2,4-dinitrophenyl disulphide; SDS, sodium dodecyl sulphate.

is abolished in the presence of Ca^{2+} by the second component which binds tightly Ca^{2+}. It was suggested by Greaser & Gergely (1972) to call these components TN-I, and TN-C, respectively. The third component, with a high affinity towards tropomyosin and possibly towards actin was proposed by us to call TN-B (Drabikowski *et al.*, 1972) and these terms will be used throughout the paper.

The results of our further studies on troponin and the properties of its individual components are the subject of the present paper. The previous results have been briefly reported (Drabikowski *et al.*, 1971a,b).

MATERIAL AND METHODS

Preparation of proteins. All proteins were obtained from rabbit leg and back muscles.

Troponin. Three methods of preparation of troponin were used at various steps of these studies. In two of them troponin was obtained from its complex with tropomyosin; the complex was isolated either essentially according to Ebashi & Ebashi (1964) or from alcohol-ether dried muscle powder obtained after Bailey (1948), as described previously (Drabikowski *et al.*, 1969).

The solutions containing troponin-tropomyosin complex obtained in either way were adjusted to pH 4.5 in 1 M-KCl at 0°C and after one hour the precipitate containing tropomyosin was removed by centrifugation. Supernatant, after adjustment to pH 7.0 with KOH and dialysis against 1 mM-$NaHCO_3$ - 1 mM-2-mercaptoethanol, yielded crude troponin. When the viscosity of obtained solution was higher than 0.10 dl/g indicating the presence of some tropomyosin, the isoelectric fractionation at pH 4.5 in 1 M-KCl was repeated. Throughout the whole procedure thymol was added to avoid bacterial contamination.

Crude troponin preparations, especially those obtained from alcohol-ether powder, contained usually some ribose (as an average about 2 mol per 10^5 g of protein) indicating the presence of ribonucleic acid contaminations which could be separated on Sephadex G-100 or G-200. Also salting out of troponin at 0.7 saturation of ammonium sulphate removed nucleic acids, and the latter procedure was routinely used in this work.

Troponin was also prepared by the recently described method of Ebashi *et al.* (1971). Minced muscles after removal of myosin with Guba-Straub solution were washed three times with 3 vol. of 0.05 M-KCl solution containing 0.3 mM-$NaHCO_3$, twice with 3 vol. of 0.3 mM-$NaHCO_3$ and finally with 0.5 vol. of 1.2 M-LiCl. To the residue an equal volume of 0.4 M-LiCl was added and pH was adjusted to 4.5. After two hours standing in cold the precipitate was centrifuged off. Supernatant was adjusted to pH 7.5 and salted out with ammonium sulphate. Fraction between 0.4 - 0.7 saturation was collected, dissolved in 1 mM-$NaHCO_3$ and 1 mM-2-mercaptoethanol and dialysed against the same solution.

Tropomyosin was prepared according to Bailey (1948) using the precipitate obtained at pH 4.5 in the course of preparation of troponin. Two or three cycles

of purification by repeated precipitation at pH 4.5 in 1 M-KCl and fractionation with ammonium sulphate between 0.4 - 0.6 saturation were applied. Finally the precipitate between 0.53 and 0.6 saturation was collected. Ammonium sulphate was removed by dialysis. Purity of tropomyosin was checked by SDS-polyacrylamide disc electrophoresis.

Actomyosin was obtained by combining myosin and actin in the 3:1 weight ratio. Myosin was prepared by the method of Seraydarian *et al.* (1967). Actin was obtained as previously described (Drabikowski & Gergely, 1962) and its purity was checked by SDS-polyacrylamide disc electrophoresis.

Polyacrylamide gel electrophoresis. a) *Without SDS.* It was performed on 7.5% polyacrylamide gels essentially according to Davis (1964) with tris-glycine buffer, pH 8.5, in the presence of 4 M-urea. Usually samples of 30 - 50 μg of protein were applied. Electrophoresis was performed for 4 h at 3 mA per tube. Gels were stained with 0.05% Coomassie blue in 18% trichloroacetic acid for at least 3 h and destained by prolonged washing in 7% (v/v) acetic acid containing 5% (v/v) methanol.

b) *With SDS.* It was performed on 10% polyacrylamide gels containing 0.1% SDS, in phosphate buffer containing 0.1% SDS according to Weber & Osborn (1969). Protein samples were usually dialysed for at least 2 h at room temperature against 20 mM-sodium phosphate buffer, pH 7.0, 1% SDS and 0.05 M-DTT or 1 mM-2-mercaptoethanol. When protein did not contain salt, samples were heated for 5 min at 100°C in the presence of 2% SDS and 1 mM-2-mercaptoethanol (Spudich & Watt, 1971). The current used was 7 mA per tube. Staining as above.

Gels were scanned in a Zeiss densitometer at 550 nm. Relative quantities of each band present were determined planimetrically.

The SDS gels were calibrated for molecular weight determinations with α-actinin (kindly provided by Dr. D. E. Goll), bovine serum albumin, catalase, tropomyosin, ovalbumin, pepsin, chymotrypsinogen, lysozyme and cytochrome *c*.

Chromatography. For separation of troponin on Sephadex G-50, G-100 and G-200 columns (100×2.5 cm) about 25 mg protein was applied and it was eluted with solution containing 0.3 M-KCl, 20 mM-tris-HCl, pH 7.5, and 0.1 mM-2-mercaptoethanol.

DEAE-Sephadex A-50 was equilibrated with 50 mM-tris-HCl, pH 7.5, containing 6 M-urea, 0.1 mM-2-mercaptoethanol and 0.1 mM-EDTA. About 300 mg of troponin was applied on the 100×2.5 cm column. The linear 0 - 1 M-KCl gradient was applied in a total volume 1000 ml of the above buffer solution.

Distribution of protein in the eluates was determined by measuring the extinction at 278 nm and concentration of salt was monitored by titration with $AgNO_3$. Protein in the eluates was concentrated by the "vacuum dialysis" and, when necessary, dialysed against 1 mM-$NaHCO_3$ - 1 mM-2-mercaptoethanol and, if needed, 0.5 M-KCl.

Viscosity was measured using an Ostwald type viscometer with an outflow time of 50 sec for water at 21°C.

ATPase assay. Adenosinetriphosphatase activity was measured at 25°C in a system containing 20 mM-KCl, 20 mM-tris-HCl, pH 7.5, 2 mM-$MgCl_2$, actomyosin (usually

0.25 mg/ml), required amounts of tropomyosin and troponin or its components and 2 mM-EGTA if necessary. The reaction was started by addition of 2 mM-ATP. The samples were usually removed after 5 min and the reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid. After removal of the precipitated protein by centrifugation the amount of inorganic phosphate was measured by the method of Fiske & Subbarow (1925).

Binding of Ca^{2+}. Samples of troponin or its components (about 2 mg/ml) after a 2 min treatment with Dowex 50 in order to remove contaminating Ca^{2+}, were incubated for 20 min with 0.1 mM-$^{45}CaCl_2$ solution. After subsequent Dowex 50 treatment and removal of the resin, radioactivity was measured. The amount of exchangeable calcium was calculated as described previously (Drabikowski & Baryłko, 1971).

Amino acid analysis was carried out by using a Beckman model 120B amino acid analyser, following hydrolysis of proteins in 6 N-HCl at 110°C for 20 h.

SH-groups. Protein samples were preincubated for 24 h with 0.1 M-DTT and 6 M-urea. The excess of DTT was removed on Sephadex G-25 column equilibrated with 6 M-urea and 10 mM-tris-HCl, pH 7.5. The content of SH groups was immediately determined with HEDD (Drabikowski & Nowak, 1965).

Tryptophan content was measured according to Carpenter (1948).

Protein concentration was determined by the biuret method (Gornall *et al.*, 1949) or, occasionally, according to Lowry *et al.* (1951).

Ribose concentration was determined according to Mejbaum (1939).

Chemicals. DEAE-Sephadex, Sephadex G-50, 100 and 200 were purchased from Pharmacia (Uppsala, Sweden), ATP disodium salt from Merck (Darmstadt, West Germany), EDTA from Ciech (Poland), EGTA from Sigma Chemical Company (St. Louis, Mo., U.S.A.). Trypsin and soya-bean trypsin inhibitor, both twice-crystallized, were supplied by Sigma Chemical Co. Deionized water was used throughout the whole procedure.

RESULTS

Electrophoretic and chromatographic characterization of troponin and its constituents. As previously briefly described (Drabikowski *et al.*, 1971a) disc electrophoresis of troponin reveals at least four bands irrespective of the presence of urea. This pattern is not changed by 1 mM-DTT but is different when electrophoresis is conducted in the absence of Ca^{2+}. In this case instead of the two fastest bands a new one appears with intermediate mobility (Drabikowski *et al.*, 1970).

In the presence of denaturing agent, SDS, three major components are seen, corresponding to TN-B, TN-I and TN-C. Their corrected molecular weights, calculated at present as the average of at least 60 determinations, are presented in Table 1. Beside major components two minor ones are often present, one with a molecular weight of about 30 000 daltons and the second one moving usually as a broad band with the average molecular weight of 13 000 daltons (Table 1). Both minor components

were suggested to be the products of proteolytic splitting of TN-B and TN-I, respectively (Drabikowski *et al.*, 1971b, 1972; Dąbrowska *et al.*, 1973a,b).

The relative amounts of particular component depend on the method of preparation of troponin (Fig. 1). Troponin obtained according to Ebashi *et al.* (1971) with LiCl is very rich in TN-B and has virtually no minor components. On the other hand, the latter proteins are present in large amounts in other types of troponin preparations.

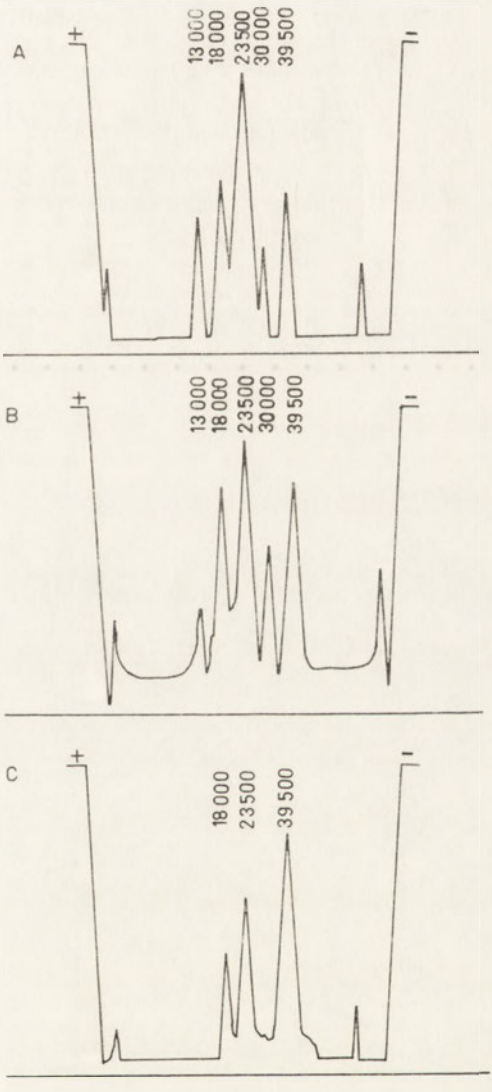


Fig. 1. Densitometric tracing of electrophoretograms of troponin prepared by different methods. Electrophoretic runs in SDS gels. Preparations of troponin from: *A*, alcohol-ether powder; *B*, low ionic strength extract; *C*, LiCl extract. For details see Methods. Protein (30 μg) was applied to the gels. Numbers over the peaks denote the estimated molecular weights.

Crude troponin, non-fractionated with ammonium sulphate, gives on Sephadex G-100 column two peaks (Fig. 3A), the main one composed of all constituents, containing traces of ribose (0 - 0.2 mol per 10^5 g protein), and the second more

retarded peak composed of small amount of protein and practically all ribose of initial crude preparation.

Troponin purified by ammonium sulphate fractionation reveals on Sephadex G-50, G-100 or G-200 one fairly symmetrical peak, which has throughout the same composition, as shown by electrophoretic analysis (Fig. 3B). A similar pattern is obtained in the presence of 0.1 mM-EDTA. These results indicate a strong interaction of all troponin constituents.

Table 1

Molecular weights of components of the troponin complex

Standard deviations and in parentheses numbers of determinations made with separate gels are given.

Component	Molecular weight (daltons)
TN-B	40 200±2 180 (62)
TN-I	23 500±2 000 (69)
TN-C	18 300±1 550 (70)
"13 000 component"	13 000±1 200 (56)
"30 000 component"	29 900±1 850 (25)

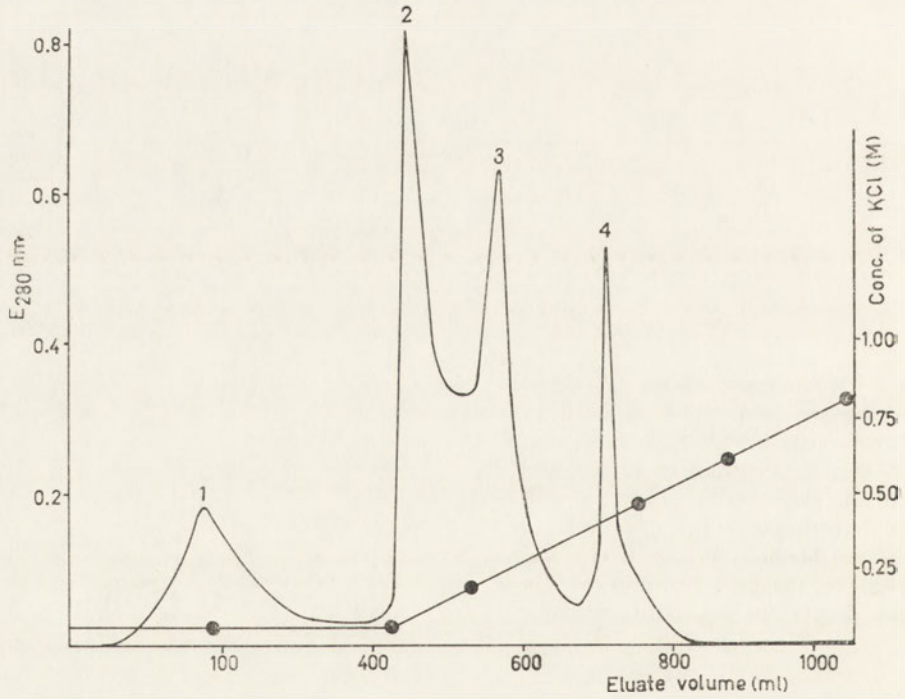


Fig. 2. Chromatography of troponin on DEAE-Sephadex. Protein (200 mg) was loaded on the DEAE-Sephadex A-50 column (100 cm×2.5 cm) equilibrated with 50 mM-tris-HCl, pH 7.5, containing 6 M-urea and 0.1 mM-EDTA. Elution was by the KCl gradient (●).

Fractionation of troponin on Sephadex G-100 in the presence of urea reveals heterogeneity of troponin (Fig. 3C). A complete separation of the components is achieved, in agreement with earlier results (Drabikowski *et al.*, 1971a) by chromatography on DEAE-Sephadex A-50 (Fig. 2). The fraction eluted before application of KCl gradient as one or sometimes as two peaks, reveals in SDS-electrophoresis a broad band corresponding to molecular weight from 10 000 to 14 000 daltons. The second fraction contains mainly TN-I, the third one mainly TN-B and the fourth one, TN-C component. The addition of EDTA results in a better separation of the components on DEAE-Sephadex column.

Electrophoretic patterns of the troponin components obtained by chromatography on DEAE-Sephadex A-50 are shown in Fig. 4. In the absence of SDS and in the

Table 2

Effect of pH, *salts and temperature on troponin complex*

Supernatants (s) and precipitates (p) obtained at pH 1.0 and 5.5 were neutralized and the "pH preparations" dialysed additionally against 0.1 mM-$NaHCO_3$. The "Ca preparations" were treated with Dowex 50. $\Delta\eta_{spec}$ is the excess of specific viscosity over the viscosity of tropomyosin. Concentration of tropomyosin and troponin fractions, 1 mg/ml. The inhibition of ATPase was calculated as follows:

$$100 - \left(\frac{\text{ATPase activity in the presence of EGTA}}{\text{ATPase activity in the presence of } Ca^{2+}} \times 100 \right)$$

Treatment	Protein in supernatant (%)	E_{278}/E_{260}		Interaction with tropomyosin $\Delta\eta_{spec}$				Inhibition of ATPase (%)		Ca exchangeable (mol/10^5 g protein)	
				without KCl		0.1 M-KCl					
		s	p	s	p	s	p	s	p	s	p
pH 1.0, 1.2 M-KCl	68.0	1.32	1.08	1.83	0	0.40	0	—	—	0.33	1.95
	70.0	1.43	1.08	1.20	0	0.48	0	78.0	0	0.22	2.80
	69.0	1.47	1.18	—	—	—	—	73.0	2.0	0.62	1.72
	70.0	1.56	1.02	2.40	0	0.62	0	70.0	5.0	—	—
	60.0	—	—	1.72	0	0.40	0	71.0	2.0	0.59	1.95
pH 5.5, without salt	32.0	1.44	1.43	1.04	0.87	0.13	—	54.0	32.0	0.70	2.24
	22.0	1.55	1.22	2.50	0.50	0.60	—	49.0	39.0	0.56	1.59
	25.0	1.59	—	1.10	0.90	0.20	—	60.0	36.0	0.70	2.28
	18.0	1.44	1.28	1.45	0.37	0.35	—	42.0	13.0	0.52	2.54
pH 7.5, 2.5 mM-$CaCl_2$	59.0	—	—	0.88	0.15	0.27	0.07	42.0	32.0	1.29	2.74
	68.4	1.70	1.25	0.55	0.12	0.24	0.01	—	—	—	—
	70.0	1.59	1.28	—	—	0.27	0.14	62.0	52.0	1.80	1.95
	68.5	1.54	1.20	0.41	0.17	0.27	0.12	28.0	—	1.44	3.02
10 min incubation at 100°C	55.0	1.20	—	>5.0	—	0.88	—	63.0	—	2.66	—
	51.0	1.28	—	gel	—	—	—	42.0	—	3.25	—
	49.5	1.35	—	gel	—	2.19	—	51.0	—	3.40	—
	42.0	1.22	—	gel	—	1.96	—	30.0	—	3.14	—
	43.0	1.40	—	gel	—	1.20	—	47.0	—	2.41	—
Untreated troponin (average values)		1.50		1.80		0.40		60.0		2.50	

presence of urea, independently of Ca^{2+} concentration, TN-B and TN-I move very slowly, and the "13 000 component" does not practically enter into separating gel. The fastest migrating component with the mobility equal to that of the marker dye corresponds to TN-C. Occasionally this component moves as a double band. The mobility of TN-C becomes significantly lower when 1 mM-EGTA is added to the protein sample and to the buffer solutions. This phenomenon is fully reversible.

Our attempts to employ isoelectric focusing for separation of troponin into its components were only partially successful, probably due to aggregation of some components even in the presence of urea. As it was reported (Drabikowski, 1972), the isoelectric point for TN-C is at pH about 3.8. TN-I and TN-B exhibit under these conditions usually few bands from pH 5.6 to 7.9, whereas the "13 000 component" has an isoelectric point at pH 8.4 - 9.0.

Effect of pH, *salts and temperature on troponin complex and its components.* Figure 5 shows the behaviour of troponin at various pH values in the presence and in the absence of salt. In the presence of 1.2 M-KCl the maximum precipitation takes place at pH 3.5, whereas at pH higher than 4.5 troponin remains soluble. In the absence of KCl troponin does not precipitate up to pH about 4.5 whereas 90% of protein precipitates at pH 5.5. Above pH 7.0 troponin is soluble even at low ionic strength. Fractions obtained in the presence of salt at pH 1.0 under the conditions used by Hartshorne *et al.* (1968) appear to be heterogeneous on electrophoresis. Supernatant, corresponding to troponin B of Hartshorne, consists chiefly of TN-B, TN-I and the minor components, whereas in the precipitate (troponin A) TN-C is the predominant species (Fig. 5). Raising pH to 3.5 at high salt concentration causes partial precipitation of TN-B and TN-I and an enrichment of supernatant in the "13 000 component."

Table 2 shows the properties of fractions obtained upon treatment of troponin at pH 1.0 in 1.2 M-KCl and at pH 5.5 at low ionic strength. The precipitate obtained at pH 1.0 has a very low E_{278}/E_{260} ratio (av. 1.0), does not interact with tropomyosin, binds exchangeable Ca^{2+} and has no effect on the actomyosin ATPase activity.

Fig. 3. Chromatography of troponin on Sephadex G-100. *A*, Crude troponin obtained from alcohol-ether powder. Numbers over the peaks denote the estimated extinction ratio E_{278}/E_{260}. *B*, Troponin purified by fractionation at 0.7 ammonium sulphate saturation (see Methods). Protein (25 mg) of either preparation was dissolved in 0.3 M-KCl - 0.02 M-tris-HCl, pH 7.5, applied to the column (100 cm × 2.5 cm) and eluted with the same solution. *C*, The same protein preparation as in *B*, except that the eluent contained 6 M-urea. Fractions (1,2,3) indicated in the Figure were analysed electrophoretically.

Fig. 4. Gel electrophoresis of troponin components separated by DEAE-Sephadex chromatography. Electrophoresis was run in *A*, 6 M-urea gels without or with 1 mM-EGTA; *B*, SDS gels. Protein (50 μg) was applied to the gels. Figures denote the successive peaks eluted from DEAE-Sephadex column.

Fig. 5. Precipitation of troponin at various pH values: (●) without KCl, (○) in 1.2 M-KCl; pH was adjusted with HCl. Temperature 20°C. SDS-polyacrylamide electrophoretic pattern of precipitates (p) and supernatants (s) obtained at indicated pH values is also given.

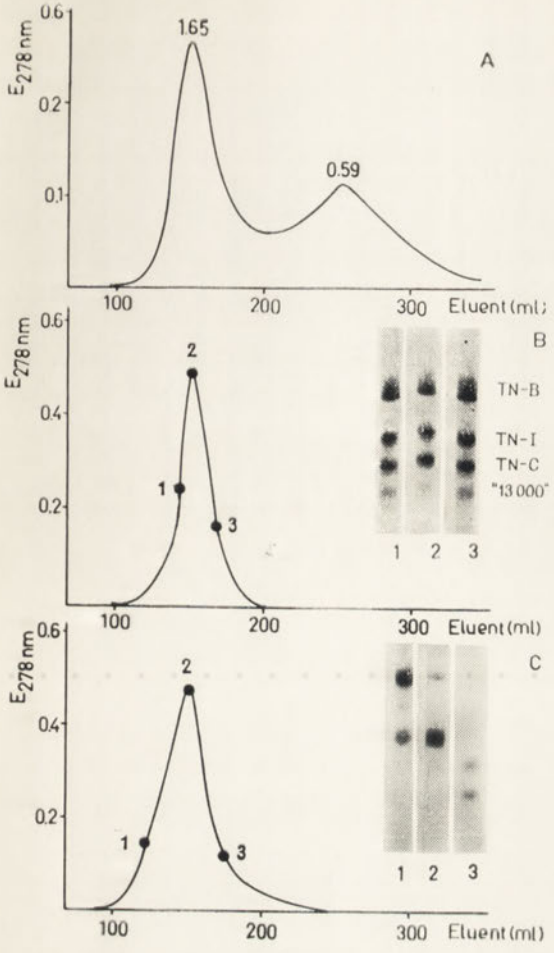


Fig. 3

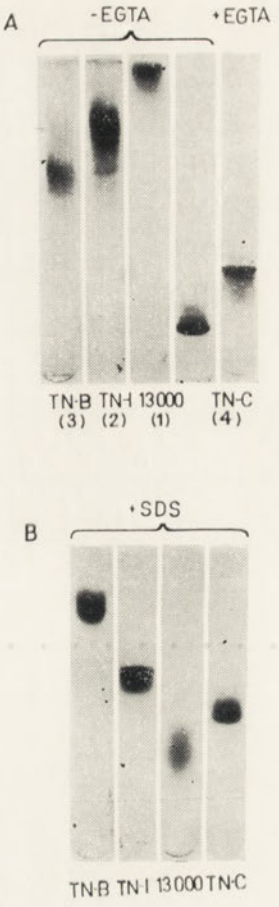


Fig. 4

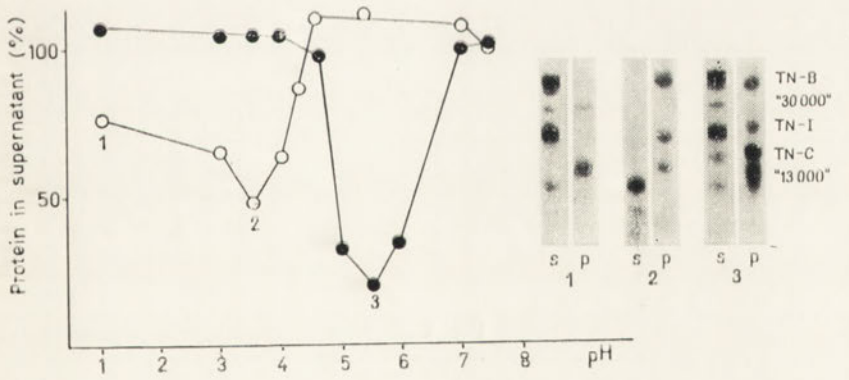


Fig. 5

W. Drabikowski *et al.* (*facing* p. 188)

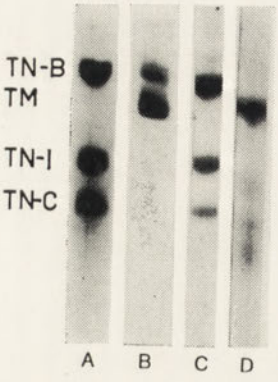


Fig. 6

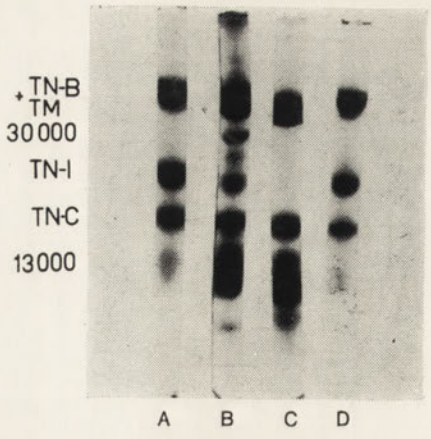


Fig. 7

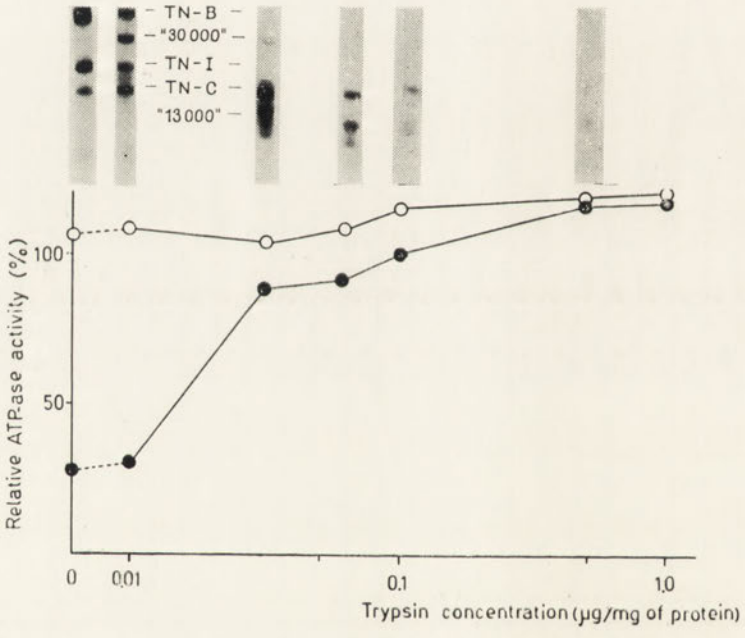


Fig. 8

W. Drabikowski *et al.* (*facing* p. 199)

Contrary to that the corresponding supernatant has an inhibitory effect on the actomyosin ATPase, practically does not bind Ca^{2+} and interacts with tropomyosin. The "pH 5.5 supernatant" is rich in TN-B and TN-I whereas the precipitate contains predominantly TN-C and the "13 000 component". As a result, the increase of the viscosity of tropomyosin in the absence of salt and inhibition of Mg^{2+}-stimulated ATPase activity of actomyosin by the precipitate is lower, whereas the binding of Ca^{2+} is higher than that of supernatant. Upon the addition of the "pH 5.5 precipitate" to the tropomyosin a coagulation is observed probably due to the effect of the "13 000 component" (Drabikowski *et al.*, 1971a).

The isolated, purified TN-I and TN-B are fairly soluble in KCl above 0.4 M, but when KCl is absent over 90% of either protein precipitates; a mixture of both proteins shows the same solubility. On the other hand, the "13 000 component" and TN-C are fully soluble in water and in KCl solutions at all the concentrations tested. The mixtures of TN-C with TN-I or with TN-B (1:1, w/w) are also soluble in the absence of salt, indicating that TN-C interacts with TN-I and TN-B to form complexes showing similar solubility as those of the intact troponin.

From the troponin solutions up to 50% of protein can be precipitated by 2.5 mM-$CaCl_2$ or $MgCl_2$. No further precipitation of protein occurs on increasing the concentration of either cation. SDS-disc electrophoresis does not reveal any differences between supernatant and precipitate, except that in the supernatant usually a new band appears with the molecular weight of about 50 000 (Fig. 9B) absent in the initial troponin preparation and in the precipitate obtained therefrom (Fig. 9A). As shown in Table 2 the effect of the supernatant on the viscosity of tropomyosin is greater than that of the precipitate.

During heating of troponin at low ionic strength at 100°C for 5 - 10 min about 30% of protein precipitates. The SDS-polyacrylamide gel electrophoresis indicates that the precipitate consists almost entirely of TN-B component (Fig. 9C). In the supernatant, besides TN-I, TN-C and small amounts of TN-B, usually a new band appears corresponding to the protein of molecular weight of about 50 000 (Fig.

Fig. 6. Isoelectric separation of troponin and tropomyosin (TM) at pH 4.5. Fraction salting out at 0.4 - 0.6 saturation with ammonium sulphate from the high ionic strength extract of the alcohol-ether powder was used. Samples in: 1 M-KCl (*A*, *B*) or 1 M-LiCl (*C*, *D*) were incubated at pH 4.5 for 1 h and subsequently separated by centrifugation. *A*, *C*, supernatants; *B*, *D*, precipitates.

Fig. 7. Degradation of troponin constituents on incubation of tropomyosin-troponin complex at pH 4.5. Troponin-tropomyosin complex (see Fig. 6) in 1 M-KCl was incubated at pH 4.5 for *A*, 0 h; *B*, 3 h; *C*, 24 h; *D* as *C* with the exception that before adjustment of pH to 4.5 the sample was heated at 100°C for 5 min.

Fig. 8. Effect of the trypsin-digested troponin on Mg^{2+}-stimulated ATPase activity of actomyosin. Samples of troponin in 20 mM-tris-HCl, pH 7.5, were digested at 20°C for 20 min by trypsin in concentrations given on the abscissa. Digestion was stopped by the addition of soya-bean trypsin inhibitor. ATPase activity was measured in the reaction mixture containing 0.30 mg/ml of actomyosin, 60 μg of digested troponin and 60 μg of tropomyosin, 0.03 M-KCl, 0.01 M-tris-HCl, pH 7.5, 2 mM-ATP, 2 mM-$MgCl_2$. Solid symbols denote samples in which 1 mM-EGTA was present. SDS-gel electrophoretic pattern of the trypsin-digested samples is included.

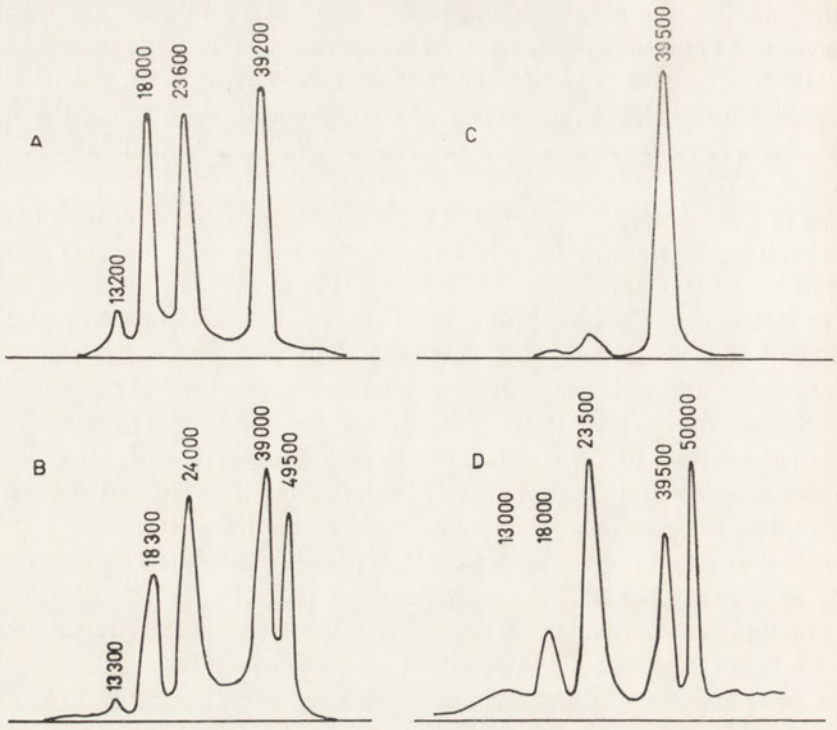


Fig. 9. Densitometric tracing of electrophoretograms of troponin fractions obtained by precipitation with $CaCl_2$ (*A*, *B*) and by heating (*C*, *D*). Electrophoretic runs in SDS gels. *A*, *C*, precipitates; *B*, *D*, supernatants. Protein (30 μg) was applied to the gels. Numbers over the peaks denote the estimated molecular weights.

9D) similarly as after precipitation with $CaCl_2$. Somewhat suprisingly, heating of troponin does not abolish its main function, i.e. its effect on ATPase activity of actomyosin and binding of Ca^{2+}. The supernatant also enhances viscosity of tropomyosin much more than the untreated troponin (Table 2).

Amino acid composition of troponin components. Contrary to other components, TN-C exhibits in addition to the tyrosine peak at 278 nm four other peaks, characteristic for phenylalanine residue (Fig. 10). In accordance this protein contains an unusually large number of phenylalanine residues (52 mol per 10^5 g protein, Table 3). TN-I, TN-B and "13 000 component", but not TN-C, show a shoulder in the region of 290 nm indicating the presence of tryptophan. TN-C is rather poor in basic residues (84 mol per 10^5 g protein) and rich in aspartic and glutamic acids (total acid residues — 321 mol/10^5 g protein). In consequence TN-C has an extremely high negative charge. TN-B and TN-I have lower content of acid residues than TN-C and the amount of basic residues is considerably higher (160 - 180 mol per 10^5 g protein). Both components show also much higher content of proline than TN-C.

Polyanionic character of TN-C is most probably responsible for the metachromatic property of this protein. Among all constituents only TN-C shifts the maximum

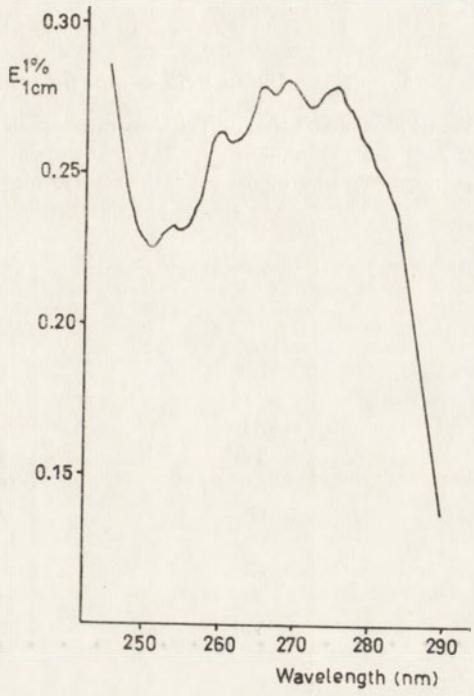


Fig. 10. Absorption spectrum of TN-C. Measurements made in 20 mM-tris-HCl, pH 7.2.

of absorption of toluidine blue to lower wavelength (Fig. 11); this observation was made by Hartshorne *et al.* (1968) with respect to troponin A. Divalent cations like Ca^{2+} or Mg^{2+} abolish this property, probably as a result of neutralization of the negative charge of TN-C. EDTA is ineffective.

The other differences concern cysteine content of troponin constituents. Data given in Table 3 show the absence of SH groups in TN-B component and relatively high content in TN-I (4.0 SH groups per mol). The content of SH groups in TN-C is 1 mol per mol.

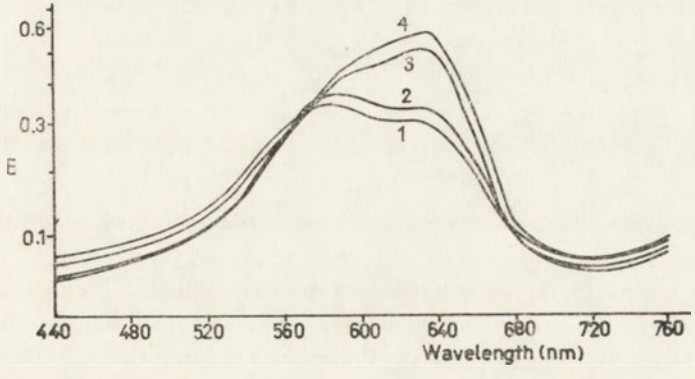


Fig. 11. Metachromatic properties of TN-C. All samples contained 2×10^{-5} M-toluidine blue. In addition they contained: *1*, 0.5 mg/ml of TN-C; *2*, 0.5 mg/ml of TN-C and 0.1 mM-EGTA; *3*, 0.5 mg/ml of TN-C and 0.1 mM-$CaCl_2$; *4*, toluidine blue alone.

Table 3

Amino acid composition of the troponin components

Values for TN-B, TN-I and TN-C are means of the determinations made with 3 different preparations of each component. As in the "13 000 component" the content of some amino acids varies significantly the results of determinations performed on two individual preparations are given. The results are expressed in mol per mol of protein.

Amino acid	TN-B	TN-I	TN-C	"13 000 component"	
Lys	47.6	24.7	9.4	14.1	11.3
His	8.0	3.8	1.1	1.5	1.9
Arg	27.6	14.1	5.9	8.0	5.3
Asp	26.4	19.2	24.4	6.8	7.5
Thr	8.1	5.1	5.0	2.2	2.6
Ser	10.3	10.2	6.0	3.4	2.9
Glu	73.0	34.2	34.4	16.0	16.7
Pro	17.2	8.8	3.5	3.3	2.3
Gly	13.1	9.5	12.8	5.4	5.4
Ala	33.8	18.2	12.5	8.3	7.9
Val	15.2	7.9	7.0	4.2	3.1
Met	5.6	9.4	8.2	0.8	0.7
Ile	10.1	5.4	7.7	4.0	4.1
Leu	24.4	19.8	9.0	11.1	9.2
Tyr	4.7	2.1	1.5	1.8	0.7
Phe	5.6	3.6	9.5	1.7	1.7
Trp[a]	—	3.3	0	—	—
SH-equivalents[b]	0	3.7	1.4	1.7	1.0

[a] Determined according to Carpenter (1948).
[b] Determined according to Drabikowski & Nowak (1965).

The "13 000 component" has an amino acid composition similar to that of TN-I. One has to mention, however, that the content of some amino acids, e.g. cysteine, varies significantly from preparation to preparation of the "13 000 component". Therefore in Table 3 instead of the mean values the results of determination of two separate preparations of the "13 000 component" are presented.

The reported values of the amino acids content of troponin components are in good agreement with the values reported recently by other authors (Hartshorne & Pyun, 1971; Schaub *et al.*, 1972; Wilkinson *et al.*, 1972).

The role of troponin components; the effect on the activity of actomyosin ATPase, binding of calcium and interaction with tropomyosin. Examination of the effect of troponin components on actomyosin ATPase (Drabikowski *et al.*, 1971a) has been extended in the present work using individual constituents, purity of which was checked with SDS-electrophoresis. Fractions TN-I and the "13 000 component" inhibit the Mg^{2+}-stimulated ATPase activity of actomyosin although the effect of TN-I is usually much higher. The inhibitory effect in both cases is not sensitive to Ca^{2+} (Fig. 12A). TN-B, as well as TN-C, have no effect on the ATPase activity

of actomyosin. Inhibition produced by either TN-I or the "13 000 component" in the presence of tropomyosin is progressively abolished by the increasing amounts of TN-C. The course of neutralization of TN-I activity by TN-C in the presence of tropomyosin and Ca^{2+} indicates that about one mol of TN-C is required to "neutralize" one mol of TN-I (Fig. 12B). In the absence of Ca^{2+} TN-C also partially abolishes inhibition of ATPase activity by TN-I, and TN-B has no effect on this process.

Even prolonged incubation with 6 M-urea and 0.1 mM-EDTA does not change the effect of troponin on the actomyosin ATPase. After a subsequent removal of urea and EDTA by dialysis troponin confers the same Ca^{2+} sensitivity to the actomyosin system as the control, untreated troponin.

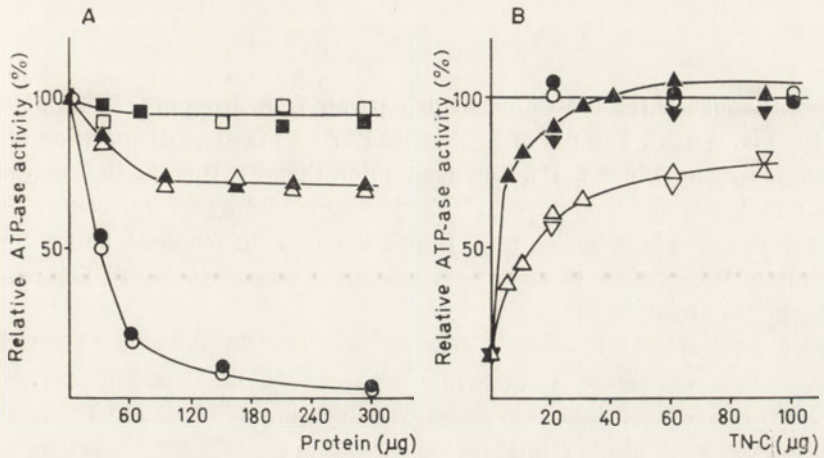


Fig. 12. Effect of troponin components of the Mg^{2+}-stimulated ATPase activity of actomyosin. *A*: TN-B (□, ■); TN-I (○, ●); the "13 000 component" (△, ▲) added in the amounts indicated on the abscissa. *B*: TN-I (△, ▲); TN-I+TN-B (▽, ▼); control without TN-B or TN-I (○, ●). Each component added in the amount of 60 µg/ml and TN-C as indicated on the abscissa. The reaction mixture contained: 0.30 mg/ml of actomyosin, 0.03 M-KCl, 0.01 M-tris-HCl, pH 7.5, 2 mM-ATP, 2 mM-$MgCl_2$, 60 mg/µl of tropomyosin and the troponin components in the indicated amounts. Solid symbols denote samples in which 1 mM-EGTA was present.

Troponin binds about 2.5 - 3.0 mol of calcium with a binding constant above 10^6 M^{-1} (Drabikowski & Baryłko, 1971). TN-C is the only component which binds calcium with a similar binding constant. Table 4 shows that TN-C contains about 4 mol of exchangeable calcium per 10^5 g of protein, not removable on a short treatment with Dowex 50. Preliminary experiments indicate that the maximum number of binding sites extrapolated from Scatchard plot is somewhat above 5 mol of Ca^{2+} per 10^5 g of TN-C. The ability to bind calcium is unaffected not only by heating but also by prolonged treatment of troponin with urea and EDTA. TN-C is also resistant to proteolysis. Its digestion with trypsin in concentration 1:100 (w/w) does not induce any visible degradation or any decrease in Ca^{2+} binding.

The recent results suggest that TN-B is the only component responsible for the interaction of troponin moiety with tropomyosin (Greaser & Gergely, 1972; Drabikowski *et al.*, 1972). Consistent with this view is our observation that TN-B is the

Table 4

Binding of exchangeable Ca *by the components of troponin*

The results are expressed as mol/10^5 g protein.

No. of troponin preparation	TN-B	TN-I	TN-C	"13 000 component"
1	0.74	0	4.74	0
2	—	0	4.65	0
3	0.17	0.10	3.72	0.04
4	0.45	0	4.82	0
5	—	0	4.18	—
6	0.46	0.30	4.40	0
7	—	0	5.70	0

component which is the most difficult to separate from tropomyosin at pH 4.5 in 1 M-KCl (Fig. 6 A,B). The use of LiCl instead of KCl favours dissociation of TN-B from tropomyosin (Fig. 6 C,D). The assumption that TN-B is the link between the troponin molecule and tropomyosin is further supported by the correlation between disappearance of TN-B during mild tryptic digestion of troponin and the loss of the ability of this protein to increase viscosity of tropomyosin (R. Dąbrowska & A. Szpacenko, unpublished).

Minor constituents of troponin preparations. The 30 000 and 13 000 daltons proteins — the two minor components — present in the troponin preparations are supposed to be the products of proteolytic digestion of TN-B and TN-I, respectively (Drabikowski *et al.*, 1971b, 1972; Dąbrowska *et al.*, 1973 a, b). It has been shown now (Fig. 8) that an extremely mild proteolysis of troponin devoid of these minor components results in the formation of "30 000 component" concomitantly with a parallel disappearance of TN-B. Simultaneous examination of the effect of digested troponin on the Mg^{2+}-stimulated ATPase activity of actomyosin shows that degradation of the TN-B to "30 000 protein" does not change the ability to inhibit ATPase in the absence of Ca^{2+}. The inhibition gradually decreases in the course of TN-I digestion.

Figure 8 shows that the increase of the trypsin concentration results in further degradation of both the "30 000 component" and TN-I to lower molecular weight products (from 11 000 to 14 000 daltons). Similarly, after adjustment of troponin preparation containing the "30 000 component" to pH 4.5, splitting of this protein to the low molecular weight products can be observed (Fig. 7). All these results seem to indicate that the fragments with molecular weight ranging from 11 000 to 14 000 daltons appear as the intermediate products of proteolytic digestion of both TN-B and TN-I.

DISCUSSION

The detailed examination of the composition of commonly used troponin preparations performed in this work clearly indicates differences in the relative proportions of the individual components. These differences are mainly due to the

effect of endogenous proteases: lysosomal cathepsin D active at acid pH and neutral protease(s) tightly adsorbed on myofibrils and responsible for degradation of TN-B and TN-I (Drabikowski *et al.*, 1971b; Dąbrowska *et al.*, 1973a,b). Under favourable conditions a large amount of these components can be decomposed at two steps of the isolation procedure either during a prolonged extraction in the neutral medium and/or during separation of troponin from tropomyosin at pH 4.5. The method recently developed by Ebashi *et al.* (1971) based on short extraction at pH 4.5 with LiCl of muscle mince devoid of myosin and of sarcoplasmic proteins enables to minimize proteolytic splitting of troponin. The use of LiCl instead of KCl, because of its more powerful effect on dissociation of troponin from tropomyosin, is also advantageous.

The results of this work furnish additional evidence for our previous suggestions (Drabikowski *et al.*, 1972) that the "13 000 component" derives from TN-I. Inhibition of Mg^{2+}-stimulated ATPase activity of actomyosin, specific for TN-I, is also observed with the "13 000 component". Another proof is the similarity in the amino acid composition of this degradation product and TN-I. At variance with the results of Wilkinson *et al.* (1972) our results indicate the presence in the "13 000 component" of that fragment of TN-I which contains at least one cysteine residue. Splitting of TN-I to form the "13 000 component" results in changes of isoelectric point, solubility and interaction with tropomyosin. The results of this paper also support the assumption (Dąbrowska *et al.*, 1973a,b) that "30 000 protein" is the first product of TN-B degradation by neutral proteases. During incubation of troponin at pH 4.5 further degradation of "30 000 component" seems to occur yielding fragments of similar molecular weight as the "13 000 component".

Preparations of crude troponin are contaminated by ribonucleoproteins, which can be easily removed by salting out with ammonium sulphate or, alternatively, by chromatography on Sephadex G-100 or DEAE-Sephadex. Ribonucleoproteins are responsible for the low E_{278}/E_{260} ratio in crude troponin. This could be also the reason of a relatively high content of carbohydrates found by Schaub *et al.* (1972). The mean content of sugars per 10^5 g protein determined in our laboratory in troponin preparations (H. Walkowiak, unpublished) is as follows: 1.2 mol of hexoses, 0.7 mol of hexosamines, 0.5 mol of methylpentoses and 0.08 mol of sialic acid. Although these sugars were found in all individual troponin components they were present in molar ratio below 1:1. This indicates that none of troponin constituents is a glycoprotein.

The components of troponin form a tightly bound complex. The sum of estimated mol. wt. of these components agrees well with the mol. wt. of 85 000 daltons obtained for intact troponin on calibrated Sephadex G-100 column (Drabikowski *et al.*, 1972). This suggests that the molar ratio of the components in the troponin complex is 1:1:1.

TN-B and TN-I exhibit an extremely high tendency to aggregate. Even in the presence of urea the dissociation of aggregates is not complete as one can judge from the results of polyacrylamide disc electrophoresis or isofocusing electrophoresis (Drabikowski, 1972). A "50 000 protein" appearing in the presence of few milli-

molar $CaCl_2$ or as a result of heating may be considered to be a product of aggregation of TN-I. The relation of this "50 000 protein" to tropocalcin (Greaser *et al.*, 1972) is now under study in our laboratory.

Interaction of TN-C with either TN-I or TN-B seems to be chiefly of electrostatic type. In the electric field even in the absence of urea partial dissociation of TN-C from the rest of the complex occurs, due to a considerable difference in the net charge between TN-C and other components. The separation of TN-C from the remaining troponin components at pH 1.0 is in favour of the electrostatic interaction.

Another factor influencing aggregation of the troponin components are Ca^{2+} ions. Extending our previous observations on the whole troponin (Drabikowski *et al.*, 1970) to the individual components we have found that EDTA decreases considerably the mobility of only TN-C. This is assumed to be the result of aggregation as suggested by Wakabayashi & Ebashi (1968). Contrary to these results, Schaub *et al.* (1972) found an increase in mobility of TN-C in the presence of EGTA. The reason for this discrepancy is not clear at present.

Wilkinson *et al.* (1972) found that TN-I and TN-C were sufficient for reconstitution of the activity of intact troponin, i.e. for conferring Ca^{2+}-sensitivity to actomyosin system, whereas Greaser & Gergely (1971) reported that TN-B is also necessary. The results of this paper are in favour of the view of former authors. Troponin devoid of TN-B by heating or by short trypsin treatment has the same activity as the whole troponin. Moreover, on examination of the effect of various troponin preparations on ATPase activity of actomyosin in the presence of EGTA, we did not find any relationship between the extent of inhibition and the amount of TN-B. Consequently, the addition of TN-B has no effect on inhibition produced by TN-I and tropomyosin system.

According to Greaser & Gergely (1971) the reconstruction of troponin complex is possible only when its components are mixed in the presence of urea; after subsequent removal of urea, the activity of original troponin can be recovered. Our observations and those of Eisenberg & Kielley (1972) suggest, however, that the treatment with urea is not necessary and that TN-C interacts with TN-I or TN-B also when the latter proteins are in the aggregated state. Probably TN-I and/or TN-B dissociate when they are solubilized by TN-C, so that another disaggregating agent like urea is not needed.

One has to mention, however, that the activity of the system containing TN-I, TN-C and tropomyosin differs somewhat from that of the intact troponin. The inhibitory effect of TN-I is partially abolished by increasing concentrations of TN-C also in the absence of Ca^{2+}. This phenomenon seems not to be due to the lack of TN-B. Similarly Schaub *et al.* (1972) noted that inhibition of the ATPase activity by a reconstituted troponin system in the presence of EGTA was often not as great as could be demonstrated on the intact myofibrils. The authors postulated the appearance of a modified form of TN-C on prolonged exposure to EGTA. This modified form of TN-C would be still able to neutralize the inhibitory effect of TN-I but the whole system would be no more sensitive to Ca^{2+}. However, we did

not find any effect of prolonged incubation with EGTA or EDTA and urea on the activity of the intact troponin to confer Ca^{2+}-sensitivity to actomyosin.

Our values for binding of Ca^{2+} by TN-C agree well with those given by Greaser & Gergely (1970) suggesting 1:1 molar ratio, but are considerably lower than the values given by Hartshorne & Pyun (1971) and Ebashi *et al.* (1971). In spite of different values reported by various authors for calcium binding by the intact troponin (Ebashi *et al.*, 1968; Fuchs & Briggs, 1968; Hartshorne & Pyun, 1971; Drabikowski & Baryłko, 1971; Bremel & Weber, 1972) it is rather evident that the amount of calcium bound by isolated TN-C can account only for a part of Ca^{2+} bound to the whole troponin complex. It is possible that TN-C is modified during preparation, and that *in vivo* it binds 2 mol of Ca^{2+} per mol. The second possibility postulated by Potter & Gergely (1972) would be that another component of troponin complex has an enhancing effect on the binding of Ca^{2+} by TN-C.

Most of the workers agree that there are two classes of binding sites in troponin. We have found that only a part of Ca^{2+} bound to troponin could be removed by EGTA or fragmented sarcoplasmic reticulum (Drabikowski *et al.*, 1970; Drabikowski, 1972). These observations in turn suggest that only the removable part of Ca^{2+}, corresponding most probably to the lower affinity binding sites, takes part directly in the contraction-relaxation cycle. A similar view was recently expressed by Bremel & Weber (1972).

The role of TN-B component is less known. The results of Greaser & Gergely (1972), Drabikowski *et al.* (1972), as well as those presented in this paper indicate that TN-B is that component of troponin which interacts with tropomyosin. The fact that *in vitro* the presence of TN-B seems not to be necessary for conferring Ca^{2+}-sensitivity to actomyosin might suggest that under these conditions TN-I and TN-C can affect the actin-myosin interaction not being bound to tropomyosin. This assumption needs, however, further support.

Troponin binds also to F-actin, even in the absence of tropomyosin. This binding seems to depend on the relative proportions of the constituents, namely only those preparations of troponin which are rich in TN-B and poor in TN-C cause precipitation of F-actin. Pure TN-B also results in the formation of F-actin paracrystals. The precipitated actin can be dissolved by the excess of TN-C. It is noteworthy that TN-B is the component which is the most difficult to remove from F-actin during purification of this protein (E. Nowak, unpublished).

All these observations strongly support the view that TN-B is the link between troponin moiety and both tropomyosin and F-actin. This was the reason for naming this component TN-B (B for binding). On the basis of the observations *in vitro* one can speculate about the role of TN-B *in vivo*. In thin filaments composed of F-actin backbone and the regulatory proteins, i.e. tropomyosin and troponin complex, TN-B might be responsible for a spatial arrangement of the other components of the regulatory protein system.

The authors are grateful to Dr. S. Buraczewski from the Institute of Animal Physiology and Nutrition, Jabłonna, for making available the Beckman Amino Acid Analyser; Mrs. E. Nowak and Dr. H. Walkowiak for determination of carbohydrates; Mrs. Z. Jeżyńska for excellent technical assistance. The work was supported in part by Foreign Research Agreement no. 05-015-1 of N.I.H. under PL 480.

REFERENCES

Bailey K. (1948). *Biochem. J.* **43**, 271 - 279.
Bremel R. D. & Weber A. (1972). *Nature* **238**, 97 - 101.
Carpenter D. C. (1948). *Anal. Chem.* **20**, 536 - 538.
Davis B. J. (1964). *Ann. N. Y. Acad. Sci.* **121**, 404 - 427.
Dąbrowska R., Dydyńska M., Szpacenko A. & Drabikowski W. (1973a) *Intern. J. Biochem.* (in press).
Dąbrowska R., Baryłko B., Nowak E. & Drabikowski W. (1973b) *FEBS Letters* **29**, 239—242.
Drabikowski W. (1972). *IV Intern. Biophys. Congress, Moscow. Symposium Papers Studia Biophysica.* (in press).
Drabikowski W. & Baryłko B. (1971). *Acta Biochim. Polon.* **18**, 353 - 366.
Drabikowski W., Baryłko B., Dąbrowska R. & Sarzała G. (1970). *Life Science* **9**, 1225 - 1233.
Drabikowski W., Dąbrowska R. & Baryłko B. (1971a). *FEBS Lett.* **12**, 148 - 152.
Drabikowski W., Dąbrowska R. & Nowak E. (1969). *Acta Biochim. Biophys. Acad. Sci. Hung.* **4**, 112 - 129.
Drabikowski W. & Gergely J. (1962). *J. Biol. Chem.* **237**, 3412 - 3417.
Drabikowski W. & Nowak E. (1965). *Acta Biochim. Polon.* **12**, 61 - 71.
Drabikowski W., Nowak E., Baryłko B. & Dąbrowska R. (1972). *Cold Spring Harbor Symposium on Quantitative Biology* **37**, 245—248.
Drabikowski W., Rafałowska U., Dąbrowska R., Szpacenko A. & Baryłko B. (1971b). *FEBS Lett.* **19**, 259 - 263.
Ebashi S. & Ebashi F. (1964). *J. Biochem.* (*Tokyo*) **55**, 604 - 613.
Ebashi S. & Endo M. (1968). *Progr. Biophys. Mol. Biol.* **18**, 123 - 183.
Ebashi S. & Kodama A. (1965). *J. Biochem.* (*Tokyo*) **58**, 107 - 108.
Ebashi S., Kodama A. & Ebashi F. (1968). *J. Biochem.* (*Tokyo*) **64**, 465 - 477.
Ebashi S. & Ohtsuki I. (1972). *XXXVII Cold Spring Harbor Symposium on Quantitative Biology* 215 - 225.
Ebashi S., Wakabayahi T. & Ebashi F. (1971). *J. Biochem.* (*Tokyo*) **69**, 441 - 445.
Eisenberg E. & Kielley W. W. (1972). *Fed. Proc.* **31**, 502.
Fiske C. H. & Subbarow Y. (1925). *J. Biol. Chem.* **66**, 375 - 400.
Fuchs F. & Briggs F. N. (1968). *J. Gen. Physiol.* **51**, 655 - 675.
Gornall A. G., Bardawill C. J. & David M. M. (1949). *J. Biol. Chem.* **177**, 751 - 766.
Greaser M. L. & Gergely J. (1970). *Fed. Proc.* **29**, 463.
Greaser M. L. & Gergely J. (1971). *J. Biol. Chem.* **246**, 4226 - 4233.
Greaser M. L. & Gergely J. (1972). *XXXVII Cold Spring Harbor Symposium on Quantitative Biology* 235 - 245.
Greaser M. L., Gergely J., Han M. H. & Benson E. S. (1972). *Biochem. Biophys. Res. Commun.* **48**, 358 - 360.
Hartshorne D. J. (1972). *XXXVII Cold Spring Harbor Symposium on Quantitative Biology* 225 - 235.
Hartshorne D. J. & Pyun H. Y. (1971). *Biochim. Biophys. Acta* **229**, 698 - 711.
Hartshorne D. J., Threiner M. & Mueller H. (1968). *Biochim. Biophys. Acta* **175**, 320 - 330.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265 - 275.
Mejbaum W. (1939). *Z. Physiol. Chemie* **258**, 117 - 120.
Murray A. C. & Kay C. M. (1971). *Biochem. Biophys. Res. Commun.* **44**, 237 - 244.
Potter J. & Gergely J. (1972). *Fed. Proc.* **31**, 501 - 501.

Schaub M. C., Perry S. V. & Häcker W. (1972). *Biochem. J.* **126**, 237 - 249.
Seraydarian K., Briskey E. J. & Mommaerts W. F. H. M. (1967). *Biochim. Biophys. Acta* **133**, 399 - 411.
Spudich J. A. & Watt S. (1971). *J. Biol. Chem.* **246**, 4866 - 4871.
Wakabayashi T. & Ebashi S. (1968). *J. Biochem.* (*Tokyo*) **64**, 731 - 732.
Weber K. & Osborn M. (1969). *J. Biol. Chem.* **244**, 4406 - 4412.
Wilkinson J. M., Perry S. V., Cole H. A. & Trayer I. P. (1972). *Biochem. J.* **127**, 215 - 228.

WŁASNOSCI TROPONINY I JEJ SKŁADNIKÓW

Streszczenie

1. Trzy główne składniki troponiny zostały otrzymane w stanie czystym drogą frakcjonowania na DEAE-Sephadexie A-50 w obecności mocznika, EDTA i przy zastosowaniu gradientu stężenia KCl.

2. Dwa składniki: białko o ciężarze cząsteczkowym 23 500 daltonów (TN-I) i białko o ciężarze cząsteczkowym 18 300 daltonów (TN-C) są wystarczające dla zapewnienia aktomiozynie czułości na Ca^{2+}. Spośród składników troponiny tylko TN-C posiada wysokie powinowactwo do Ca^{2+}. Trzeci składnik o ciężarze cząsteczkowym 40 200 daltonów (TN-B) wykazuje interakcję z tropomiozyną i F-aktyną.

3. TN-C wyróżnia się wysoką zawartością fenyloalaniny i wysokim ładunkiem ujemnym (punkt izoelektryczny — pH 3.8). Poszczególne składniki troponiny różnią się zawartością grup tiolowych.

4. Białka o ciężarach cząsteczkowych 30 000 i 13 000 daltonów obecne zazwyczaj w preparatach troponiny są produktami degradacji proteolitycznej TN-B i TN-I. W celu zmniejszenia degradacji podczas otrzymywania troponiny rekomenduje się procedurę Ebashiego i wsp. (1971).

Received 7 December, 1972.

ACTA BIOCHIMICA POLONICA
Vol. 20 1973 No. 2

A. JERZMANOWSKI and K. TOCZKO

EFFECT OF DEOXYADENOSINE AND CYCLOHEXIMIDE ON BIOGENESIS OF CHROMATIN IN PEA ROOT TIPS

Department of Biochemistry, Warsaw University, Al. Żwirki i Wigury 93, 02-089 *Warszawa, Poland*

1. The DNA: histone ratio in chromatin synthetized under conditions of DNA synthesis partly inhibited (75%) by deoxyadenosine was the same as in control chromatin. 2. This ratio was also maintained when synthesis of cytoplasmic protein was inhibited in 50 - 60% by cycloheximide. 3. Chromatin synthetized in the presence or absence of cycloheximide showed the same susceptibility to DNase I. 4. Coupling of nucleohistone formation and DNA replication in pea roots has been suggested.

Histone turnover and the relationship between histone and nuclear DNA synthesis has been studied in several laboratories (Gurley & Hardin, 1968; Balhorn *et al.*, 1972). The results concerning hepatoma tissue culture cells seem to support the view that chromatin histones are as stable in the cell as the DNA strands (Hancock, 1969; Balhorn *et al.*, 1972).

The available data indicate that the synthesis of histones is not coupled in an absolute and obligatory way with the synthesis of DNA (Littlefield & Jacobs, 1965; Sadgopal & Bonner, 1969). Recently Jones & Irvin (1972) studying the effect of hydrocortisone on the synthesis of DNA and histones in regenerating rat liver noticed that liver nuclei are able to accumulate histones synthetized under conditions of inhibited DNA synthesis. On the other hand it is known that DNA synthesis is closely coupled to the concomitant cytoplasmic synthesis of certain proteins and among them basic proteins (Wanka *et al.*, 1972).

Therefore it seemed of interest to investigate the effect of partial inhibition of either DNA or protein synthesis on the formation of DNA-histone complex in chromatin, i.e. when the normal ratio of DNA to basic proteins is affected. In this work the effect of deoxyadenosine and cycloheximide, inhibitors of DNA and protein synthesis, on biogenesis of pea chromatin is reported.

MATERIALS AND METHODS

Material. For all experiments root tips, 0.3 - 0.5 cm long, cut from 3-day-old pea seedlings (*P. sativum*, var. Kujawski) germinated in the dark at 22°C, were used.

Labelling with [^{3}H]*thymidine and* [^{14}C]*amino acids.* Root tips were placed in aqueous solution containing 5 μCi/ml of [^{3}H]thymidine-methyl (spec. radioactivity 18.0 Ci/mmol) and 2.5 μCi/ml of ^{14}C-labelled protein hydrolysate from algae (spec. radioactivity 1.0 mCi/mg) and incubated at 27°C for appropriate period of time. After incubation the root tips were washed with ice-cold water and immediately processed.

Inhibition of DNA and protein synthesis. To inhibit DNA synthesis freshly isolated root tips before labelling with isotopes were preincubated for 2 h in aqueous solution containing 2 mg/ml of deoxyadenosine. To inhibit protein synthesis preincubation was carried out for 1 h with 250 μg/ml of cycloheximide. The control material was preincubated in water.

Isolation of nuclei was performed according to the method of Sadgopal & Bonner (1970) and purity of the preparation was checked by light-microscopy.

Preparation of soluble chromatin. Freshly isolated pea nuclei were washed twice with cold 0.01 M-tris-HCl buffer, pH 8.0, and suspended in the same buffer. This suspension was sheared at 0°C in the Unipan type 203 homogenizer for 2×90 sec at 150 V, centrifuged for 30 min at 15 000 *g* and the supernatant was used for analysis.

Fractionation of chromatin into DNA, histones, non-histone proteins and RNA was carried out as described by Ochalska-Czepulis & Toczko (1972).

Determination of DNA, RNA and protein. DNA was determined by the method of Burton (1956) with calf thymus DNA as a standard. RNA was determined by the method of Mejbaum (1939) with yeast RNA as a standard, and protein by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Determination of radioactivity. The measurements involved: *a*, total cell proteins [2% NaOH extract of the 10% trichloroacetic acid (TCA) precipitate of the whole tissue homogenate]; *b*, acid-extractable nuclear proteins (0.4 N-HCl extract; double extraction); *c*, total DNA, isolated according to Schneider (1946); *d*, chromatin DNA; *e*, chromatin proteins (histones and the non-histone proteins non-extractable with 0.4 N-HCl). The examined samples were collected on filter paper discs which were washed sequentially with 10% TCA, 95% ethanol and anhydrous ethyl ether, air dried and placed in scintillation vials to which 10 ml of toluene containing POP and POPOP was added. Discs were counted for ^{3}H radioactivity (DNA) and ^{14}C radioactivity (proteins) in Packard Liquid Scintillation Counter.

Digestion of chromatin by DNase I. Solution of [^{3}H]thymidine-labelled chromatin (0.6 - 0.8 E units at 260 nm) in 0.01 M-tris-HCl - 3 mM-$MgCl_2$ buffer, pH 8.0, was digested with DNase (18 μg/ml) at 37°C for 30 min, cooled and centrifuged at 12 000 *g* after the addition of TCA to 5% final concentration. Radioactivity of the DNA in pellet was determined after hydrolysis in 5% TCA (90°C, 15 min).

Reagents. Acrylamide, *N,N*-bisacrylamide, TEMED, sodium persulphate, deoxyadenosine and cycloheximide were from Serva (Heidelberg, G.F.R.); DNase

I and calf thymus DNA were from Worthington Biochem. Corp. (Freehold, N.J., U.S.A.), yeast RNA from Light and Co. Ltd (Colnbrook, England) and bovine serum albumin from Michrome (London, England). Labelled compounds were from UVVR (Czechoslovakia). Other reagents were from P.O.Ch. (Gliwice, Poland).

RESULTS

The experiments were performed with the root tips excised from 3-day-old pea seedlings. Incubation period did not exceed 4 h. It has been proved beforehand that a 6 - 8 h incubation of the excised tips in water disturbed neither DNA nor protein synthesis (Fig. 1).

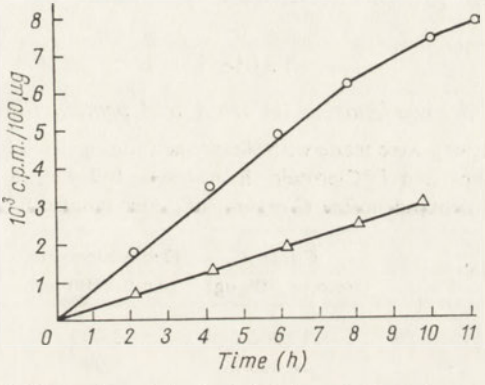


Fig. 1. Biosynthesis of DNA and protein in isolated root tips of pea. ○, DNA; △, protein.

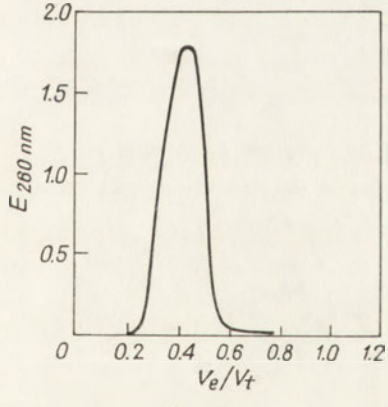


Fig. 2

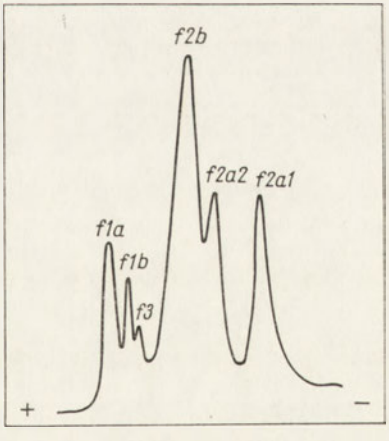


Fig. 3

Fig. 2. The elution profile of pea chromatin on Sepharose 4B. Solubilized chromatin, 5 ml, containing 10 E units at 260 nm was applied to the column (1.5×20 cm) and chromatographed as described by Ochalska-Czepulis & Toczko (1972).

Fig. 3. Densitometric scan of electrophoretic pattern of histones from pea chromatin.

Chromatin isolated by our procedure from pea roots appeared as a single sharp peak when chromatographed on Sepharose 4B column (Fig. 2). The absorption of chromatin at 320 nm did not exceed 2 - 3% of that at 260 nm and the value E_{280}/E_{260} was approx. 0.65. Weight ratios of DNA, protein and RNA in chromatin were: DNA, 1.0; histones, 0.98 - 1.08; non-histone proteins, 0.38 - 0.45; RNA, 0.08 - 0.13. Densitogram of proteins extracted from chromatin with 0.4 N-HCl and fractionated by gel electrophoresis showed the pattern typical for histones (Fig. 3). These data proved that the obtained chromatin preparation was not contaminated with cytoplasmic proteins or RNA.

A 2 h preincubation of pea roots with deoxyadenosine (2 mg/ml) — an inhibitor of DNA synthesis — resulted in an about 75% decrease in specific radioactivity of total and chromatin DNA (Table 1). The specific radioactivity of both total and acid-extractable nuclear proteins decreased concomitantly by only 10%. In chroma-

Table 1

Effect of deoxyadenosine on DNA and protein biosynthesis

Measurements of radioactivity were made with the preparations isolated from pea roots incubated for 1 h with [^{3}H]thymidine and [^{14}C]protein hydrolysate following a 2 h preincubation with deoxyadenosine (2 mg/ml) or water (control).

Preparation	Control (c.p.m./100 µg)	Deoxyadenosine (c.p.m./100 µg)	Inhibition (%)
Total DNA	12 900	3 000	77
Total protein	6 500	5 900	9
Acid-extractable nuclear proteins	2 900	2 550	12
Chromatin:			
DNA	15 200	3 400	78
histones	3 100	800	75
non-histone proteins	35 700	30 200	15

Table 2

Effect of cycloheximide on DNA and protein biosynthesis

Details as in legend to Table 1, except that preincubation was carried out with cycloheximide (250 µg/ml) for 1 h.

Preparation	Control (c.p.m./100 µg)	Cycloheximide (c.p.m./100 µg)	Inhibition (%)
Total DNA	11 300	5 600	50
Total protein	6 500	2 350	64
Acid-extractable nuclear proteins	3 000	1 300	57
Chromatin:			
DNA	13 600	6 600	49
histones	2 400	1 150	52
non-histone proteins	31 900	20 700	35

Table 3

Digestion of chromatin DNA by DNase I

^{3}H-labelled chromatin isolated from pea root tips treated with cycloheximide as described in Material and Methods was digested with DNase I (18 μg/ml) at 37°C for 30 min. The radioactivity was measured in the acid precipitable DNA before and after digestion.

Chromatin from pea roots	^{3}H c.p.m./100 μg DNA		Digestion (%)
	Before digestion	After digestion	
Control	12 000	6 300	48
Cycloheximide-treated	5 300	2 600	51

tin, however, a specific radioactivity of histones was lowered exactly as in DNA, while the decrease in specific activity of non-histone proteins followed that of total cell proteins.

Data given in Table 2 proved that on 1 h preincubation with 250 μg/ml of cycloheximide the synthesis of total cell proteins and the acid-extractable nuclear proteins was inhibited by about 60%. In chromatin the specific radioactivity of histones was decreased to a similar extent, while that of non-histone proteins seemed to be somewhat less affected by cycloheximide (35%). It is noteworthy that DNA synthesis was also decreased to an extent similar to that observed with chromatin-bound histones.

To examine the effect of decreased synthesis of nuclear basic proteins on the character of DNA-histone complex, chromatins synthetized in the presence and absence of cycloheximide were digested with DNase I (Table 3). No difference in digestibility of DNA in the respective preparations of chromatin was observed.

DISCUSSION

To study chromatin biosynthesis in pea root tips deoxyadenosine and cycloheximide, inhibitors of DNA and protein synthesis, were employed. Deoxyadenosine was chosen because of its selective action on DNA synthesis (Larsson & Reichardt, 1966). Cycloheximide, which acts primarily on protein synthesis, affects also DNA synthesis possibly due to deficiency of histones. According to Wanka *et al.* (1972) synthesis of histones is very sensitive to cycloheximide whereas the activity of enzymes involved in DNA synthesis remain unchanged. Recently Weintraub (1972) provided some experimental data suggesting that histones could play the role of "chain elongating proteins" in DNA replication.

The presented data show that deoxyadenosine and cycloheximide did not alter the ratio of specific radioactivity of DNA and histones in chromatin. These results seem to suggest that both under conditions of DNA or protein deficiency the formation of DNA-histone complex was not disturbed. This was confirmed by the same digestibility of DNA in chromatins synthetized in the presence and absence of cycloheximide. The constant ratio of specific radioactivity in DNA and histones in chromatin synthetized in the presence of deoxyadenosine indicates the lack of

exchange of histones accumulating under these conditions with the histones of preformed chromatin. On the contrary, as no such relation was observed in the case of DNA and non-histone proteins, one can assume that these proteins exchange actively with the corresponding proteins of chromatin.

The presented evidence is in favour of the Weintraub (1972) hypothesis on direct coupling of nucleohistone formation and DNA replication.

This work was supported by the Polish Academy of Sciences within the project 09.3.1. The authors are grateful to Professor I. Chmielewska for her encouragement and critical review of the manuscript, to Professor H. Teleżyński for helpful suggestions and discussion and to Miss A. Pawełkiewicz for skillful assistance.

REFERENCES

Balhorn R., Oliver D., Hohmann P., Chalkley R. & Granner D. (1972). *Biochemistry* **11**, 3915 - 3921.
Burton K. (1956). *Biochem. J.* **62**, 315 - 323.
Gurley L. R. & Hardin J. M. (1968). *Arch. Biochem. Biophys.* **128**, 285 - 292.
Hancock R. (1969). *J. Mol. Biol.* **40**, 457 - 466.
Jones R. B. & Irvin J. L. (1972). *Arch. Biochem. Biophys.* **152**, 828 - 838.
Larsson A. & Reichardt P. (1966). *J. Biol. Chem.* **241**, 2540 - 2549.
Littlefield J. W. & Jacobs P. S. (1965). *Biochim. Biophys. Acta* **108**, 652 - 658.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265 - 274.
Mejbaum W. (1939). *Z. Physiol. Chem.* **258**, 117 - 120.
Ochalska-Czepulis M. & Toczko K. (1972). *Acta Biochim. Polon.* **19**, 347 - 351.
Sadgopal A. & Bonner J. (1969). *Biochim. Biophys. Acta* **186**, 349 - 357.
Sadgopal A. & Bonner J. (1970). *Biochim. Biophys. Acta* **207**, 206 - 226.
Schneider W. C. (1946). *J. Biol. Chem.* **164**, 747 - 751.
Wanka F., Moors J. & Krijzer F. N. C. M. (1972). *Biochim. Biophys. Acta* **269**, 153 - 161.
Weintraub H. (1972), *Nature* **240**, 449 - 453.

WPŁYW DEZOKSYADENOZYNY I CYKLOHEKSYMIDU NA BIOGENEZĘ CHROMATYNY W WIERZCHOŁKACH WZROSTU KORZENI GROCHU

Streszczenie

Wykazano, że stosunek DNA do histonów w chromatynie syntetyzowanej w warunkach częściowego zahamowania syntezy DNA (75%) przez dezoksyadenozynę jest taki sam jak w chromatynie kontrolnej. Stosunek ten zachowany jest również, gdy cytoplazmatyczna synteza białka wyhamowana jest w 50 - 60% przez cykloheksymid. Nie zaobserwowano różnic w podatności na trawienie DNazą I DNA z chromatyny syntetyzowanej w obecności cykloheksymidu i DNA z chromatyny kontrolnej. Wysunięto sugestię o sprzężeniu syntezy nukleohistonu z replikacją DNA w korzeniach grochu.

Received 30 January, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 20 1973 No. 2

RECENZJE KSIĄŻEK

BIOLOGICAL HYDROXYLATION MECHANISMS. Biochemical Society Symposia, Number 34, Edinburgh 1971 (G. S. Boyd and M. S. Smellie, eds.) Academic Press, London, New York 1972; str. 250, cena £ 6.00.

Książka jest zbiorem 11 referatów wygłoszonych w Uniwersytecie w Edynburgu w 1971 r. na międzynarodowym sympozjum poświęconym mechanizmom hydroksylacji w układach biologicznych. Referaty dotyczą różnorodnych zagadnień z tej dziedziny — od modelowych układów monooksygenaz, poprzez badanie budowy i mechanizmów działania enzymów hydroksylujących aż do ostatnich osiągnięć w badaniach cytochromu P-450 i hydroksylacji cholesterolu w wątrobie. Wszystkie są uzupełnione obszerną bibliografią.

Referat G. S. Boyda *Biologiczne Reakcje Hydroksylacji* jest obszernym wprowadzeniem do zagadnień objętych tematyką sympozjum i jest połączeniem faktów znanych z uprzednich badań z aktualnymi danymi przedstawianymi w pozostałych referatach sympozjum. Przedmiotem referatu J. E. van Liera i wsp. jest rola wodoronadtlenków steroli w metabolizmie steroli w wątrobie i korze nadnerczy przy udziale miksosomów i mitochondriów. Charakterystykę czynnych składników (rozpuszczalny cytochrom P-450, oksydoreduktaza NADPH - cytochrom *c*), rola fosfatydylocholiny oraz mechanizm działania rozfrakcjonowanego układu enzymatycznego z wątroby, katalizującego hydroksylację kwasów tłuszczowych, węglowodorów i leków zawiera referat M. J. Coona i wsp. Zmiany spektralne w mikrosomach wątroby wywołane podawaniem zwierzętom lekarstw przedstawił S. Orenius. Referat A. G. Hildebrandta dotyczy wiązania metyraponu (2-metylo-1,2-bis-3-pirydylopropanon) przez cytochrom P-450. Metyrapon hamuje, jak wiadomo, metabolism wielu lekarstw i innych związków w wątrobie, działając na mikrosomalne oksydazy o mieszanej funkcji. Autor zidentyfikował w mikrosomach wątroby szczura formę nisko i wysoko spinową ferri-cytochromu P-450 oraz dwie formy ferro-cytochromu P-450. Sugeruje, że metyrapon i substrat są wiązane w cytochromie P-450 przez dwa różne miejsca. Związanie inhibitora uniemożliwia utworzenie aktywnego połączenia cytochromu P-450 z tlenem, które hydroksyluje substrat.

H. V. Gelboin i wsp. wykazują, że mikrosomalne hydroksylazy arylowęglowodorów są adaptacyjnym układem enzymatycznym, przy czym synteza RNA zachodzi tylko w początkowym 30 - 60 minutowym okresie. Na podstawie inhibicji 7,8-benzoflawonem stwierdzili istnienie dwu form badanego enzymu. Dane dotyczące budowy i reakcji centr aktywnych hydroksylazy (tj. cytochromu P-450 i putidaredoksyny, białka zawierającego żelazo i siarkę) przekształcającej D-kamforę w 5-*exo*-alkohol przedstawili I. C. Gunsalus i wsp. Stwierdzili m.in. niezwykle szybki (poniżej 100 msec) przebieg reakcji między zredukowaną formą putidaredoksyny a cytochromem P-450.

Podsumowaniem danych odnośnie mechanizmu reakcji cytochromu P-450 z substratem, tlenem i donatorem elektronów w reakcjach hydroksylacji jest referat R. W. Estabrooka i wsp. Interesujące są podobieństwa i różnice w działaniu cytochromu P-450 związanego z błoną, jak to ma miejsce w tkankach zwierzęcych, oraz rozpuszczalnego cytochromu P-450 izolowanego z bakterii. Warto dodać, że schemat cyklicznych przekształceń oksydoredukcyjnych cytochromu P-450 w reakcjach hydroksylacji zaproponowany przez autorów, znalazł się również na obwolucie książki. Badaniom interakcji cytochromu P-450 (w jego formie Fe^{2+} i Fe^{3+}) z kory nadnerczy ze steroidami i inhibitorami ich metabolizmu poświęcony jest referat H. Schleyera i wsp. Zamykający sympozjum

referat (G. S. Boyda) dotyczy hydroksylacji cholesterolu w wątrobie i w tkankach wytwarzających hormony sterydowe, co prowadzi do syntezy kwasów żółciowych lub pregnenolonu.

Książka jest cenną pozycją dla tych wszystkich, którzy zajmują się lub interesują zagadnieniami hydroksylacji w układach biologicznych.

Zbigniew Kaniuga

CLINICAL IMMUNOLOGY. World Health Organization Technical Report Series, no. 496, 1972; str. 50, cena $ 1,0, fr. szw. 4.-

Sprawozdanie Naukowego Komitetu Ekspertów Światowej Organizacji Zdrowia z 1971 r. przedstawia aktualne problemy immunologii klinicznej — dziedziny obejmującej schorzenia charakteryzujące się nienormalną czynnością tkanki limfoidalnej oraz schorzenia, w których jakość odpowiedzi immunologicznej odgrywa podstawową rolę.

Dzięki postępowi w ostatnim dziesięcioleciu wiele zagadnień immunologicznych zostało wyjaśnionych, np. czynność grasicy, natura współudziału komórek w procesach odpowiedzi immunologicznych, mechanizmy tworzenia przeciwciał oraz biologiczna funkcja i nieprawidłowości różnych immunoglobulin.

Sprawozdanie to przedstawia aktualny stan wiedzy w zakresie immunologii oraz sposoby praktycznego jej zastosowania w problemach klinicznych. Zawiera propozycje tworzenia organizacyjnych jednostek immunologii klinicznej w instytutach akademickich, sugerując pilną konieczność tworzenia takich jednostek z odpowiednim personelem i pełnym zapleczem laboratoryjnym. Główne dziedziny wymagające podstawowych badań immunologicznych są to: choroby immunoproliferacyjne, infekcyjne, alergiczne, hematologiczne oraz transplantologia.

Takie jednostki immunologii klinicznej byłyby także zaangażowane w kliniczne badania problemów takich, jak zapobieganie anemii hemolitycznej u noworodków, wykrywanie infekcji wewnątrzmacicznych, wczesna diagnostyka chorób nowotworowych oraz ocena podatności danej populacji na choroby infekcyjne.

Aneks 1 zawiera szczegółowy przegląd problemów klinicznej immunologii oraz testów diagnostycznych mających zastosowanie w poszczególnych typach schorzeń. Osobne podrozdziały są poświęcone zagadnieniom mechanizmów odpowiedzi immunologicznych, chorobom infekcyjnym i czynnikom immunizacyjnym, zjawiskom nadwrażliwości, immunohematologii, schorzeniom połączonym z autoimmunizacją, przeszczepom, supresji efektów immunopatologicznych i wreszcie aktywności immunologicznej guzów nowotworowych.

Barbara Kwiatkowska-Patzer

ORAL ENTERIC BACTERIAL VACCINES. World Health Organization Technical Report Series, no 500; 1972, str. 34, cena $ 1,0.

Doustne szczepienia przeciwko chorobom infekcyjnym jelit po raz pierwszy wprowadzono 70 lat temu. Nie były one jednak powszechnie zaakceptowane, ponieważ nie powodowały znamiennego wzrostu poziomu przeciwciał w surowicy i wobec tego wnioskowano, że nie powodują powstania odporności.

Obecnie rozważane są inne kryteria pozwalające stwierdzić wartość doustnych czynników uodparniających.

Badania nad szczepionkami *Shigella* i *Salmonella* oraz postępy immunologii na nowo postawiły problem dostnego uodparniania przeciwko głównym schorzeniom bakteryjnym przewodu pokarmowego, jak dur brzuszny, czerwonka, cholera oraz zakażenia patogennymi szczepami *Escherichia coli.*

Sprawozdanie Komitetu Ekspertów WHO z 1971 r. przedstawia aktualny stan badań i doświadczenia kliniczne nad profilaktyką doustną infekcji jelitowych. Zwraca szczególną uwagę na znaczenie lokalnych mechanizmów immunologicznych w przewodzie pokarmowym. Mechanizmy te nie są jeszcze całkowicie poznane.

Sprawozdanie zwraca uwagę na pilną potrzebę badań tych mechanizmów, bowiem pełna ich znajomość pozwoli ocenić wartość doustnej szczepionki.

Podkreślono wyraźnie, że żadna ze szczepionek parenteralnych stosowanych obecnie nie daje dobrych wyników, ponieważ dają one krótki okres odporności, objawy uboczne, są kosztowne i stwarzają znaczne trudności użycia dla zabezpieczenia całej populacji od ryzyka zachorowania.

Dla wszystkich tych powodów sprawa oceny wartości szczepionki doustnej, a szczególnie czasu trwania uodpornienia przez nią wywołanego, wydaje się być sprawą pilną.

Barbara Kwiatkowska-Patzer

KOMUNIKAT

Komitet Mikrobiologiczny Polskiej Akdaemii Nauk i Redakcja „Postępów Mikrobiologii" uprzejmie zawiadamiają PT Czytelników, że od roku 1974 „Postępy Mikrobiologii" ukazywać się będą jako kwartalnik.

Zamówienia i prenumeratę przyjmują wszystkie urzędy pocztowe i listonosze. Wpłaty można również uiszczać na konto PKO nr 1-6-100020 RSW „Prasa-Książka-Ruch", Centrala Kolportażu Prasy i Wydawnictw, ul. Towarowa 28, 00-839 Warszawa.

Cena zł 25—.

ACTA BIOCHIMICA POLONICA

PRENUMERATA KRAJOWA

Cena prenumeraty krajowej

rocznie 100,—

półrocznie 50,—

Instytucje państwowe, społeczne, zakłady pracy, szkoły itp. mogą zamawiać prenumeratę wyłącznie w miejscowych Oddziałach i Delegaturach RSW „Prasa-Książka-Ruch".

Prenumeratorzy indywidualni mogą opłacać w urzędach pocztowych i u listonoszy, lub dokonywać płat na konto PKO Nr 1-6-100020 RSW „Prasa-Książka-Ruch", Centrala Kolportażu i Wydawnictw, Warszawa, ul. Towarowa 28 (w terminie do 10 dnia miesiąca poprzedzającego okres prenumeraty).

PRENUMERATA ZAGRANICZNA

Prenumeratę za zleceniem wysyłki za granicę, która jest o 40% droższa od prenumeraty krajowej, przyjmuje RSW „Prasa-Książka-Ruch", Biuro Kolportażu Wydawnictw Zagranicznych, Warszawa, ul. Wronia 23, konto PKO Nr 1-6-100024.

Bieżące i archiwalne numery można nabyć lub zamówić we Wzorcowni Wydawnictw Naukowych PAN-Ossolineum-PWN, 00-901 Warszawa, Pałac Kultury i Nauki (wysoki parter) oraz w księgarniach naukowych „Domu Książki".

Sprzedaż egzemplarzy zdezaktualizowanych, na uprzednie pisemne zamówienie, prowadzi RSW „Prasa-Książka-Ruch", Centrala Kolportażu Prasy i Wydawnictw, 00-958 Warszawa, skr. poczt. 12.

SUBSCRIPTION ORDERS CAN BE SENT DIRECTLY TO:
„ARS POLONA — RUCH"
WARSZAWA 1
P.O. BOX 154
SENDING REMITTANCE OF $ 12.— THROUGH THE
BANK HANDLOWY, WARSZAWA, ul. TRAUGUTTA 7

Acta bioch. pol. Vol. 20, No. 1, s. 113—206, Warszawa, kwiecień—czerwiec 1973.
Indeks 35202