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ACTA BIOCHIMICA POLONICA

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ZOFIA KURATOWSKA, E. KOWALSKI, B. LIPIŃSKI and ELIGIA MICHALAK

PREPARATION OF THE ERYTHROPOIETIC FACTOR FROM HUMAN BLOOD PLASMA*

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In recent years numerous reports on the isolation and chemistry of erythropoietin, the humoral factor stimulating erythropoiesis, have been published [7, 8, 12]. Goldwasser and coll. [12, 4] starting with plasma from anaemic sheep succeeded in concentrating the erythropoietic activity by chromatographic techniques. They demonstrated that this activity was connected with the α_2-glycoprotein fraction of plasma proteins. Lowy *et al.* [6] obtained similar results with rabbit blood plasma using ammonium sulfate fractionation and adsorption on suitable adsorbents.

In this report some findings concerning the presence of erythropoietin in human blood and the possibility of its isolation are described.

MATERIAL AND METHODS

Materials and chemicals. Blood from polycythaemic patients was obtained by venipuncture, and plasma was separated by centrifugation. Cohn fractions, obtained by the Cohn's method 6 [1] from 80 lit. of plasma of regular blood donors, were kindly supplied by the Blood Derivatives Division of the Institute of Haematology, Warszawa. Placental blood and placental Cohn Fraction IV were supplied by Warszawska Wytwórnia Surowic i Szczepionek.

Neuraminidase preparation of bacterial origin was a generous gift from Prof. Dr. H. F. Schultze and Dr. G. Schwick (Marburg a. L.), and methoxyneuraminic acid from Prof. Dr. E. Klenk (Köln).

^{59}Fe citrate was purchased from the Radiochemical Centre, Amersham, England. DEAE-cellulose (Serva, Heidelberg) and Amberlite IRC-50, mesh size about 100 (Rohm & Haas Co., U.S.A.) were used.

* Preliminary data were presented at the Conference on Erythropoietin and the regulation of Erythropoiesis, San Francisco, U.S.A., January 21 - 23, 1962.

Methods. Erythropoietic activity was estimated in three ways after subcutaneous injections of the substance tested.

1. Reticulocyte counts. Normal adult male mice were injected with 0.5 ml. per animal on three successive days. Six mice were used for each experiment. Reticulocytes were counted before the first injection (0 day) and on the 4th, 6th and in some experiments on the 10th day.

2. Determination of the erythromyeloid ratio in the bone marrow of mice femurs. It was determined on the 7th to 10th day after the first injection. Glass slide films were stained with Giemsa's.

3. Determination of ^{59}Fe incorporation according to the method of Fried *et al.* [3]. Rats were injected with 1 ml. of the solution tested on two successive days. On the 3rd day 1 μc of the isotonic solution of ^{59}Fe citrate, pH 7.0, was injected intravenously. After 20 hr. the radioactivity of 1 ml. haemolysed blood samples was estimated with a scintillation counter (EKCO, type N-550 *A* with a crystal of 0.5 inch in diameter). The accuracy of counting was 3.5%.

In some experiments the fractions were tested by all three methods, in other only the incorporation of ^{59}Fe was determined on starved for 48 hr. as well as on non-starved animals.

The effect of neuraminidase on the erythropoietic active substance was tested as follows: 20 mg. of active fraction was dissolved in 5.5 ml. of 0.9% NaCl and 2 ml. of 0.1 M-$CaCl_2$ and 1.5 ml. of neuraminidase (containing 100 units per ml.) were added. After adjustment of the solution with 0.01 N-KOH to pH 5.6, the mixture was incubated at 37° for 1.5 hr. and then dialysed for 24 hr. against water. One ml. portions of the solution of the digested substance were injected into rats.

Sialic acid was determined by the method of Werner & Odin [11] using methoxyneuraminic acid as a standard, hexoses according to Weimer & Moshin [10] and hexosamines by the Elson-Morgan method as described by Rondle & Morgan [9]. Uronic acid was determined according to Dische [2] and total N by the micro-Kjeldahl method.

Electrophoresis on the cellulose-acetate strips was carried out in barbital buffer, pH 8.6, at 100 v, for 1 hr., staining with Amido-Black 10 B. Chromatography on ion exchange columns was performed as described by White *et al.* [12].

UV spectrophotometry was carried out with a Unicam SP 500 instrument.

RESULTS

Blood plasma fractions

It was possible to separate erythropoietic activity from polycythaemic plasma by ammonium sulfate fractionation. The scheme of fractionation is presented in Table 1, the results of biological assays of the

respective fractions are shown in Table 2. It can be seen that by 50% saturation with ammonium sulfate the inactive proteins are removed, while erythropoietic activity is precipitated by 75% saturation.

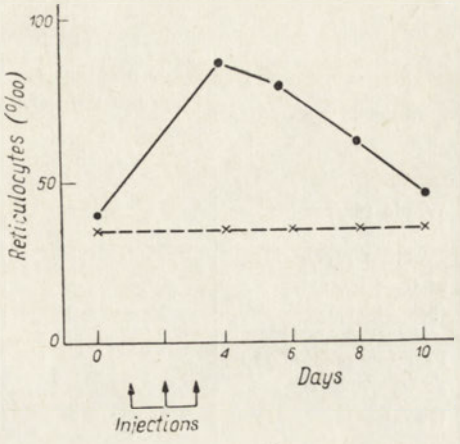

Fig. 1. The effect of Cohn Fraction IV_4 from human blood plasma on reticulocytosis in mice

It was found that erythropoietic activity is concentrated in Cohn Fraction IV_4 obtained from human blood donors' plasma. The activity of the whole plasma and of its fractions is presented in Table 3 and in Fig. 1, and the corresponding chemical data in Table 4.

Table 1

Scheme of ammonium sulfate fractionation of polycythaemic or placental blood plasma

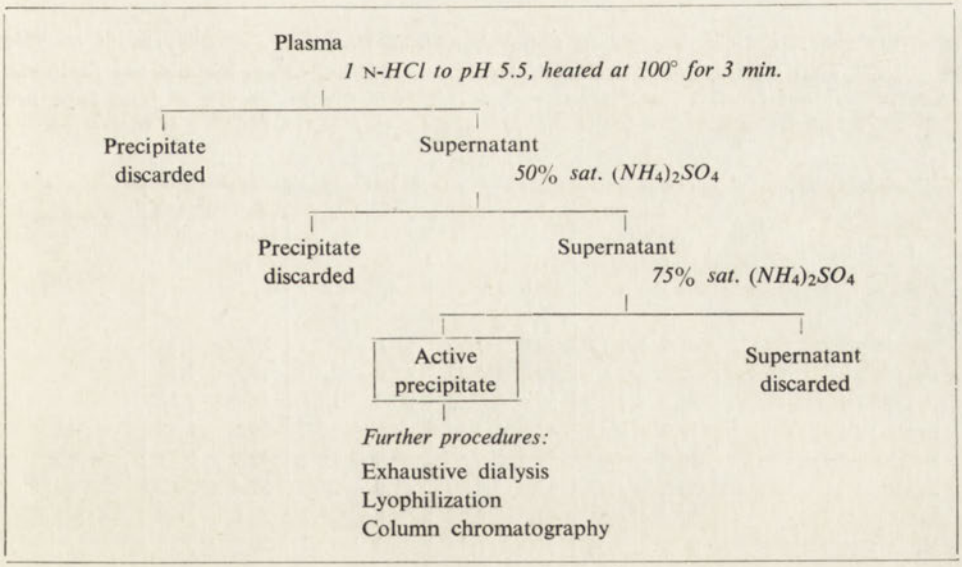

Table 2

Effect of polycythaemic plasma fractions on erythropoiesis

Average values of 6 animals, ± S.D.M. are given. Details see Methods

Material injected	Incorporation of ^{59}Fe into red cells of rats (% of the dose)	Reticulocytosis in mice (‰) 0 day	Reticulocytosis in mice (‰) 6th day	Erythroblasts in mice bone marrow (%)
Whole plasma	52.2 ± 6.25	30.0 ± 6.52	65.0 ± 17.2	32.4 ± 5.21
Fraction precipitated at 0—50% sat. $(NH_4)_2SO_4$	25.0 ± 4.25	36.0 ± 8.16	35.7 ± 10.2	23.3 ± 4.12
Fraction precipitated at 50—75% sat. $(NH_4)_2SO_4$	64.8 ± 3.31	38.0 ± 11.2	98.4 ± 13.5	41.2 ± 3.51
Supernatant after prepitation at 75% sat. $(NH_4)_2SO_4$	24.3 ± 4.38	37.2 ± 8.31	38.0 ± 12.6	22.2 ± 6.12
None (control)	26.6 ± 5.61	36.5 ± 6.51	35.3 ± 6.93	20.9 ± 3.53

Table 3

Effect of Cohn fractions of human plasma on erythropoiesis

For the fractionation the pooled donors' plasma was used. Incorporation of ^{59}Fe was estimated on rats, other estimations were made on mice. Details see Methods. Average values from 6 experiments, ± S.D.M. are given. Values in bold type are statistically significant in respect to the control ($p < 0.01$)

Fraction injected	^{59}Fe incorporation (% of the dose)	Reticulocytosis (‰) 0 day	Reticulocytosis (‰) 4th day	Erythroblasts in bone marrow (%)	Erythropoietic activity
Whole plasma	**40.0** ± 2.12	32.0 ± 7.32	**53.1** ± 7.32	**29.0** ± 3.31	+
Fraction I	26.4 ± 2.42	40.8 ± 7.12	45.0 ± 3.40	19.6 ± 7.82	—
Fraction II/III	26.2 ± 3.43	37.6 ± 9.11	33.0 ± 13.1	16.9 ± 3.22	—
Fraction IV_1	**39.5** ± 4.52	41.6 ± 7.33	**58.0** ± 12.7	**29.6** ± 2.28	+
Fraction IV_4	**68.8** ± 3.01	39.4 ± 7.58	**86.0** ± 18.4	**42.9** ± 5.77	+++
Fraction V	**35.2** ± 4.63	33.2 ± 18.7	41.0 ± 21.8	**32.4** ± 5.43	+
Fraction VI	20.5 ± 4.51	40.7 ± 22.0	54.6 ± 21.2	23.0 ± 3.39	—
None (control)	26.6 ± 5.61	36.5 ± 6.51	36.4 ± 7.62	20.9 ± 3.53	

Table 4

Estimation of some components in blood plasma Cohn fractions

Percent values of dry wt. are given. Details see Methods

Fraction	Total nitrogen	Sialic acid	Hexosamine	Hexoses	Uronic acids	Erythropoietic activity
I	13.5	0.70	1.6	1.3	0.0	—
II/III	11.0	0.96	2.1	1.9	0.3	—
IV_1	8.2	1.22	2.4	2.3	0.0	+
IV_4	11.5	1.58	3.5	2.4	0.0	+++
V	12.5	0.62	0.4	0.3	0.0	+
VI	10.2	0.73	2.6	0.5	0.2	—

Attempts to purify erythropoietin from Fraction IV_4 were undertaken. For this purpose the fraction precipitated by 50 - 75% sat. ammonium sulfate was subjected to ion-exchange chromatography on DEAE-cellulose. The second fraction from DEAE-cellulose was further chromatographed on Amberlite IRC-50. The chromatograms are presented in Fig. 2 and 3. The activities tested by ^{59}Fe incorporation into red blood cells of starved rats, and the results of sialic acid and hexosamines estimations are given in Table 5.

Table 5

Erythropoietic activity, and sialic acid and hexosamines content in Fraction IV_4 of human plasma and in preparations obtained from this fraction

The material was injected in 0.5% solution. The values of ^{59}Fe incorporation are averages of 6 experiments, ± S.D.M.

Material injected	Sialic acid (% of dry wt.)	Hexosamines (% of dry wt.)	Incorporation of ^{59}Fe into red cells of starved rats (% of the dose)
Fraction IV_4	1.58	3.5	25.8 ± 3.23
Fraction precipitated at 50—75% sat $(NH_4)_2SO_4$	3.5	7.8	32.7 ± 3.48
Amberlite IRC-50 eluate, no. 30—50	5.4	8.8	45 ± 4.02
None (control)			9.5 ± 1.08

Placental blood plasma fractions

Fractionation of placental plasma was performed with ammonium sulfate as shown in Table 1. Further purification has been carried out by ion-exchange chromatography on DEAE-cellulose followed by chro-

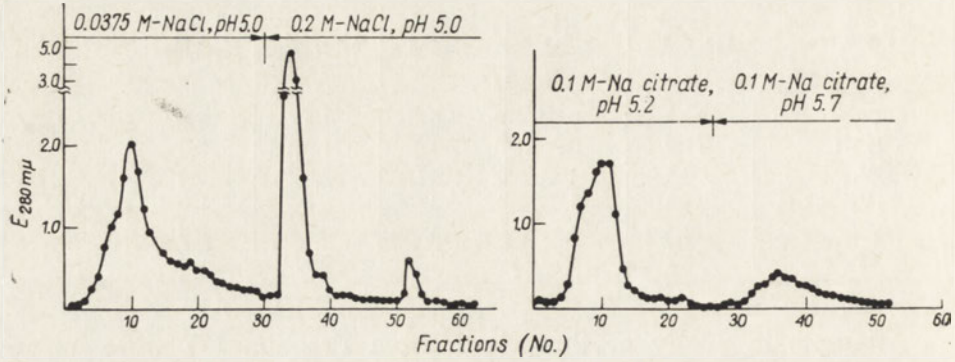

Fig. 2. DEAE-cellulose chromatography of fraction 50 - 75% sat. $(NH_4)_2SO_4$ from Cohn fraction IV_4 of human blood plasma; 10 ml. portions of effluent were collected

Fig. 3. Amberlite IRC-50 chromatography of fractions no. 32 - 38 from DEAE-cellulose; 10 ml. portions of effluent were collected

matography on Amberlite IRC-50 as described above for Cohn Fraction IV_4. The activity and the sialic acid and hexosamine content of whole placental plasma and its fractions are given in Table 6. The

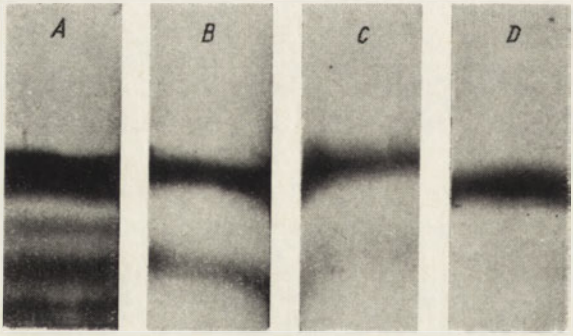

Fig. 4. Electrophoresis on cellulose-acetate strips of (A), placental blood plasma; (B), 50 - 75% sat. $(NH_4)_2SO_4$ fraction; (C), eluate from DEAE-cellulose; (D), eluate from Amberlite IRC-50

respective chromatograms were similar to those presented in Fig. 2 and 3. Paper electrophoresis (Fig. 4) of these fractions showed a high degree of purification after the last step.

Since Fraction IV_4 of normal pooled plasma is enriched in erythropoietic activity, also fraction IV of placental plasma was fractionated.

Table 6

Erythropoietic activity, and sialic acid and hexosamines content in fractions obtained from placental plasma

^{59}Fe incorporation was tested on rats starved for 48 hr. Average values from 6 experiments, ± S.D.M. are given. The differences between control value of ^{59}Fe incorporation and experimental values are statistically significant ($p < 0.001$).

Fraction injected	Sialic acid (% of dry wt.)	Hexosamines (% of dry wt.)	Incorporation of ^{59}Fe into red cells (% of the dose)
Whole plasma	0.19	0.25	19.2 ± 1.31
Precipitate at 50—75% sat. $(NH_4)_2SO_4$	3.6	7.2	41.7 ± 2.82
Amberlite IRC-50 eluate no. 30—50	8.3	10.2	48.0 ± 3.02
None (control)			9.5 ± 1.08

Table 7

Erythropoietic activity of Fraction IV from human placental plasma and the effect of neuraminidase

For experiments rats starved for 48 hr. were used. Average values from 6 animals, ± S.D.M. are given

Material injected	Incorporation of ^{59}Fe (% of the dose)	p (in relation to control value)
Cohn Fraction IV	14.5 ± 1.57	<0.001
Eluate from Amberlite IRC-50 at pH 5.7	22.7 ± 2.38	<0.001
Eluate after treatment with neuraminidase	10.5 ± 1.22	<0.8
None (control)	9.5 ± 1.08	

It was found that the erythropoietic activity can also be isolated by ammonium sulfate fractionation, column chromatography, or by a combination of both these methods. The activities of placental plasma Cohn Fraction IV and of the final product are given in Table 7.

The active material obtained in this way became completely inactive after 1.5 hr. incubation with neuraminidase (Table 7).

DISCUSSION

The above presented experiments give clear-cut evidence that it is possible to obtain a partially purified erythropoietic factor from human blood. It was shown that this factor is present in the highest amount in Fraction IV_4 obtained by Cohn's method 6 from a pool of 80 lit. of plasma. The plasma was prepared from the blood of 400 - 600 regular donors. It contained the active factor, while plasma of single donors who gave blood for the first time was inactive, as was shown in the previous paper [5].

It is known from other works that placental blood possesses increased erythropoietic activity. Our experiments confirm the possibility of purification of this factor.

The data in literature concerning the increase of erythropoietic activity in polycythaemic blood are controversial. In the previous work [5] it was shown that the blood of patients with *polycythaemia vera* contained markedly more erythropoietic activity than the blood of normal subjects. Therefore, plasma of such patients was used and we were able to separate the erythropoietic activity.

The methods of purification of the active fraction consisting in ammonium sulfate fractionation and ion-exchange chromatography were found to be suitable independent of the starting material. The highest degree of purification was obtained by chromatography on Amberlite IRC-50 preceded by ammonium sulfate precipitation and DEAE-cellulose adsorption. In cellulose-acetate electrophoresis this fraction showed a single band.

The sugar components, hexosamines, hexoses and sialic acid, were present in all active fractions. The activity could be abolished by the action of neuraminidase.

The preparation of purified erythropoietic factor from human blood opens possibilities for therapeutic use of this substance, which probably will be devoid of antigenicity in humans. Fraction IV_4 is a by-product in Cohn's fractionation and the placental blood is a material easily available although usually not utilized. Both sources may be used as starting material for the preparation of the active erythropoietic factor.

SUMMARY

1. Erythropoietin preparation was obtained from the plasma of polycythaemic patients, Cohn Fraction IV_4 of normal subjects and placental plasma or placental plasma Cohn Fraction IV.

2. Methods of isolation and purification consisted in $(NH_4)_2SO_4$ fractionation followed by chromatography on DEAE-cellulose and Amberlite IRC-50.

3. The active fraction was electrophoretically homogeneous (on cellulose-acetate strips), contained neuraminic acid and hexosamines. Neuraminidase abolished the activity of the preparation.

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OSOCZE LUDZKIE JAKO ŹRÓDŁO OTRZYMYWANIA CZYNNIKA ERYTROPOETYCZNEGO

Streszczenie

1. Otrzymano preparat o aktywności erytropoetycznej z osocza chorych na czerwienicę, z frakcji IV_4 Cohna osocza ludzi zdrowych, z krwi łożyskowej, oraz z frakcji IV osocza łożyskowego.

2. Oczyszczanie erytropoetyny polegało na frakcjonowanym strącaniu $(NH_4)_2SO_4$ oraz chromatografii na kolumnach z DEAE-celulozą i Amberlitem IRC-50.

3. Aktywny preparat końcowy był jednorodny w elektroforezie na paskach acetylo-celulozy i zawierał kwas neuraminowy i heksozaminy. Neuraminidaza całkowicie inaktywowała preparat.

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PURIFICATION AND PROPERTIES OF CATALASE FROM *MYCOBACTERIUM SMEGMATIS*

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The study of catalases from mycobacteria may help to establish the biological and biochemical differences between the saprophytic and virulent strains. Therefore the isolation of pure enzyme from both kinds of mycobacteria and the comparison of their properties would be of great value. The mycobacteria catalase is very interesting because of the correlation between the enzymic activity and bacterial virulence. Kreis & Joubioux [5] reported such a correlation, although the pathogenicity of the bacteria was not proportional to the catalase content. The simultaneous increase of resistance to isonicotinhydrazide and decrease of catalase activity may be connected with these correlations.

Till now, however, no crystalline mycobacteria catalase has been obtained, mainly because of much smaller quantities of this enzyme in mycobacteria than in the liver [7], *Micrococcus lysodeicticus* [3], or *Rhodopseudomonas spheroides* [1], from which the pure enzyme had been already obtained.

This report deals with the purification and some properties of catalase from saprophytic *Mycobacterium smegmatis*.

EXPERIMENTAL AND RESULTS

Special reagents. Ethylcellulose for column chromatography was prepared from cotton-wool defatted with a mixture of chloroform and ether (1 : 1, v/v), then heated for 20 hr. with 1 N-HCl in anhydrous ethanol. 6.6 mM - K - Na-phosphate buffer, pH 6.8, according to Michaelis was used. Isonicotinhydrazide (INH) was USP XV grade, sodium azide chemically pure, and streptomycin was POLFA (Tarchomin) product.

Growth of the organisms. Mycobacterium smegmatis was grown on solid medium of Kirchner [2] for 6 days at 37°, then the formed pellicle was transferred to the Sauton liquid medium [10] and incubated again

for 6 days at 37°. The organisms were harvested by filtering, and stored at -10°.

Determination of protein. The turbidimetric method of Clayton [1] was used. To 2 ml. suitably diluted sample, containing 0.2 to 3.2 mg. of protein, 8 ml. of the precipitating solution was added. (The solution was obtained by adding 2 ml. of sat. $(NH_4)_2SO_4$ to 100 ml. of 1.25% trichloroacetic acid). The sample was mixed and after 20 min. the extinction at 610 mμ, in 2 cm. cuvette was measured using Pulfrich photometer.

Determination of catalase activity. This was done by the method of Jolles as modified by Summer & Dounce [7]. As substrate 10 mM-hydrogen peroxide in 6.6 mM-phosphate buffer, pH 6.8, was used. The reaction was stopped by adding 2 N-H_2SO_4 and the remaining hydrogen peroxide estimated iodometrically. The activity was expressed as the velocity constant extrapolated to the zero time (K_o) or average velocity constant (K_s) calculated from three velocity constants determined at 3,6 and 9 min.

To check the presence of catalase in the course of column chromatography, a simplified procedure was used. To 2 ml. of hydrogen peroxide solution one drop of the effluent from the column was added. After 10 min. 2 ml. of 2 N-H_2SO_4, 0.25 ml. of 5% potassium iodide, 0.2 ml. sat. solution of molybdic acid, and starch solution were added. No colour reaction appeared when catalase was present.

Purification of catalase

The procedure was based on the method described for animal [7] and bacterial [3, 1] catalase. The modifications applied were: precipitation of the enzyme with manganeous chloride, lyophilization, and column chromatography on ethylcellulose. Besides, it was observed that after storing the crude bacterial extract for about two months, the catalase activity increased several times; therefore the purification was carried out on 60-day-old extracts.

The frozen mycobacteria cells, 25 g., were thawed, then ground with an equal volume of glass powder (particles passing a 0.15 mm. mesh sieve). The cells were ground for 10 min., 80 ml. of 6.6 mM-citrate-phosphate buffer, pH 6.8, being added stepwise. After centrifuging, the sediment was washed twice with buffer. The pooled supernatants (170 ml.) were stored at 0 - 4° for 60 days. The extract was then treated at room temperature with 81.6 ml of ethanol - chloroform mixture (1 : 1, v/v), shaken for 1 min. and centrifuged at 17 000 *g* for 10 min. To the aqueous solution 1/100 of the volume of 1 M-$MnCl_2$ was added and after 2 - 3 min. the formed precipitate was spun down at 12 000 *g*. From the sediment the enzyme was extracted by several portions of 0.5 M-phosphate buf-

Table 1

Purification of catalase from Mycobacterium smegmatis

Stage	Volume (ml.)	Activity ($K_s \times 10^3$)	Protein (ml.)	Purity ($K_s \times 10^3$/ mg./protein)	Purification	Yield (%)
Fresh crude extract	170	11 900	1250	9		30
Extract stored for 60 days	170	39 440	1258	31	1	100
Treated with Et OH-$CHCl_3$ mixture	170	17 000	27.2	625	20	42
Extract from $MnCl_2$ precipitate	35	15 400	14.0	1100	35	39
Dialysis	56	12 880	—	—	—	33
Lyophilization	10	12 000	14.0	587	28	30
Eluate from ethylcellulose	11	10 560	4.4	2400	77	27

Table 2

The effect of temperature on the activity of purified catalase preparation from M. smegmatis

The temperature was measured inside the incubation vessels. K_0 was calculated by extrapolation, using the diagram of functions at time intervals. The mean values of 5 separate estimations, ± S.D. are given

Temp.	$K_0 \times 10^3$	log $K_0 \times 10^3$
0°	120±5.1	2.0792
10°	165±9.2	2.2175
20°	194±7.3	2.2878
30°	321±9.7	2.3636
40°	210±3.5	2.3222
50°	195±2.0	2.2900
60°	105±5.1	2.0212

Table 3

The effect of isonicotinhydrazide, NaN_3 and streptomycin on the activity of M. smegmatis *catalase*

Inhibitor	Final concentration (M)	Inhibition (%)
Isonicotin-hydrazide	1×10^{-3}	0
	1×10^{-2}	8
	1.6×10^{-1}	19
NaN_3	1×10^{-6}	30
	2×10^{-6}	52
	1×10^{-5}	76
Streptomycin	1×10^{-3}	4.8
	3×10^{-2}	24

fer, pH 7.4. The extracts were combined and dialysed at 4° for 8 hr. against water. After dialysis the preparation was concentrated to 10 ml. by lyophilization, and passed through a column (8×10 mm.) filled with ethylcellulose moistened with 6.6 mM-phosphate buffer, pH 6.8. The column was eluted with the same buffer and 1 ml. fractions were collected with a fraction collector. The flow rate was 1 ml./3 min. Eleven 1 ml. fractions which contained catalase were pooled and used for further experiments. The procedure of purification is shown in Table 1.

Properties of the purified enzyme

The optimum pH of the catalase preparation was found to be 7 (Fig. 1), the optimum temperature about 30° (Table 2), and the activation energy 3600 cal./mole (Fig. 2). The enzymic activity was inhibited

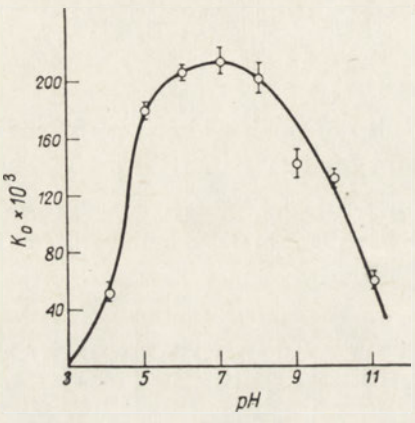

Fig. 1

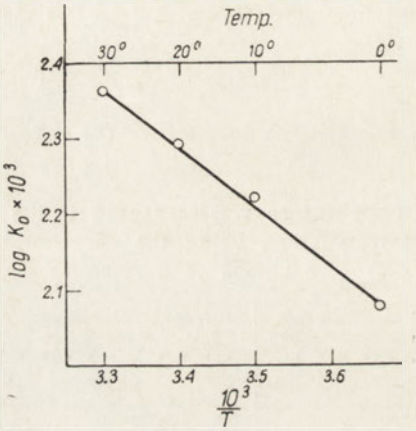

Fig. 2

Fig. 1. The effect of pH on the activity of catalase from *M. smegmatis*. As substrate 10 mM-hydrogen peroxide dissolved in 6.6 mM-phosphate buffer, pH 6.8, was used. Appropriate pH was obtained by adding phosphoric acid or sodium hydroxide, and it was checked with indicators. The mean and limit values of 5 separate estimations are given

Fig. 2. The effect of temperature on catalase activity

in 30% by 10^{-6} M-sodium azide. INH and streptomycin at similar concentrations were without effect. Slight inhibiting effect of INH was observed at the concentration 1.6×10^{-1} M, and of streptomycin at 3×10^{-3} M (Table 3).

DISCUSSION

The adopted method of catalase preparation from disintegrated *M. smegmatis* cells allowed to obtain 80-fold purification of the enzyme. The modification applied to the commonly used methods for catalase

preparation consisted in precipitation with manganeous chloride, the enzyme being precipitated together with nucleic acid, and then extracted with concentrated buffer solution. It was observed that during storage of the crude cell-free extract at about 0° the enzymic activity increased 3 times. The Euler phenomenon which then appeared was similar to the influence of butanol and other solvents on catalase, as reported by Kaplan & Woon-Ki-Paik [4]. The mechanism of this activation has not been studied. It may be due to the splitting during storage at low temperature of the lipid-protein complexes, concomitant with the release of enzymic activity.

The studied properties of the purified enzyme can be compared with those of the crude extract [6, 8]. The activation energy was found to be similar: 3600 ± 200 cal./mole for the purified, and 4300 ± 500 cal. for the crude preparation. There were some differences in the influence of sodium azide. At the concentration of 10^{-6} M, the enzymic activity was inhibited in about 80% in the crude, and only in about 30% in the purified preparation. This difference may perhaps be explained by the different effect of the inhibitor on catalase before and after the Euler's phenomenon.

The inhibiting effect of INH and streptomycin was observed only at very high concentrations (about 10^{-1} M).

The optimum pH for the purified catalase was about 7. It was similar to the optimum pH for the catalase from *Mycobacteria tuberculosis* H 37 Rv. [9].

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SUMMARY

A method of 80-fold purification of catalase from *M. smegmatis* is described. The optimum pH of the purified enzyme is 7, optimum temperature 30° and the activation energy 3600 cal./mole. The 10^{-6} M-NaN_3 causes a 30% inhibition of the enzymic activity. Similar concentrations of isonicotinhydrazide and streptomycin have no inhibiting effect.

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OCZYSZCZENIE I WŁAŚCIWOŚCI KATALAZY Z *MYCOBACTERIUM SMEGMATIS*

Streszczenie

Opisano metodę 80-krotnego oczyszczania katalazy prątkowej. Optimum działania otrzymanego preparatu było przy pH 7 i temperaturze 30°; energia aktywacji wynosiła 3600 cal. NaN_3 10^{-6} M hamował w 30% aktywność enzymu; izonikotynohydrazyd i streptomycyna w podobnych stężeniach nie miały wpływu.

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STUDIES ON HYDROLASES OF DIGESTIVE JUICES

VII. PROTEOLYTIC ACTIVITY OF CANINE INTESTINAL JUICE*

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It has long been thought that the final breakdown of ingested protein takes place in the lumen of the small intestine, free amino acids being absorbed into the blood stream. This view, however, was recently questioned as it has not been definitely established whether the hydrolysis of tri- and dipeptides is accomplished by the enzymes present in the *succus entericus* or within the cells of intestinal mucosa.

Some data indicate that the enzymes are present in the epithelium shed by the intestinal mucosa, and diffuse therefrom into the solution [34, 7, 21]. Nevertheless the enzymic composition of the intestinal juice has not been thoroughly studied. Experiments of Shlygin [21] and Kuvayeva [14] on the duodenal juice of dog and man were concerned with a limited number of enzymes, enterokinase being the only proteinase studied. Phaneuf [18] demonstrated the absence of proteinases in sheep intestinal juice, but Alexander & Chowdhury [2] observed the enzymic hydrolysis of peptone and casein by horse ileal juice.

On the other hand the experiments of Newey & Smyth [15, 16], Wiggans & Johnston [33], and Robinson & Shaw [19] indicate that dipeptides may pass across the wall of the mucosa and undergo hydrolysis intracellularly.

As this problem has not been definitely elucidated it is not yet possible to evaluate the extent to which the digestion of peptides takes place in the lumen of the intestine. In this paper some experiments are presented concerning the enzymic activity of canine intestinal juice obtained from the Thiry-Vella fistula towards some peptides, amino acid derivatives and proteins.

* Previous papers of this series: [11, 12, 30, 31, 29, 17].

MATERIALS AND METHODS

Collection of the intestinal juice. Dogs with the Thiry-Vella fistula made in the upper part of the small intestine, about 40 cm. from pylorus, and in one case in the distal part of the small intestine, about 300 cm. from pylorus, were used. The total length of the small intestine was about 330 cm. The juice secreted in response to mechanical irritation was collected, centrifuged for 15 min. at 2500 r.p.m. and used for enzymic assays without further treatment, or after 14 hr. dialysis in cellophane bags against 1 mM-ethylenediaminetetra-acetate (EDTA) solution or tridistilled water. All procedures were performed at 0 to 4°.

Substrates and reagents. L-leucyl-glycine, D-leucyl-glycine, glycyl-L-tyrosine, D-leucyl-L-tyrosine and chloroacetyl-L-tyrosine (Hoffman La Roche); glycyl-L-leucine, DL-alanyl-glycine, diglycyl-glycine and protamine sulphate ex salmon roe (salmine sulphate) (L. Light); glycyl-DL-alanine (Reanal, Hungary), L-carnosine (Schuchardt) and haemoglobin (Difco) were used. Other substrates were prepared according to the following methods: glycyl-glycine [5], L-leucinamide [10], L-lysine ethyl ester [32], and L-tyrosine ethyl ester [1]. Tris (2-amino-2-hydroxymethylpropane-1,3-diol) was obtained from L. Light. Other reagents used for the preparation of buffer solution as well as chlorides of activating cations were of analytical purity ($CoCl_2$, Merck; $MnCl_2$ and $MgCl_2$, Fabryka Odczynników Chemicznych, Gliwice; $ZnCl_2$, Kahlbaum).

Determination of enzymic activity. The hydrolysis of dipeptides and diglycyl-glycine was measured as follows: 0.6 ml. of suitably diluted intestinal juice was incubated with 0.2 ml. of the solution of the activating cation (final concentration 0.005 M) or 0.2 ml. of water for 25 min. at 37°, and then 0.6 ml. of 0.2 M-tris-HCl buffer, pH 8.0, and 0.6 ml. of 0.03 M-substrate solution were added. Immediately after the addition of the substrate and after a determined period of incubation 0.5 ml. samples were taken and the enzymic reaction stopped by the addition of alkali and cobaltous phosphate suspension and subsequent immersion in boiling water bath according to the procedure of Crook & Rabin [4]. The rate of substrate decomposition was estimated by measuring the decrease of extinction of peptide cobalt complexes at 560 mμ. The enzymic hydrolysis of L-leucinamide was estimated according to the procedure of Hill *et al.* [10] for the determination of leucine aminopeptidase activity.

The method of Hestrin [8] was used for the measurement of L-lysine ethyl ester and L-tyrosine ethyl ester hydrolysis. The Rosen ninhydrin method [20] for chloroacetyl-L-tyrosine, and the Anson method [3] for haemoglobin hydrolysis were used.

For the determination of protamine sulphate hydrolysis 0.4 ml. of intestinal juice was incubated with 0.3 ml. of 0.2 M-phosphate buffer, pH 8.0, and 0.3 ml. of 2% (w/v) substrate solution. After a determined period of time, protein and undigested protamine were precipitated by the addition of 1 ml. of saturated picric acid solution, the formed precipitate centrifuged, and hydrolysis products present in the supernatant analysed by paper chromatography. For this purpose 10 µl. samples were applied on discs of Whatman no. 1 paper, 18 cm. in diameter, and developed with butan-1-ol - acetic acid - water (200 : 30 : 75, by vol.) as a solvent mixture. After separation was completed the dried chromatograms were cut into appropriate sectors, amino acids and peptides detected with ninhydrin and isatin, and arginine detected with 8-oxyquinoline reagent according to Jepson & Smith [13]. For semiquantitative evaluation the ninhydrin-stained spots corresponding to arginine were cut out and eluted with 4 ml. of acetone - ethanol - water (1 : 1 : 1, by vol.) mixture. The extinction of the eluates was read in a Coleman Junior spectrophotometer at 575 mµ.

Enterokinase activity was determined by following the rate of trypsinogen activation measured by corresponding changes in the rate of lysine ethyl ester hydrolysis, lyophilized dog pancreatic juice being used as the source of trypsinogen.

Protein determination. In all samples of intestinal juice protein was estimated with the Folin & Ciocalteu phenol reagent [6] according to the procedure already described [12]. Protein nitrogen was calculated with the assumption that the average nitrogen content in proteins amounts to 16%.

RESULTS

Intestinal juice obtained from the Thiry-Vella fistula exhibited a marked enzymic activity toward 7 out of 15 substrates tested (Table 1). Under the conditions used no hydrolysis of D-leucyl-glycine, glycyl-L--tyrosine, D-leucyl-L-tyrosine, L-carnosine, and chloroacetyl-L-tyrosine was found.

Dialysis of intestinal juice against EDTA solution caused a decrease of dipeptides and L-leucinamide hydrolysis. The most marked decrease was observed in the case of L-leucyl-glycine, whereas L-leucinamide hydrolysis was comparatively less affected (Table 2). The preincubation of the dialysed juice with divalent cations restored the activity partially or completely, and in some cases a marked increase of activity above the original level was observed. Co^{2+} ions activated specifically the hydrolysis of glycyl-glycine, whereas Mn^{2+} ions the hydrolysis of L-leucyl-glycine and L-leucinamide. The splitting of the latter substrate was also specifically activated by Mg^{2+} ions. Zn^{2+} ions restored

Table 1

The enzymic hydrolysis of some peptides, amino acid derivatives and proteins by canine intestinal juice

The proteolytic coefficient C_1 is derived from the first order velocity constant $\left(K_1 = \frac{1}{t} \log \frac{100}{100 - H}\right.$, where t = time of incubation, and H = per cent of substrate decomposition$\left.\right)$ according to the equation: $C_1 = \frac{K_1}{\text{mg. of protein N/ml.}}$

Substrate	No. of experiments	Activity, ±S.D.
Glycyl-glycine	6	$C_1 = 3.03 \pm 0.88 \times 10^{-2}$
L-Leucyl-glycine	6	$C_1 = 2.80 \pm 0.64 \times 10^{-2}$
D-Leucyl-glycine	4	no hydrolysis
Glycyl-L-leucine	5	$C_1 = 2.83 \pm 1.12 \times 10^{-2}$
DL-Alanyl-glycine	5	$C_1 = 9.26 \pm 3.70 \times 10^{-2}$
Glycyl-DL-alanine	5	$C_1 = 8.50 \pm 1.00 \times 10^{-2}$
Glycyl-L-tyrosine	4	no hydrolysis
D-Leucyl-L-tyrosine	4	no hydrolysis
L-Carnosine	3	no hydrolysis
L-Leucinamide	6	$C_1 = 1.81 \pm 0.31 \times 10^{-2}$
Chloroacetyl-L-tyrosine	4	no hydrolysis
Protamine sulphate	5	0.72 ± 0.06 mg. arginine/ml./hr.
L-Lysine ethyl ester	5	$0.05 \pm 0.04 \times 10^{-4}$ Anson
L-Tyrosine ethyl ester	5	$0.03 \pm 0.02 \times 10^{-4}$ units/
Haemoglobin	5	$0.03 \pm 0.02 \times 10^{-4}$ mg protein

Table 2

Effect of dialysis and divalent cations on hydrolysis of dipeptides by canine intestinal juice

Intestinal juice was dialysed for 14 hr. against 0.001 M-EDTA solution and then for 7 hr. against tridistilled water. Dialysed juice was preincubated with solution of chlorides of respective cations for 25 min. at 37°. The final concentration of cations was 0.005 M. Comparative percentage values of enzymic activity are given (untreated juice = 100)

Substrate	No. of experiments	Untreated juice	Dialysed juice				
			Alone	Preincubated with			
				Mg^{2+}	Mn^{2+}	Co^{2+}	Zn^{2+}
Glycyl-glycine	6	100	24	—	76	195	62
L-Leucyl-glycine	6	100	6	—	237	37	54
L-Leucinamide	6	100*	65	130	250	59	24
DL-Alanyl-glycine	5	100	28	—	84	111	68
Glycyl-DL-alanine	5	100	19	—	59	104	33
Glycyl-L-leucine	5	100	23	—	85	101	88

* The activity obtained with undialysed juice preincubated with Mn^{2+} ions was 471.

only partially the activities diminished by dialysis, displaying no specific activation, and even inhibiting the hydrolysis of L-leucinamide. The hydrolysis of DL-alanyl-glycine, glycyl-DL-alanine, and glycyl-L--leucine was restored partially by Mg^{2+} and Zn^{2+} ions, and completely by Co^{2+} ions, but none of the cations increased the activity above the original level.

The effect of dialysis and divalent cations on diglycyl-glycine hydrolysis was also observed. The enzymic breakdown of this substance showed a two-stage course of reaction (Fig. 1). In the first stage the splitting of one peptide bond produced glycine and glycyl-glycine; the dipeptide cobalt complex, formed when Crook & Rabin [4] method was used, gave higher extinction values than the similar complex of tripeptide. In the second stage glycyl-glycine was hydrolysed to free glycine

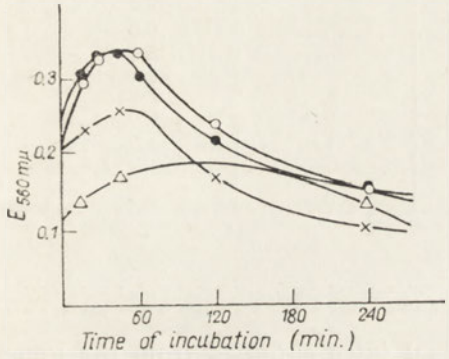

Fig. 1. Enzymic hydrolysis of diglycyl--glycine by canine intestinal juice. The extinction of coloured cobalt complexes formed with peptide mixture was measured at 560 mμ (Crook & Rabin method [4]). (●), Untreated juice; (○), juice dialysed for 18 hr. against tridistilled water; (×), juice preincubated with Co^{2+}; (△), juice preincubated with Mn^{2+} for 20 min. at 37°. The final cation concentration was 2×10^{-3} M

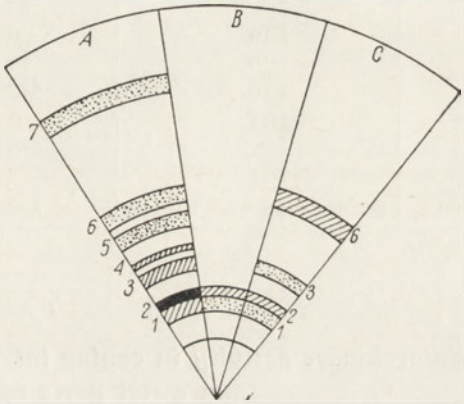

Fig. 2. Chromatogram of the digestion products of protamine sulphate treated with canine intestinal juice. Stained with: (*A*), ninhydrin; (*B*), 8-oxyquinoline (Jepson & Smith method for arginine detection [13]); and (*C*), isatin. Identification of the spots: (*1*), arginine--containing peptide; (*2*), free arginine; (*3*), serine and/or glycine; (*6*), proline; (*4*), (*5*), (*7*), nonidentified

and the fall of extinction was observed. Dialysis diminished slightly the activity responsible for the second stage of reaction. The curve of the reaction progress is in this case displaced to the right indicating the slower hydrolysis of dipeptide formed in the initial stage. Mn^{2+} ions seemed to inhibit both stages of the reaction, whereas Co^{2+} ions activated the second stage causing much quicker disappearance of dipeptide.

Table 3

Amount of free arginine and arginine-containing peptide in digestion products of protamine sulphate treated with canine intestinal juice

The values given for arginine-containing peptide were obtained with standard solution of arginine; they are only approximate because of the unknown character of the peptide. The preincubation with EDTA solution was performed for 60 min. at 37°, final concentration being 0.004 M. The results of individual estimations are given. Samples no. 9 - 11 of intestinal juice from dog no. I were used

Intestinal juice sample no.	Time of incubation (min)	Arginine in the peptide (mg./ml.)	Free arginine (mg./ml.)
I/9	0	—	0.0
I/9	60	—	0.5
I/10	0	—	0.0
I/10	60	—	0.8
I/10	240	—	3.3
I/11	0	0.0	0.0
I/11	240	1.0	1.7
I/11, preincubated with EDTA	240	0.9	0.9

Table 4

Enterokinase activity in canine intestinal juice obtained from the upper and distal parts of the small intestine

Enterokinase activity is expressed as activity of trypsin formed during the incubation of lyophilized pancreatic juice with intestinal juice. The activity of trypsin was measured with lysine ethyl ester as substrate and the values are given in Anson units. The intestinal juice was obtained from the Thiry-Vella fistula made about 40 cm. from the pylorus, upper part, and about 300 cm. from the pylorus, distal part of the small intestine. The results of individual estimations are given. Samples no. 9 - 11 of intestinal juice from dog no. I, and no. 19 - 20 from dog no. II were used

Segment of the intestine	Intestinal juice, sample no.	Dilution of the intestinal juice	Activity (units per mg. of protein $\times 10^4$)
Upper	I/9	1 : 2	39.8
	I/9	1 : 4	11.2
	I/10	1 : 12	19.0
	I/10	1 : 15	2.7
	I/11	1 : 3	22.9
	I/11	1 : 6	0.6
Distal	II/19	undiluted	0.0
	II/20	undiluted	0.0

Considerable degradation of protamine sulphate incubated with intestinal juice was observed. Chromatographic analysis of the digestion products indicated the presence of free arginine as well as other amino acids. On the chromatograms stained with ninhydrin at least 7 different spots could be detected (Fig. 2). Using standard solutions of amino acids and different staining methods, several amino acids were identified, i.e. arginine, proline, glycine and/or serine. The two latter amino acids could not be distinguished one from another since they had the same mobility under the conditions applied. Besides free arginine a peptide containing arginine, but giving a smaller R_F value than arginine, was also found on the chromatograms. This peptide disappeared quickly from the digestion mixture if intestinal juice had been preincubated with Mn^{2+} ions. Hydrolysis of protamine by the intestinal juice was inhibited by EDTA which simultaneously changed the ratio of free arginine to the peptide in favour of the peptide (Table 3). The hydrolysis of haemoglobin, L-lysine ethyl ester and L-tyrosine ethyl ester by the intestinal juice was negligible. The splitting of chloroacetyl-L-tyrosine did not exceed the limits of experimental error.

The intestinal juice obtained from the upper part of the small intestine showed well pronounced enterokinase activity. This activity could not be detected in the juice from the distal part of the intestine (Table 4).

DISCUSSION

The hydrolysis of various substrates as well as the changes of activity caused by dialysis or treatment with divalent cations indicated the presence in canine intestinal juice of several proteolytic enzymes. Since a mixture of proteins was used the possibilities to characterize and identify individual enzymes were limited. The attempt to identify several peptidases was based on the observed facts and known properties of the purified enzymes, its results, however, should be treated with circumspection.

The experimental data demonstrated the presence of some enzymes of the exopeptidase group. One of them was probably leucine aminopeptidase. This enzyme could be responsible for the hydrolysis of leucinamide and leucyl-glycine, which proceeded very quickly especially in the presence of Mg^{2+} and Mn^{2+} ions. These two cations are known to be essential for the activity of purified leucine aminopeptidase from intestinal mucosa [22] and kidney [27].

The hydrolysis of glycyl-glycine and the second stage of diglycyl-glycine breakdown could be due to the presence of glycyl-glycine dipeptidase. The presence of Co^{2+} ions was required for its full activity.

This cation was reported to be an activator of glycyl-glycine dipeptidase obtained from hog intestinal mucosa and other sources [23].

It was not clear whether the observed hydrolysis of other dipeptides tested was related to the action of glycyl-glycine dipeptidase or leucine aminopeptidase. The inability of Mn^{2+} and Co^{2+} ions to enhance considerably this activity seemed, however, to indicate that separate enzymes were responsible for the hydrolysis of DL-alanyl-glycine, glycyl-DL-alanine, and glycyl-L-leucine. Dipeptidases hydrolysing alanyl-glycine and glycyl-leucine have been described as separate enzymes [24, 25]. Nevertheless the assumption that the hydrolysis of these dipeptides was catalysed by separate enzymes, or more precisely, that the observed activity was not due to the action of leucine aminopeptidase or glycyl-glycine dipeptidase, was supported only by the higher value of proteolytic coefficient and the lack of any specific effect of divalent cations.

Besides dipeptidases specific for glycyl-glycine, alanyl-glycine, and glycyl-leucine also the enzymes hydrolysing proline dipeptides, i.e. prolinase and prolidase, have been reported to occur in intestinal mucosa [23, 25]. Although no appropriate substrates were used, the appearance of free proline among the digestion products of protamine indicated that both enzymes might have been present in the intestinal juice.

The changes of extinction values of the cobalt complex in the course of diglycyl-glycine hydrolysis indicated that the breakdown in the initial stage was due to the presence of aminotripeptidase. No special requirement for divalent cations was observed, the same being reported for aminotripeptidase obtained from intestinal mucosa [25, 26].

It seems that neither of the two known carboxypeptidases was present in the intestinal juice studied, at least not in measurable amounts. This view is supported by the observed lack of chloroacetyl-L-tyrosine hydrolysis, and a different pattern of protamine hydrolysis products as compared with that obtained when pancreatic juice was used as a source of the enzymes (Z. Szafran, unpublished data). It is probable that the observed hydrolysis of protamine sulphate was mediated by leucine aminopeptidase. In fact the activity of this enzyme toward small proteins has already been described [9].

It was difficult to establish the presence in intestinal juice of endopeptidases other than enterokinase. Hydrolysis of the substrates for these enzymes was very low in comparison with that observed in the case of pancreatic juice [29], and generally exceeded only slightly the limits of accuracy of the methods used. It is not yet clear whether enterokinase is specific for trypsinogen activation or whether it can also hydrolyse lysyl-isoleucyl or similar bonds in other proteins. The

quick degradation of protamine by the intestinal juice could be alternatively explained by the action of endopeptidase in the early phase of reaction.

Whatever may be the mechanism of the secretion of enzymes by intestinal mucosa, the presented results suggest that the intestinal juice obtained from the Thiry-Vella fistula possesses a marked activity toward peptides. Taking into account the values of proteolytic coefficients for the hydrolysis of dipeptides and leucinamide, the activity of intestinal juice is comparable to that reported by Smith & Bergmann [26] for crude extracts of intestinal mucosa. It can be concluded that the final stage of protein digestion takes place, at least in part, in the lumen of the small intestine, and the digestive function of intestinal juice should not be neglected.

The authors wish to express their gratitude to Professor Dr. B. Skarżyński for his helpful advice.

SUMMARY

The hydrolysis of some dipeptides, amino acid derivatives, and proteins by canine intestinal juice obtained from the Thiry-Vella fistula has been studied. The presence of leucine aminopeptidase, glycyl-glycine dipeptidase, aminotripeptidase, and some other dipeptidases and endopeptidases in intestinal juice has been discussed on the basis of the observed rates of hydrolysis, influence of divalent cations, and known specificity of the enzymes. It was concluded that at least partial hydrolysis of dipeptides occurred in the lumen of the intestine under the action of intestinal juice peptidases.

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BADANIA NAD HYDROLAZAMI SOKÓW TRAWIENNYCH

VII. ENZYMY PROTEOLITYCZNE SOKU JELITOWEGO PSA

Streszczenie

Badano hydrolizę kilku dwupeptydów, pochodnych aminokwasów i białek pod wpływem soku jelitowego psa uzyskanego z przetok Thiry-Vella. Na podstawie obserwowanej szybkości hydrolizy różnych substratów, wpływu kationów dwuwartościowych i znanej swoistości enzymów przedyskutowano możliwość występowania w soku jelitowym aminopeptydazy leucynowej, dwupeptydazy glicylo-glicyny, aminotrójpeptydazy, a także innych dwupeptydaz i endopeptydaz. Jako ogólny wniosek przedstawiono pogląd, że hydroliza dwupeptydów zachodzi co najmniej częściowo w świetle jelita pod wpływem peptydaz zawartych w soku jelitowym.

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DIRECT TRANSFER OF ORTHOPHOSPHATE FROM ADENOSINE TRIPHOSPHATE TO MYOSIN AND H-MEROMYOSIN

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Earlier investigations from this laboratory [3, 4] demonstrated that when myosin, H-meromyosin or actomyosin were incubated with ATP [1] the amount of protein-bound P_i increased considerably; the increase was especially conspicuous during the initial period of incubation. These findings were interpreted as an indication of formation of phosphorylated proteins as intermediates arising during the splitting of ATP. Although such a hypothesis has been accepted by many authors (cf. Kalckar [15], Weber [25], Kielley [16]) the phosphorylated intermediate has never been isolated and its nature remained unknown.

In the present study an attempt was made to obtain evidence for the formation of the phosphorylated contractile proteins and to elucidate whether the increased amount of the protein-bound P_i found during ATP splitting [5, 3, 4] derived directly from the nucleotide.

The experiments were performed on myosin and H-meromyosin which were incubated with ^{32}P labelled ATP and unlabelled P_i or, on the contrary, with unlabelled ATP and radioactive orthophosphate. Using this isotope dilution method it could be supposed that if P_i was transferred directly from the hydrolysed ATP to the enzymes the specific activity of the formed protein-bound P_i would be equal with that of the added [^{32}P]ATP. If on the other hand the bound P_i was derived from the free P_i present in the medium the specific activities of these two "kinds" of orthophosphates should correspond to each other. The results of experiments presented below seem to indicate that the protein-bound P_i arises chiefly at the expense of ATP and, therefore, we have ground to believe that during enzymic breakdown of ATP

[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate; TCA, trichloroacetic acid; PCMB, *p*-chloromercuribenzoate; tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

by myosin and H-meromyosin a phosphorylated intermediate is really formed. A preliminary report of some of these results has already been published [12].

MATERIAL AND METHODS

Actin-free myosin was prepared from rabbit muscles according to Portzehl & Weber [22] and H-meromyosin by the method of Szent-Györgyi [24]. ATP labelled with ^{32}P in β and γ position was prepared from ADP and radioactive phosphate (received from the Institute of Nuclear Research, Warszawa) by means of an oxidative phosphorylation process with rat liver mitochondria according to Colowick & Kaplan [7]. The labelled product, [^{32}P]ATP, was purified by passage through a bed of the ion-exchange resin Dowex 1 by the method of Cohn & Carter [6] and then checked by paper chromatography using ethanol - 1 M-acetic acid - 1 M-NH_4OH (75 : 28 : 2, by vol.) according to Bergkvist [2]. The specific activity of γ-P in ATP was determined after hydrolysis with myosin.

Myosin, used in a gel form, and H-meromyosin were incubated (at 20° or at 0°) with ATP and P_i in conditions recorded in the Tables for each kind of experiments. ATP and P_i solutions were mixed before the experiment and added to the incubation mixture as a single solution. The enzymic process was stopped by one of the following methods. In the case of myosin gel: by prompt cooling to 0° and immediate centrifugation in cold, or by addition of ice-cold 1 M-acetate buffer solution, pH 4.6, (0.5 M final concentration) and centrifugation in cold. In case of H-meromyosin, by addition of saturated $(NH_4)_2SO_4$ up to 55% saturation and centrifugation of the precipitated protein. In each case the protein residue separated by centrifugation was immediately washed twice with appropriate solution (0.03 M- KCl or 0.2 M-acetate buffer, pH 4.6, or ammonium sulphate 55% saturation, respectively).

The supernatant fluids were diluted to an appropriate volume and samples were used for determination of the free orthophosphate and of its radioactivity. When the enzymic process was stopped simply by cooling to 0° the supernatant was immediately mixed with TCA, 10% final concentration.

In the protein residue separated by centrifugation and washed twice the bound P_i and, in some cases, the bound nucleotides were determined after extraction with cold 10% TCA or 1.5 N-$HClO_4$ according to the usual procedure in this laboratory [21].

Both the free and the protein-bound P_i were estimated by the method of Martin & Doty [20] which allowed a simultaneous determination of the radioactivity and of the content of P_i in the same sample. It was established in control experiments that no contamination of the P_i

by the radioactive ATP and ADP took place in this procedure. The nucleotides were determined according to Cohn & Carter [6] and the amount of protein by the biuret method [11].

RESULTS

In the first series of experiments myosin or H-meromyosin were incubated with radioactive ATP and unlabelled P_i. Typical results of three of such experiments are presented in Table 1. Although in these

Table 1

Specific activity of the protein-bound and of the free P_i after incubation of myosin and H-meromyosin with [^{32}P]ATP and unlabelled P_i

Conditions: Expt. (1). H-meromyosin, 25 mg.; $MgCl_2$, 2.7 μmoles; 0.05 M-glycine - KCl buffer, pH 9.0; [^{32}P]ATP, 2.8 μmoles, specific activity 43 500 counts/min./μmole γ-P; P_i, 2.9 μmoles; total volume, 4 ml.; temp. 20°.

Expt. (2). Myosin, 51 mg.; $MgCl_2$, 5 μmoles; 0.03 M-tris - maleate buffer, pH 7.0; KCl, 0.06 M; ATP, 9.7 μmoles, specific activity 38 400 counts/min./μmole γ-P; P_i, 6.8 μmoles; total volume, 10 ml.; temp. 20°.

Expt. (3). Myosin, 9.6 mg.; $MgCl_2$, 3.0 μmoles; 0.03 M-tris - maleate buffer, pH 7.0; KCl, 0.06 M; ATP, 3.0 μmoles, specific activity 44 300 counts/min./μmole γ-P; P_i, 2.4 μmoles; total volume, 6 ml.; temp. 0°.

To the control samples [^{32}P]ATP and P_i were not added. Specific activity is expressed as counts per min. per μmole inorganic orthophosphate.

Expt. no.	Sample		Incubation (min.)	Interruption of enzymic process	Protein-bound P_i		Free P_i in the solution	
					(μmoles/g. protein)	(counts/min./μmole P_i)	(μmoles)	(counts/min./μmole P_i)
1	H-meromyosin	Control	—	$(NH_4)_2SO_4$: 55% sat.	0.29	—	0	—
		With [^{32}P]ATP and P_i	1		0.92	31 600	3.14	7 300
			5		1.26	32 600	3.81	14 400
			10		0.86	38 400	4.50	16 300
			15		0.73	27 000	5.60	17 400
2	Myosin	Control	—	Separation at 0°	0.46	—	0	—
		With [^{32}P]ATP and P_i	1		1.76	25 500	13.1	11 000
			5		1.00	23 500	14.5	13 900
			10		0.89	21 500	16.0	16 600
			20		0.50	20 400	17.6	18 000
3	Myosin	Control	—	Acetate buffer pH 4.6	0.25	—	0	—
		With [^{32}P]ATP and P_i	1		11.0	32 200	2.90	6 100
			1		5.7	31 000	2.64	5 900
			1		13.2	35 400	2.78	5 700
			1		5.3	38 200	2.78	6 800
			1		6.2	39 400	2.71	5 900

experiments the conditions and the method of interruption of the enzymic process were different we can see that in all cases the specific activity of the protein-bound P_i formed during incubation was not much lower than the activity of γ-P of the added [^{32}P]ATP. At the same time it was much higher than the activity of the free P_i which was initially present and which accumulated in the medium as the result of [^{32}P]ATP hydrolysis. The amount of the bound P_i was, however, small and variable; in some cases (Table 1, Expt. 2) during a more prolonged incubation period it showed a clear tendency to diminish and its specific activity became lower.

In the next series of experiments the enzymes were incubated in the presence of unlabelled ATP and radioactive P_i. Table 2 shows the results of two experiments performed with myosin. We see that under such conditions, in accordance with the hypothesis of a phosphorylated intermediate, the specific activity of the protein-bound P_i was lower than the activity of the free P_i in the medium. This was especially

Table 2

Specific activity of the protein-bound and of the free P_i after incubation of myosin with unlabelled ATP and $^{32}P_i$

Conditions: Expt. (1). Myosin, 23 mg.; $MgCl_2$, 4.0 μmoles; 0.03 M-tris - maleate buffer, pH 7.0; KCl, 0.06 M; ATP, 4.0 μmoles; P_i, 4.0 μmoles; specific activity 90 000 counts/min./μmole P_i; total volume 4 ml.; temp. 20°.

Expt. (2). Myosin, 20 mg.; $MgCl_2$, 3.0 μmoles; 0.03 M-tris - maleate buffer, pH 7.0; KCl, 0.06 M; ATP, 3.0 μmoles; P_i, 2.0 μmoles, specific activity 54 000 counts/min./μmole P_i; total volume 3 ml.; temp. 0°.

To the control samples ATP and $^{32}P_i$ were not added. Specific activity is expressed as counts per min. per μmole inorganic orthophosphate.

Expt. no.	Sample	Incubation (min.)	Interruption of enzymic process	Protein-bound P_i		Free P_i in the solution	
				(μmoles/g. protein)	(counts/min./μmole P_i)	(μmoles)	(counts/min./μmole P_i)
1	Control	—	Cooling at 0°	0.42	—	0	—
	With ATP and $^{32}P_i$	1		1.05	24 300	6.05	63 000
		5		1.62	31 000	6.85	60 500
		15		2.29	31 000	7.3	49 500
		20		2.10	34 700	7.3	49 000
2	Control	—	Acetate buffer pH 4.6	0.5	—	0	—
	With ATP and $^{32}P_i$	1		5.5	3 200	2.8	38 000
		1		8.0	4 500	2.7	40 000
		1		5.8	2 900	2.9	37 000
		1		6.0	3 500	2.5	42 000

conspicuous when the reaction was interrupted with acetate buffer, pH 4.6 (Expt. 2). In both series of experiments (Tables 1 and 2), when the enzymic process was stopped simply by cooling to 0° (Table 1, Expt. 2; Table 2, Expt. 1) the difference in the specific radioactivities between the free and the bound P_i was, however, not very great.

In the course of incubation the difference in the radioactivities diminished not only as the result of the naturally expected shift of the specific activity of the free P_i in the medium but also in consequence of a distinct change, in opposite direction, of the activity of the bound P_i. In this way the results of both series of experiments confirm the high probability of a direct transfer of P_i from ATP to the enzyme and of the formation of a phosphorylated product.

Table 3

The amount of protein-bound nucleotides and the specific activity of the free and the protein-bound P_i after incubation of myosin with [^{32}P]ATP and unlabelled P_i

Conditions: Myosin, 38 mg.; $MgCl_2$, 10.0 μmoles; 0.03 M-tris-maleate buffer, pH 7.0; KCl, 0.06 M; ATP, 10.0 μmoles, specific activity 44 300 counts/min./μmole γ-P; P_i, 8.0 μmoles; total volume, 22 ml.; temp. 0°. To the control samples [^{32}P]ATP and P_i were not added. The enzymic reaction was interrupted with acetate buffer (pH 4.6) after 1 min. incubation. Specific activity is expressed as counts per min. per μmole inorganic orthophosphate

Sample	Bound nucleotides		Bound P_i		Free P_i in the solution		
	ADP (μmoles/g. protein)	ATP (μmoles/g. protein)	(μmoles/g. protein)	(counts/ min./ μmole P_i)	(μmoles)	(counts/ min./ μmole P_i)	split from ATP (μmoles)
Control	—	—	0.25	—	0	—	0
With [^{32}P]ATP and P_i	2.3	8.9	3.6	35 600	8.10	5 800	0.10
	1.1	8.8	4.6	33 800	8.17	7 300	0.17

In the third series of experiments (the results of one of which are presented in Table 3) after incubation with [^{32}P]ATP and unlabelled P_i the separated and washed protein was examined for the presence and radioactivity of the bound P_i and also for the content of the bound nucleotides. These experiments were performed to elucidate whether it was possible that additional P_i was released from ATP, present in the separated and washed enzyme, as a result of an incomplete inhibition of the enzymic activity. If this were so, a corresponding amount of ADP would accumulate in the protein residue. As can be seen, however, from Table 3 such a subsequent P_i release could hardly take place. This point will be discussed later.

A supplementary proof that the enzymic process, at least after addition of acetate buffer (pH 4.6) or $(NH_4)_2SO_4$, was completely stopped was given by the following experiments: H-meromyosin and myosin were incubated with [^{32}P]ATP in the presence of $(NH_4)_2SO_4$ (55% saturation) and 0.5 M-acetate buffer, respectively. It appeared that after 20 min. incubation no radioactivity in the P_i of the medium was present.

Table 4

Influence of some inhibitors upon the specific activity of the protein-bound and the free P_i during incubation of myosin with unlabelled ATP and $^{32}P_i$

Conditions: Myosin, 20 mg.; 0.03 M-tris-maleate buffer, pH 7.0; KCl, 0.06 M, ATP, 6.0 μmoles; P_i, 2.5 μmoles, specific activity 62 000 counts/min./μmole P_i; total volume 10 ml.; temp. 0°. To the control samples ATP and $^{32}P_i$ were not added. The enzymic reaction was interrupted with acetate buffer, pH 4.6. Specific activity is expressed as counts per min. per μmole inorganic orthophosphate

Sample	Inhibitor	Incubation (min.)	Protein-bound P_i		Free P_i in the solution		
			(μmoles/g. protein)	(counts/min./μmole P_i)	(μmoles)	(counts/min./μmole P_i)	split from ATP (μmoles)
Control	None	—	2.1	—	0	—	—
With ATP and $^{32}P_i$	None	1	7.2	1920	2.6	62 000	0.1
		10	7.1	3500	4.1	38 000	1.6
	$MgCl_2$, 20 μmoles	1	5.8	2230	2.8	54 000	0.3
		10	5.1	1460	3.3	44 500	0.8
	$MgCl_2$, 20 μmoles PCMB, 5 μmoles	1	7.6	1740	2.4	63 000	0.0
		10	10.0	2880	2.8	54 200	0.3
	$MgCl_2$, 20 μmoles $CuSO_4$, 10 μmoles	1	3.7	1240	2.5	62 000	0.0
		10	6.0	2230	2.6	60 000	0.1

In the last series of experiments myosin was incubated with unlabelled ATP and radioactive P_i in the presence of $MgCl_2$, PCMB or $CuSO_4$ used as inhibitors. These experiments were performed in relation to recent findings of Gergely & Maruyama [10] some of which seemed to disagree with our results. Although the hydrolysis of ATP in these experiments was considerably reduced, from the data given in Table 4 it can be seen that the specific activity of the increased amount of the protein-bound P_i was many times lower than the activity of the free P_i. Thus, also in this case, the P_i found as protein-bound evidently derived not from the medium but from ATP which was split on a very small scale.

DISCUSSION

The conception of phosphorylation of muscle proteins was expressed already in 1941 by Kalckar [15] who supposed that myosin in the contracted state might act as a phosphate acceptor and during the relaxation process as a phosphate donor. Later on, Weber [25] worked out a hypothesis in which he postulated phosphorylation of contractile protein as one of the first reactions in the process of contraction.

The hypothesis of Weber seemed to be supported satisfactorily, although indirectly, by experiments of Levy *et al.* [17, 18, 19] in which myosin and actomyosin were incubated with ATP in the presence of $[^{18}O]H_2O$. A high level of ^{18}O found in the P_i after breakdown of ATP was interpreted by these authors as evidence of formation and subsequent splitting of a phosphorylated protein intermediate. Recent findings of Dempsey & Boyer [8] have shown, however, that myosin and actomyosin in the presence of ATP may catalyse an exchange of the oxygens of P_i of the medium with water oxygens. The existence of such an exchange indicates that the evidence of experiments of Levy *et al.* in favour of the phosphorylation hypothesis is not so convincing as it seemed to be, but the hypothesis itself is of course far from being disproved by these findings.

To elucidate whether the increased amount of protein-bound P_i found during interaction of contractile proteins with ATP [3, 4, 5] can serve as an indication of formation of phosphorylated intermediates it is, first of all, necessary to study the origin of the bound P_i. Otherwise, the sole fact of an increase of the bound P_i may be misleading and its meaning may be interpreted differently. Quite recently Gergely & Maruyama [10] reinvestigated the relationship of P-binding and ATPase activity. These authors confirmed the earlier findings from our laboratory [3, 4] as regards the increase of the protein-bound P_i during the course of the ATPase reaction; they were, however, of opinion that this could be ascribed to the binding of orthophosphate from the medium and not to a direct transfer from ATP.

The evidence presented by Gergely & Maruyama was based chiefly on results of two kinds of experiments. 1°, An increase of P_i binding under influence of ATP was found also in the presence of inhibitors (Mg^{2+}, PCMB and $CuSO_4$) when hardly any splitting of ATP took place. 2°, In presence of added $^{32}P_i$ and unlabelled ATP the specific activity of bound P_i was not lower than that of the total P_i.

According to Gergely & Maruyama these two lines of evidence suggested that the observed P_i-binding was only enhanced by the presence of ATP and to a lesser degree by the presence of some other nucleotides.

Although a certain discrepancy, which will be discussed below, appears to exist between the findings of Gergely & Maruyama [10] and our results we believe that the presented experiments provide evidence that at least a very considerable part of the protein-bound P_i derives directly from the ATP hydrolysed during interaction with myosin or H-meromyosin. In experiments in which the enzymes were incubated with [^{32}P]ATP and unlabelled P_i the specific activity of protein-bound P_i was found to be of the same order as γ-P in the added nucleotide and much higher than that of the P_i in the medium (Table 1). On the other hand, when the incubation was performed with radioactive P_i and unlabelled ATP the specific activity of the protein-bound P_i was distinctly lower and sometimes much lower than that of the free P_i in the medium (Table 2).

It seemed, however, necessary to take into consideration the following possibility. It might have occurred that the enzymic reaction was not yet completely inhibited when, after incubation, the cleavage of ATP was supposed to be stopped. If this were so, already after centrifugation and washing of the protein, some additional amount of P_i could possibly be split from ATP attached to the precipitated protein, thus imitating a real phosphorylation [2].

The evidence provided by the present experiments indicates, however, that such an additional enzymic release of P_i can have only a very limited significance.

First, such a release should be accompanied by a corresponding increase of the amount of ADP. Yet, the determination of ADP in the protein after its separation and washing demonstrated clearly (Table 3) that even the total amount of ADP present could be responsible for not more than a small part of the increased quantity of the protein-bound P_i found at the same time. When a suitable correction for the presence of the ADP was made the specific activity of the bound P_i still appeared to be about four times higher than the activity of the free P_i in the medium.

Secondly, as was already shown, virtually no enzymic splitting of ATP was observed in the presence of either $(NH_4)_2SO_4$ at 55% saturation or acetate buffer, pH 4.6. Therefore the suspected splitting of ATP, already after centrifugation of the protein, might occur only when the enzymic action was stopped by prompt separation of the enzyme at 0°: as a result, a somewhat larger amount of P_i than in the other experiments should have been present in the separated protein. It is therefore noteworthy that the highest increase of protein-bound P_i was found

[2] We are greatly obliged to Prof. Dr. H. H. Weber who in a private letter drew attention to this possibility.

not with this method of inhibition but in the experiments (Table 3, Expt. 3) in which the enzymic process was completely interrupted by addition of acetate buffer (pH 4.6). Here the specific activity of the bound P_i was also only slightly lower than the activity of γ-P in the added [^{32}P]ATP.

All these findings strongly support the view that during the enzymic cleavage of ATP at least a very considerable part of the protein-bound P_i originates directly from the nucleotide. In some of the experiments (Table 1, Expt. 3) the quantity of P_i found as protein-bound formed 20 to 30% of the total orthophosphate released from ATP during one minute of incubation. According to a rough calculation these highest figures suggest that 4 to 5 moles of orthophosphate were found per mole of the isolated myosin.

The earlier observations from this laboratory [21, 9, 23] indicate clearly that a certain part of the protein-bound P_i originates from binding of free P_i present in the medium. This fact explains why in experiments with unlabelled ATP and $^{32}P_i$ the specific activity of the bound P_i never could be reduced to zero and in experiments with [^{32}P]ATP it never reached the activity of γ-P of the nucleotide. After a short incubation period, with radioactive P_i the specific activity of the bound P_i was much lower than the activity of the free P_i. We may suppose therefore that the fact that in similar experiments Gergely & Maruyama [10] were unable to find any difference in the specific activity of the bound and the free P_i is likely to be due to a longer incubation period and to a higher concentration of P_i used by these authors.

The recent results of Dempsey & Boyer [8] seem to indicate that the P_i present in the phosphorylated enzyme may exchange with the free P_i. If this is correct, the higher concentrations of orthophosphate in experiments of Gergely & Maruyama could shift the equilibrium of the exchange and thus minimize the difference in the radioactivity of the bound and the free P_i.

It remains to discuss the experiments in which myosin was incubated with ATP in the presence of inhibitors and added P_i. As mentioned above Gergely & Maruyama [10] found under these conditions a distinct P-binding with only a slight P_i liberation or even without it. They conclude therefore that the phosphate binding could not be directly correlated with the ATPase activity. To elucidate the reason of this discrepancy we performed analogous experiments using radioactive orthophosphate. From the results presented in Table 4 it may be seen that ATP hydrolysis was highly inhibited but like in our former experiments the increased amount of protein-bound P_i derived almost entirely directly from ATP and not from the inorganic phosphate. Thus,

the specific activity of the bound P_i appeared to be 20 to 50 times lower than the activity of the added orthophosphate.

According to Kielley [16], if we postulate the existence of a temporarily phosphorylated enzyme we may think that "the phosphate group can shift tautomerically between paired equivalent groups of the enzyme". We can suppose that the inhibitory effect of Mg^{2+}, PCMB and $CuSO_4$ might consist not so much in the prevention of formation of the phosphorylated intermediate but rather in the interference with the processes of its dephosphorylation. Such a view, in so far as Mg^{2+} is concerned, is consistent with earlier findings of Brahms & Kąkol [3] who showed that the initial increase of protein-bound P_i during ATP hydrolysis by myosin was somewhat higher in the presence of Mg^{2+} than in its absence. The strikingly high values of protein-bound P_i obtained in the experiments in which the enzymic process was stopped by addition of acetate buffer, pH 4.6 (Table 1, Expt. 3; Table 2, Expt. 2), may perhaps also be significant as they indicate a possibility of selective inhibition of some steps in the enzymic cleavage of ATP, especially of the steps connected with the dephosphorylation of the enzyme.

From the results presented above we may conclude that both the origin and the lability of the supposed phosphorylated enzyme indicate that it can be assumed to be an intermediate which is formed and subsequently split in the course of ATP hydrolysis. In connection with the findings of Gergely & Maruyama [10], especially with their observation of an increase of P-binding by myosin under influence of ADP when (provided that myokinase is absent) a direct transfer of P_i from the nucleotide is impossible, the following supposition can be made. It is not to be excluded that during ATP hydrolysis two kinds of processes may occur. A temporarily phosphorylated enzyme is formed as shown above and at the same time the P-binding may be enhanced under influence of the nucleotides.

From evidence that has accumulated over many years it seems likely that as a result of even slight changes in environment the enzymic activity of contractile muscle proteins and its mechanism may be considerably modified (cf. the interesting study of the kinetics of the fibrillar ATPase by Bendall [1]). It seems worth while to mention that in our next experiments with actomyosin some of the findings were quite different from the results presented above. When actomyosin was incubated with [^{32}P] ATP in a medium of a high ionic strength ($I > 0.15$), a direct transfer of P_i from the nucleotide was clearly seen similarly as in the case of myosin and H-meromyosin. In a medium of a low ionic strength (0.1), however, under conditions in which superprecipitation occurred, a considerable amount of protein-bound P_i appeared which, most probably, originated chiefly from an acid inso-

luble phosphorus compound present initially in actomyosin. Preliminary reports of some of these findings have already appeared [13, 14] and the investigation is continued.

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SUMMARY

1. The origin of protein-bound orthophosphate (P_i) formed during splitting of ATP by myosin and H-meromyosin was studied with isotope dilution methods.

2. When the enzymes were incubated with [^{32}P] ATP and unlabelled P_i the specific activity of the protein-bound P_i was of the same order as the activity of γ-P in the nucleotide. When incubation was performed with unlabelled ATP and radioactive P_i the specific activity of the bound P_i was always much lower than that of the free P_i in the medium. These results suggest that myosin- and H-meromyosin-bound P_i which appears during ATP hydrolysis derives directly from the nucleotide.

3. When ATPase activity was inhibited by $MgCl_2$, *p*-chloromercuribenzoate or $CuSO_4$ the protein-bound P_i likewise appeared also originating mainly from ATP.

4. It is concluded that the phosphorylated myosin and H-meromyosin may be the intermediates formed and subsequently split during hydrolysis of ATP.

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BEZPOŚREDNIE PRZENOSZENIE ORTOFOSFORANU Z KWASU ADENOZYNO-TRÓJFOSFOROWEGO NA MIOZYN I H-MEROMIOZYN

Streszczenie

Za pomocą metody rozcieńczania izotopowego badano pochodzenie związanego z białkiem ortofosforanu (P_i) zjawiającego się w czasie rozszczepiania kwasu adenozynotrójfosforowego (ATP) pod wpływem miozynu i H-meromiozynu. Przy inkubacji tych białek z [^{32}P] ATP i nieznakowanym P_i specyficzna aktywność związanego P_i była tego samego rzędu co aktywność γ-P w nukleotydzie. Natomiast w czasie inkubacji z nieznakowanym ATP i radioaktywnym P_i aktywność specyficzna P_i była (również i w obecności inhibitorów) zawsze znacznie niższa niż aktywność wolnego P_i w środowisku.

Uzyskane wyniki wskazują na to, że zjawiający się związany z białkiem P_i pochodzi bezpośrednio z nukleotydu i że ufosforylowany miozyn, względnie H-meromiozyn można uważać za produkt pośredni zjawiający się i rozpadający się w czasie enzymatycznej hydrolizy ATP.

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OXYGEN AND DOSE-RATE EFFECTS ON SURVIVAL CURVES OF γ-IRRADIATED TRANSFORMING DNA IN THE PRESENCE OF PROTECTIVE SUBSTANCES

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During the course of a previous investigation [14] on the γ-ray inactivation of dried preparations of several transforming principles, it was observed that the nature of the dose-survival curves was appreciably altered in the presence of a "protective" substance such as yeast extract. Subsequently several trial experiments on aqueous solutions of transforming DNA (T-DNA) demonstrated that, in addition to the expected protection conferred by the addition of foreign substances, there resulted not only a marked alteration in the form of the survival plots but, in the presence of various -SH compounds, also an appreciable dependance of the rate of inactivation on the dose-rate. Simultaneously a distinct oxygen effect made its appearance when the protecting substances used were some mercaptans.

The influence of ionizing radiations on T-DNA in aqueous medium has not been as extensively investigated as for dried or frozen preparations, where the primary objective is the determination of "marker sizes" by target theory. Ephrussi-Taylor & Latarjet [5] found that solutions of T-DNA were quite sensitive to X-rays and reported that, if there were any oxygen effect, it is not very marked. Drew [3] noted a rather high degree of protection in the presence of cysteine, a substance known to protect against the action of radiation-produced radicals, but no quantitative data were given. The first extensive and quantitative study was that of Defilippes & Guild [2], who also signalled the absence of an oxygen effect in solution, although Guild [7] subsequently reported an oxygen effect on dry DNA. Following completion of the present work, a paper appeared by Hutchinson [9] in which, in an extension of the earlier studies of Howard-Flanders [8] on bacteriophages, it was found that a net oxygen effect may be observed when pneumococcal T-DNA is irradiated in the presence of sulphydryl

substances, similar to the observations we report below for streptococcal T-DNA.

The foregoing is of special interest in view of the long-known fact that the sensitivity of living cells to ionizing radiations is usually oxygen dependent, the magnitude of this oxygen effect being frequently appreciable. Its precise nature and origin are still the subject of considerable speculation. More important still, an oxygen effect has been demonstrated for irradiated intracellular T-DNA [10].

On the other hand, although a number of physiological effects have been reported to be a function of dose-rate (see, e.g. Bacq & Alexander, [1]), this apparently does not apply to gene mutations which, with a few possible exceptions, are apparently dose-rate independent [11]. An examination of possible dose-rate effects for an *in vitro* system involving DNA consequently appeared to be warranted.

MATERIALS AND METHODS

Bacterial strains. The recipient organism used in transformation was a Challis strain of group *H* haemolytic streptococci, capable of giving a high yield of transformants [12]. The donor was a mutant of this strain resistant to 2 mg./ml. of streptomycin or dihydrostreptomycin. Streptomycin resistance was the only marker used in this investigation.

Transforming DNA. This was prepared as follows: The centrifuged and washed bacterial cells, in the presence of 0.1 M-sodium citrate, were submitted to the action of a concentrate of the lytic factor from a streptomyces strain, known as actinomycetin [13], and capable of lysing both living and heat-killed streptococci. Immediately following cell lysis, the DNA was precipitated with 1 vol. ethanol, washed several times with 70% ethanol and dissolved in a solution of 0.15 M-NaCl and 0.1 M-sodium versenate by agitation on a wrist-action shaker. To 15 ml. of the DNA solution was then added 1 ml. of 30% Duponol C and, after a lapse of 30 min. at 37°, the mixture was centrifuged for 30 - 40 min. at 13 000 *g* to remove a considerable amount of protein. The resulting solution was further deproteinized several times by the method of Sevag *et al.* [16], submitted to the action of ribonuclease for the purpose of removing RNA, then deproteinized once or twice more by the Sevag method and finally with water-saturated phenol. The T-DNA was then again precipitated with 1 vol. ethanol and dissolved in 0.015 M-NaCl containing also 0.015 M-sodium citrate. This procedure was repeated 3 - 4 times and the T-DNA finally dissolved in 0.15 M-NaCl to a concentration of about 0.5 mg./ml. The ultraviolet absorption spectrum of such a preparation exhibited a ratio of maximum (2600 A) to minimum (2300 A) absorption of 2.0 to 2.1.

Irradiation procedure. The source of radiation was a 49 curie disc of cobalt, the construction and use of which have been described elsewhere [18]. Solutions were irradiated in 6-mm. diameter test tubes distributed symmetrically about the source; and the radiation doses were obtained by ferrous sulphate dosimetry conducted on solutions in the same tubes, thus eliminating the effect of non-uniformity in dose for those samples irradiated close to the source.

Transformation technique. Transformation reactions were carried out with competent cells preserved in a deep-freeze, as described by Fox & Hotchkiss [6] for pneumococci. The concentration of T-DNA in the transforming medium was always 0.1 μg./ml., which is below the saturation level. The number of transformants was obtained by plating on an agar medium containing 3% defibrinated blood and 250 μg./ml. streptomycin. The recipient strain exhibited no growth in the presence of 10 μg./ml. streptomycin.

Protecting substances. These included the following macromolecular substances: hyaluronic acid from umbilical cord; thymus DNA, heated prior to use for 15-20 min. at 100° in order to prevent transformation inhibition; and the soluble fraction of dextran (polyglucoside) extracted from the culture medium of a *Streptococcus sanguis* strain cultured in broth with 5% saccharose. The foregoing were prepared in this laboratory according to standard procedures. Also employed were commercial preparations of yeast RNA and yeast extract (Difco).

The low molecular weight substances used included cysteine, thiourea and β-mercaptoethanol (Eastman).

All solutions were initially brought to neutrality with NaOH, following which they were diluted to the appropriate concentration with the T-DNA samples in 0.01 M-phosphate buffer at pH 7.0 - 7.2. Final concentration of T-DNA in all solutions was 1 μg./ml.

RESULTS

It is perhaps necessary, at the outset, to emphasize that all the results herein reported are based on the use of a single preparation of T-DNA, for which the ratio of ultraviolet extinction of maximum to minimum was 2.05. It was subsequently found that a duplicate preparation exhibited a different radiation sensitivity. For example, one T-DNA sample, for which the ratio of maximum to minimum extinction was 1.8, was found to be more radioresistant. Since a lower ratio of maximum to minimum absorption is indicative of a poorer degree of deproteinization, this would apparently imply a marked protective effect by the protein component of the nucleoprotein. This is in agreement with what might be expected from the recent findings of Emmer-

son *et al.* [4] and Peacocke & Preston [15], who report that the nucleic acid moieties of natural nucleoproteins are remarkably resistant to ionizing radiations, the primary effect involving initially the protein component alone.

It would, in fact, appear to be useful to carry out a systematic investigation of the radiosensitivity of the biological activity of transforming deoxyribonucleoprotein following stepwise deproteinization.

Fig. 1 presents the dose-survival curves for T-DNA in 0.85% NaCl buffered to pH 7, at dose-rates of 2.4, 8.3 and 33 kr./hr., and the protective effects of hyaluronic acid, yeast RNA and commercial yeast

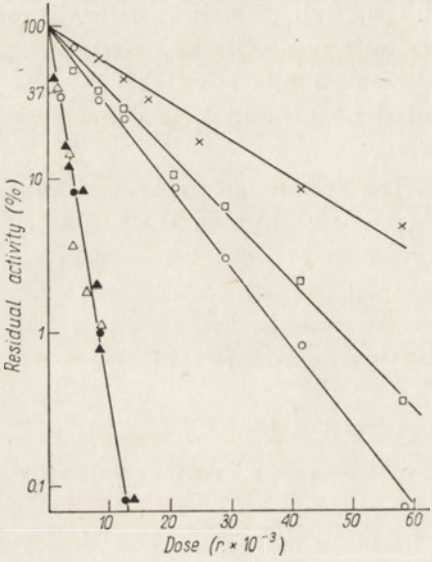

Fig. 1. Dose-survival curves in air for T-DNA, 1 μg./ml., in the absence and presence of several high molecular weight substances. (△), T-DNA only, dose-rate 2.4 kr./hr.; (●), T-DNA only, dose-rate 8.3 kr./hr.; , T-DNA only, dose-rate 33 kr./hr.; (○ T-DNA + 1 mg./ml. hyaluronic acid, dose-rate 8.3 kr./hr.; □), T-DNA + 1 mg./ml. RNA, dose-rate 8.3 kr./hr.; (×) T-DNA + 1 mg./ml. yeast extract, dose-rate 8.3 kr./hr.

extract. It will be seen that, in the absence of foreign substances, the rate of inactivation is relatively independent of the dose-rate. It should also be noted that the semi-log plots are reasonably linear to a residual activity of less than 1%.

From the same figure it will be observed that yeast extract is most effective in protecting against inactivation, on a weight basis. Furthermore, the semi-log plots remain linear under these conditions. Not shown in the figure are the results for dextran and heat-denatured thymus DNA, which were less effective as protectors than yeast extract, RNA or hyaluronic acid. In an additional experiment, not included in Fig. 1, it was found that the dose-survival curve in the presence of 1 mg./ml. yeast extract was essentially unchanged when the dose-rate was varied from 8.3 to 118 kr./hr.

With our radiation set-up, it was not feasible to run a dose-survival curve for T-DNA, in the absence of protective substances, at dose-rates

above 33 kr./hr., since the short exposure times required were not readily attainable without the introduction of considerable experimental error. We conclude, nonetheless, from the foregoing results, that with the 1 μg./ml. T-DNA concentration employed throughout this work, no dose-rate effect prevails over the entire range from 2.4 to 118 kr./hr.

The remarkably higher protective efficiency of thiourea and mercaptans, on a weight basis, is illustrated in Fig. 2. Note that, whereas for cysteine and mercaptoethanol the survival curves are linear, the

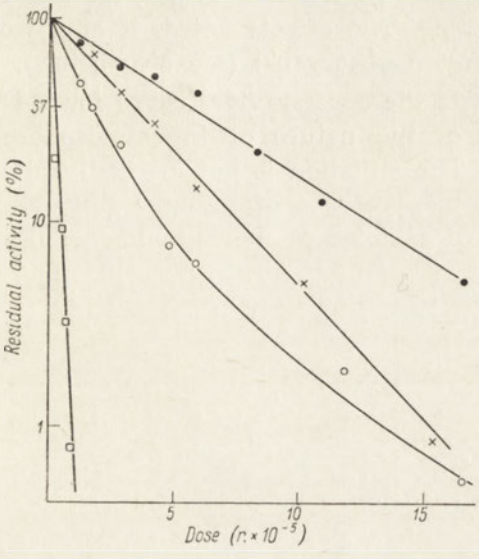

Fig. 2. Dose-survival curves in air for T-DNA, 1 μg./ml., in presence of 1 mg./ml. of thiourea and mercaptans. (□), DNA only, dose-rate 8.3 kr./hr.; (●), β-mercaptoethanol, dose-rate 118 kr./hr.; (×), cysteine, dose-rate 118 kr./hr.; (○), thiourea, dose-rate 118 kr./hr.

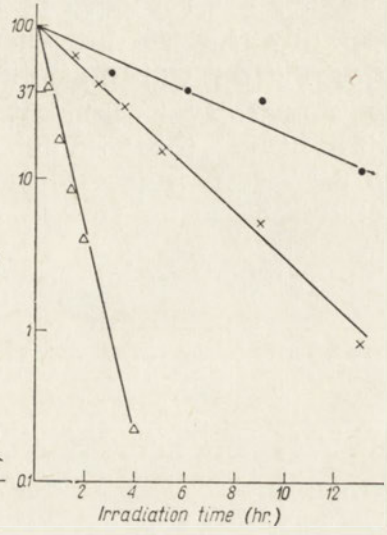

Fig. 3. Dose-survival curves in air for T-DNA irradiated in the presence of various concentrations of cysteine, dose-rate 118 kr./hr.; (●), Cysteine 2.5 mg./ml.; (×), cysteine 1.0 mg./ml.; (△), cysteine 0.25 mg./ml.

one for thiourea exhibits a gradual break at a residual activity of about 15% (cf., however, Fig. 5, below). Although, on a weight basis, mercaptoethanol appears to be more efficient as a protector than cysteine or thiourea, it is actually only slightly more effective than cysteine when the concentrations are expressed on a molar basis, as may be seen from Fig. 3, which illustrates the influence of cysteine concentration on the rate of inactivation.

Although the degree of protection conferred by cysteine in the above experiments is much greater than that reported by Hutchinson [9], the difference in results is more apparent than real. The degree of protection is, in effect, also a function of the ratio of cysteine to T-DNA

concentration, as might be expected; and Hutchinson used a concentration of crude T-DNA several hundred times higher than that employed in the present work.

All the foregoing irradiations were conducted in an atmosphere of air. Attention was now directed to the possible influence of oxygen. For this purpose the solutions to be irradiated were first saturated either with oxygen or nitrogen, and then sealed with paraffin wax. It would obviously have been desirable to pass the appropriate gas continuously through the solutions during irradiation, but, for technical reasons, this was not found possible. The effects noted, if any, would consequently represent the minimum observable (see also below).

From Fig. 4 it will be seen that the dose-survival curve for T-DNA alone is relatively independent of the nature of the atmosphere in which irradiation is carried out. The situation is not markedly altered when the T-DNA is exposed in the presence of thiourea (Fig. 5). On the other hand, in the presence of cysteine or mercaptoethanol (Fig. 6

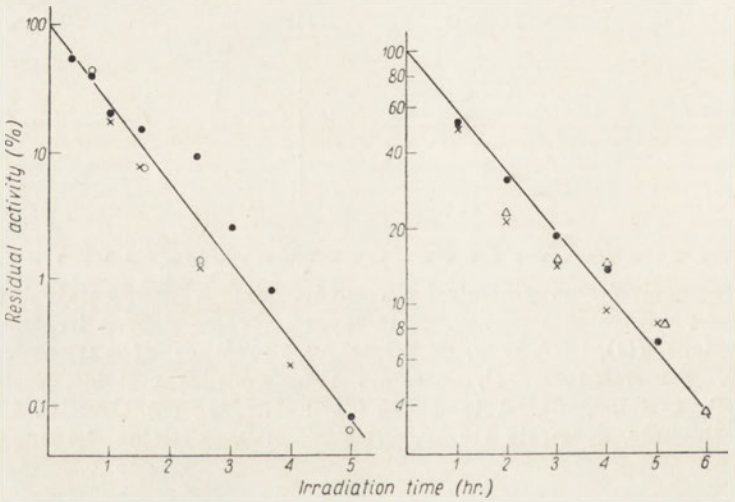

Fig. 4. Dose-survival curves for T-DNA irradiated in solution at a dose rate of 2.4 kr./hr. in an atmosphere of (●), air; (×), oxygen; (○), nitrogen

Fig. 5. Dose-survival curves for T-DNA irradiated in the presence of 0.25 mg./ml. thiourea, at dose-rate of 33 kr./hr., in an atmosphere of (●), air; (×), oxygen; (△), nitrogen

and 7), a very pronounced oxygen effect is apparent, qualitatively similar to that observed by Hutchinson [9] for pneumococcal T-DNA with glutathione as an -SH protector. The results for irradiation in an atmosphere of air fall, both for cysteine and mercaptoethanol, between those for oxygen and nitrogen, as might be anticipated.

The close resemblance between our findings and those of Hutchinson [9], who bubbled oxygen or nitrogen continuously through the solutions during irradiation, suggests that our solutions were adequately saturated with the respective gases during the period of exposure to the radiation source, demonstrating the reality of the oxygen effect.

In addition to the oxygen effect so clearly placed in evidence by Fig. 6 and 7, the presence of cysteine also provokes a marked dependance of the slopes of the dose-survival curves on the dose-rate, in an atmosphere of air. This is demonstrated quantitatively in Fig. 8, from

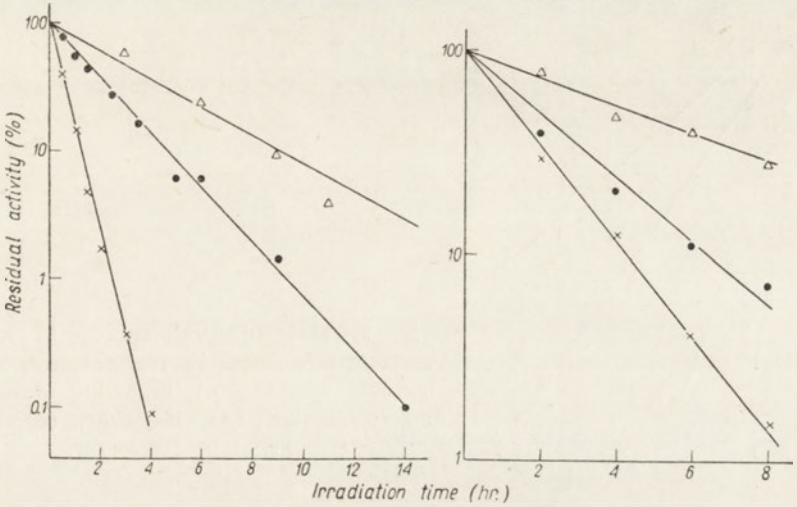

Fig. 6. Dose-survival curves for T-DNA irradiated in the presence of 0.25 mg./ml. cysteine, at a dose-rate of 33 kr./hr., in an atmosphere of (●), air; (×), oxygen; (△), nitrogen

Fig. 7. Dose-survival curves for T-DNA irradiated in the presence of 0.25 mg./ml. β-mercaptoethanol, at a dose-rate of 33 kr./hr., in an atmosphere of (●), air; (×), oxygen; (△), nitrogen

which it will be observed that, e.g., in the presence of 250 μg./ml. (2×10^{-3} M) cysteine, the $D_{37\%}$ dose for T-DNA inactivation is 88 kr. at a dose-rate of 118 kr./hr., but only 16 kr. at a dose-rate of 6.5 kr./hr. The dose-rate effect was found in separate experiments to be only slightly less pronounced in the presence of mercaptoethanol but much less so for thiourea. The specificity of this effect is clearly established by the fact that it is completely absent in the presence of yeast extract.

Attempts were subsequently made to determine to what extent the observed dose-rate effect is dependent on oxygen. Unfortunately the results obtained were not entirely unequivocal. Under nitrogen alone, no influence of dose-rate was observed, as might have been anticipated.

However, under oxygen alone the effect encountered was only 20 - 25% of that expected. The reason for this is not clear and requires further investigation. It would, perhaps, be desirable to repeat this experiment under conditions where there is a continuous flow of oxygen through the system. This was indicated by the fact that, even in an atmosphere of air, the dose-rate effect was reduced by about 35% if the tubes containing the samples were sealed off instead of being left open to the

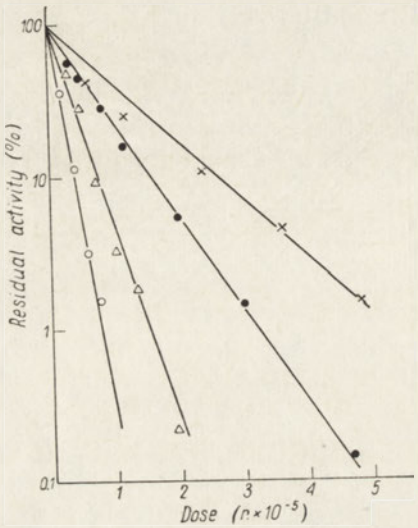

Fig. 8. Dose-survival curves in air for T-DNA irradiated in the presence of 0.25 mg./ml. cysteine at following dose-rates: (○), 6.5 kr./hr.; (△), 15 kr./hr.; (●), 33 kr./hr.; (×), 118 kr./hr.

atmosphere. On the other hand, notwithstanding the lack of continuous oxygen saturation in our experiments, as already referred to above, the oxygen effect observed (Fig. 6 and 7) is almost quantitatively similar to that reported by Hutchinson [9] under conditions which assured continuous oxygen saturation of the irradiated solutions.

DISCUSSION

From Fig. 1, 2 and 3, it is clear that the protective effect of thiourea, cysteine and mercaptoethanol is highly specific by comparison with such substances as RNA or yeast extract. This is even more strikingly apparent from the data presented in Table 1. When the irradiations are performed under nitrogen, the protective effect of cysteine and mercaptoethanol is even more pronounced.

The pronounced oxygen effect observed with cysteine and mercaptoethanol is in good agreement with that found by Hutchinson [9] for crude extracts of pneumococcal T-DNA in the presence of glutathione, notwithstanding that the dose-rates used in the latter studies were 60-fold greater than ours. The present findings are also in accord

with those of Howard-Flanders [8], who demonstrated that T-2 bacteriophage exhibits increased radiosensitivity under an oxygen atmosphere in the presence of -SH compounds. The specificity of -SH groups in this phenomenon is further attested to by the fact that, as for the T-2 bacteriophage, no oxygen effect is observed in the presence of thiourea, notwithstanding the pronounced protective effect of this compound, which is of the same order of magnitude as that for cysteine (Table 1).

Table 1

Doses required to reduce activity of transforming DNA (1 μg./ml.) in neutral solution in air, to 37% residual activity in the presence of protecting substances

Protecting substance	Dose (kr.)
None	1.6
Hyaluronic acid (1 mg./ml.)	8.0
Yeast RNA (1 mg./ml.)	10
Yeast extract (1 mg./ml.)	17
Thiourea (0.25 mg./ml.)	57
Thiourea (1 mg./ml.)	170
Cysteine (0.25 mg./ml.)	76
Cysteine (1 mg./ml.)	330
Cysteine (2.5 mg./ml.)	719
Mercaptoethanol (0.25 mg./ml.)	92
Mercaptoethanol (1 mg./ml.)	560

Additional evidence for the specificity of -SH groups in this system is the important dose-rate effect observed in air (and, albeit to a lesser extent, in oxygen) with cysteine and mercaptoethanol, but not with yeast extract, and only to a slight extent with thiourea. The nature of the dose-rate effect is rather curious. If the function of the -SH protecting groups is to trap radicals formed in aqueous medium before they can attain the T-DNA molecules, one would expect them to be less efficient when the rate of production of free radicals is increased, i.e. when the dose-rate is increased. In fact, the reverse is the case (Fig. 8). It is, of course, conceivable that the dose-rate effect on the T-DNA is more apparent than real, in that we may be dealing with a dose-rate effect on the protecting -SH groups themselves. This, however, appears rather unlikely from the findings of Swallow [17] and Hutchinson [9], which indicate that the doses used here did not result in sufficient destruction of the protective substances as to markedly change their concentration during the irradiation periods employed.

This is also supported by the fact that the survival curves do not show either pronounced breaks or curvature, which might be expected to accompany any pronounced decrease in concentration of the protective -SH groups. It would undoubtedly be of some value to examine whether a dose-rate effect exists for intracellular T-DNA, where an oxygen effect is also reasonably well established [9]; the more so in that there is no dose-rate effect in the absence of protective substances, nor in the presence of protective -SH groups under a nitrogen atmosphere. The results of such experiments might provide some clue as to the state of intracellular T-DNA.

It is perhaps of interest, in this connection, to recall what was mentioned above with regard to the differences in radiation sensitivities of different preparations of T-DNA and which are apparently dependent on their protein contents. The range of $D_{37\%}$ doses we have found in these and in previous experiments [14] is appreciable, from 800 to 3200 r. These values are to be compared with several reported by Defilippes & Guild [2], for one of whose pneumococcal T-DNA preparations the $D_{37\%}$ dose was as little as 65 r., under irradiation conditions comparable to those prevailing in the present experiments (T-DNA concentration 8 μg./ml.). It is almost essential that the source of these differences be clarified if we are to gain any further understanding of the nature of the inactivation process; this factor is also a prerequisite if a fruitful comparison of the results of different laboratories is to be made on a quantitative basis.

SUMMARY

A study has been made of the inactivation of streptococcal transforming DNA by ionizing radiations in dilute aqueous medium, and the protection conferred by the presence of various substances. A rather high degree of specific protection prevails in the presence of thiourea and some mercaptans. In the presence of the -SH compounds there is a pronounced oxygen effect. An appreciable dose-rate effect also manifests itself in the presence of the -SH compounds, but not with thiourea, yeast extract, ribonucleic acid or hyaluronic acid. The survival curves for different DNA preparations exhibit some variation in the $D_{37\%}$ dose which is, in part at least, dependent on the protein content of the transforming DNA sample; it is consequently essential to use the same preparation throughout any given series of experiments in order to obtain comparable results.

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EFEKT SZYBKOŚCI DAWKOWANIA I EFEKT TLENOWY W INAKTYWACJI TRANSFORMUJĄCEGO DNA W OBECNOŚCI SUBSTANCJI OCHRANIAJĄCYCH

Streszczenie

Zbadano przebieg inaktywacji roztworów transformującego DNA z paciorkowców przez promieniowanie jonizujące w nieobecności i w obecności substancji ochraniających. W obecności tiomocznika i niektórych związków merkaptanowych efekt ochronny jest wysoki. Stwierdzono także bardzo znaczny efekt tlenowy w obecności związków -SH. W ich obecności obserwuje się również efekt szybkości dawkowania, który nie występuje, gdy jako substancji ochronnych używa się tiomocznika, wyciągu drożdży, RNA lub kwasu hialuronowego. Dawki energii $D_{37\%}$ dla poszczególnych preparatów DNA różnią się znacznie, co przynajmniej częściowo można przypisać różnej zawartości w nich białka. Z powyższego wynika, że każdą serię badań należy przeprowadzać z tym samym preparatem DNA.

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HISTOCHEMICAL LOCALIZATION OF HYALURONIDASE AND AMYLASE BY THE FILM-SUBSTRATE TECHNIQUE

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In the film-substrate technique proposed by Daoust [2, 3] for the localization of ribonuclease and deoxyribonuclease, a tissue section is brought into intimate contact with a gelatin film containing the substrate. Following incubation, the section is removed and the film stained with an appropriate basic dye which is bound readily by the ribo- or deoxyribonucleic acids used as substrates, except for those sites where the latter have undergone enzymic hydrolysis. The stained film consequently reflects the localization of the enzyme in the tissue section. A somewhat similar technique has been independently applied by Todd [10, 11] for histochemical estimation of fibrinolytic activity by means of fibrin films, and by Adams & Tuqan [1] for localization of protease activity with the use of commercial X-ray films.

The foregoing type of technique should lend itself readily to the localization in tissue sections of a variety of other enzymes, the substrates of which are high molecular weight compounds for which suitable stains are available, and the products of enzymic hydrolysis of which (a) either lose, or undergo modification of, their staining properties, or (b) readily diffuse out of the film prior to, or during the staining procedure. We have previously reported on the application of this technique to nuclease enzymes, using as substrates some synthetic and natural oligo- and polynucleotides. The minimum chain-lengths for applicable substrates was found to be about 10 residues. Shorter chains were found to be unsuitable because of their relatively rapid rate of diffusion from the films during the incubation period or during subsequent staining [9].

In the present communication are described the results of some experiments on the extension of the film-substrate technique to two additional enzymes, amylase and hyaluronidase.

For the first of these we have made use of the variation in affinity

for iodine of polysaccharides, as a function of their degree of polymerization, to localize amylase-like enzymes which hydrolyse these polymers, the particular substrate used being water-soluble starch. The histochemical localization of amylase is a problem of some interest in view of the important physiological role of this enzyme, which has hitherto been "localized" exclusively by means of cell fractionation techniques [5, 8]. The application of the film-substrate method to this enzyme is consequently warranted, notwithstanding that the resolution attainable leaves much to be desired [9].

The procedure for hyaluronidase makes use of the metachromasy of hyaluronic acid and chondroitin sulphate. Localization data for this enzyme have likewise hitherto been based solely on cell fractionation studies [5, 8]. Two substrates were employed for hyaluronidase, with a view to determining whether there was any difference in localization of the enzyme(s) acting on these. Although we have not investigated the use of different substrates for amylase, this would most likely be worth attempting, since several amylases with different specificities are known. Attention should be drawn, in this connection, to a previous study in which a variety of polynucleotides were employed for the purpose of distinguishing between nuclease enzymes of differing specificities in tissue sections [9].

Procedure for amylase

Preparation of substrate-film. A 13% soluble starch [4] solution was prepared, the starch being initially dissolved in cold water, which was then heated to 90° with constant stirring. The solution was cooled to between 40° and 50° and combined with an equal volume of 10% Difco gelatin [1] at the same temperature. About 70 µl. of this warm mixture was deposited uniformly on a 25×25 mm. microscope slide by means of a micropipette drawn rapidly over the glass surface. The slide was then placed on a horizontal, cooled, glass plate, dried by means of a current of air and then fixed in 10% formalin (unbuffered) at 4° for at least three hours. Following fixation, the film was rinsed in running distilled water for 15 min. and once again dried in a stream of air. Such films may be kept for about 3 days, following which they become increasingly resistant to enzymic hydrolysis. Even when stored under conditions of high humidity, the films were unsuitable after 4 days. The sensitivity of these starch films was tested by exposure to a solution of saliva.

[1] Attention is drawn to the fact, previously emphasized [9], that not all commercially available gelatins are suitable.

Tissue preparation. Materials used were rat pancreas and rat parotid gland. Fresh frozen sections were cut at a thickness of about 30 μ on a Spencer freezing microtome with a knife-cooling attachment, set up in a cold room at 4°. Subsequent operations were similar to those described by Daoust [2, cf. 9]. When the sections were placed in contact with the substrate film, air bubbles were frequently found trapped in the interface. Several trials showed that this could be prevented by "breathing" on the surface of the section immediately prior to depositing it on the film. Control sections free of enzyme activity were obtained by simply heating the tissue in boiling water.

Additional controls were employed to exclude possible interference by protease activity on the gelatin of the film, with resultant removal of gelatin and modification in staining properties. For this purpose starch-free gelatin films were incubated with fresh pancreatic sections under conditions identical to those used for amylase localization. The films were then stained in alkaline toluidine solution (pH 10). Such incubated films showed only a slight, diffuse, discoloration of the entire surface, which would not sensibly interfere with the staining pattern following incubation with amylase.

Incubation. For pancreas sections, incubation times varied from 9 to 12 min. at room temperature. Because of the high amylase activity of the parotid gland, it was found more convenient in this case to incubate at temperatures of 6 - 10° for periods of 20 - 25 min.

Staining of films and sections. Substrate films were stained with normal Lugol solution for 5 min., washed in running water and finally mounted in glycerin with gelatin. The stain is not permanent and, if it is desired to examine the preparation after a lapse of several days, the cover glass must be removed and the film stained anew. The tissue sections, mounted on the gelatin blocks, were fixed and stained as described by Daoust [2].

Localization. In the pancreas (Fig. 1) the enzyme is localized in the secretory acini. The walls and lumina of blood vessels, the interlobular septa, and the islands of Langerhans, are devoid of enzymic activity.

The parotid gland (Fig. 2) exhibits amylase activity in the secretory acini. The interlobular septa are negative. The submaxillar nodes exhibit no activity. Control, heat-inactivated, sections were negative even after 2 hr. incubation.

Procedure for hyaluronidase

Preparation of substrate films. The sodium hyaluronate used was a gift of Prof. L. Rzucidło and was a commercial product of the Warsaw Serum and Vaccine Plant. It was dissolved in water at a concentration of 2.4 mg./ml. and the solution brought to pH 8 by addition of 0.1 N-

-NaOH and then mixed with an equal volume of 5% gelatin, the final solution being again brought to pH 7 - 8. The mixture was then warmed to about 40° and a film prepared on a microscope slide with about 70 µl., as described above for the amylase substrate films. The films were dried in a stream of air at a temperature not exceeding 20°. If the films are dried at higher temperatures, they may subsequently exhibit a tendency to strip away from the glass surface on which they are deposited. Following drying, the films were fixed in 10% formalin at 4° for about 18 hr., washed several times with distilled water, and finally immersed for several minutes in a 1% NaCl solution and again dried. Such films will keep for several days. Fixation of hyaluronate films in buffered formalin resulted in a decreased intensity of staining with toluidine.

For orientative purposes the hyaluronate substrate film was exposed to testicular hyaluronidase solution[2] at a concentration of 1 mg./ml. in 0.02 M-acetate buffer, pH 4.5, the buffer solution alone serving as a control. Incubation with hyaluronidase led to removal of hyaluronic acid, the control solution exhibiting no modification in stainability of the film.

Chondroitin sulphate films were prepared in an analogous manner, using 1% solutions of commercial chondroitin sulphate (Light) and an equal volume of 5% aqueous gelatin.

Tissue sections. The material used was the testis of adult rats. The sections were mounted as for amylase, using the following gelatin mounting blocks: for hyaluronic acid, a 12.5% gelatin solution in 1% NaCl; for chondroitin sulphate, the same mounting medium was employed, but brought to pH 4.5 with 1 N-HCl before solidifying.

Control sections included testis inactivated by heating in boiling water; and fresh kidney. The latter was used in order to examine the possibility of some non-specific reaction of the substrate film with a fresh section containing no detectable hyaluronidase.

Incubation. For hyaluronic acid, incubation was for 40 - 50 min. at 20 - 22° and 20 - 25 min. at 28°. For chondroitin sulphate, 1 - 1.5 hr. at 28° was necessary.

Staining. The substrate films were stained with 1% solutions of toluidine blue at pH 6 for 15 min., and tissue sections as above for amylase.

Results. Identical localization patterns were obtained with both substrates (Fig. 3 and 4). The testicular enzyme was found mainly in the concentric layer of cells adhering to the inner surface of the base-

[2] We are indebted to Mrs. F. Rzendowska, of this laboratory, for the preparation of hyaluronidase according to the method of Madenaveitio [6].

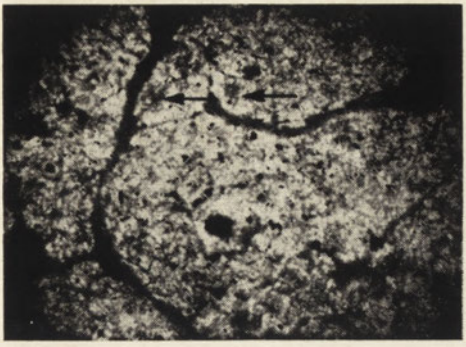

Fig. 1. Starch substrate film exposed to a section of rat pancreas for 10 min. at room temperature. Note the negative islands of Langerhans (arrows) (× 15)

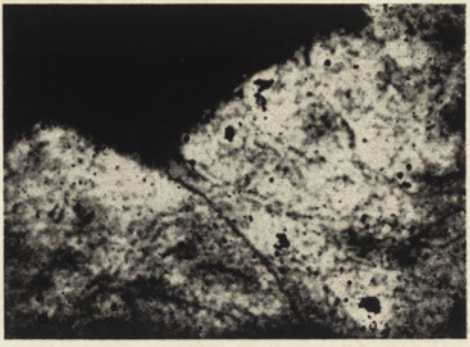

Fig. 2. Starch substrate film exposed to a section of rat parotid gland for 20 min. at 6° (× 20)

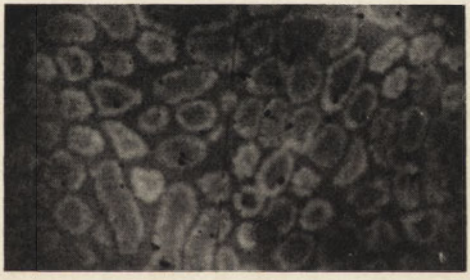

Fig. 3. Hyaluronate substrate film exposed to a section of rat testis for 20 min. at 28° (× 20)

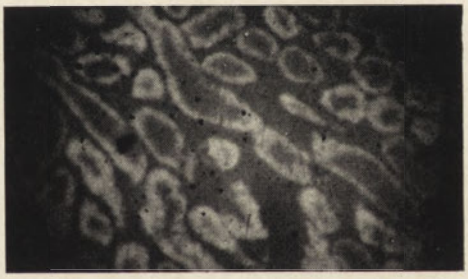

Fig. 4. Chondroitin sulphate substrate film exposed to rat testis section for 1 hr., at 28° (× 20)

ment membrane. Shorter incubation periods than those indicated above resulted in somewhat granular localization patterns, suggesting concentration of the enzyme in individual cells or clumps of cells. With prolonged incubation, a positive reaction was obtained in the entire layer of cells adhering directly to the basement membrane, with some localization of the enzyme in the tubule lumen. The enzyme appears to be most abundant in the cells adhering directly to the basement membrane, less so in the tubule lumen; while the intermediate layer, corresponding to spermatocytes, shows relatively low activity. The intertubular connective tissue, blood vessels and interstitial cells are all negative. Control, heat-inactivated, sections, as well as fresh kidney sections, were negative even after 24 hr. incubation.

Controls for potential protease interference were carried out as for amylase. No gelatin removal could be detected following incubation with testis sections under conditions similar to those for hyaluronidase localization. Prolonged incubation (2 hr.) did result in some removal of gelatin at sites corresponding to intertubular connective tissue, but not at all at those sites corresponding to hyaluronidase activity.

Incubation times could be shortened, and localization patterns somewhat improved, by increasing the gelatin concentration in the tissue supporting blocks. Activation by NaCl was also observed, in agreement with the known activating effect of NaCl on hyaluronidase activity *in vitro* [7]. Slight acidification of the tissue supporting blocks resulted in a small increase in the rate of hydrolysis of chondroitin sulphate; the failure to observe a similar increase in activity against hyaluronic acid may have been due simply to the fact that the latter substrate was more rapidly attacked.

Finally, the lack of any differences in localization for the two substrates cannot by any means be considered as conclusive, since the present method affords only gross histochemical, as contrasted to cytochemical, localization.

SUMMARY

A method is described for the gross histochemical localization of amylase and hyaluronidase, based on the use of the film-substrate technique.

The localization of amylase in rat pancreas and parotid gland, and of hyaluronidase in rat testis, has been investigated. The results are described.

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HISTOCHEMICZNA LOKALIZACJA HIALURONIDAZY I AMYLAZY PRZY UŻYCIU BŁON ZAWIERAJĄCYCH SUBSTRAT

Streszczenie

Opisano histochemiczną metodę lokalizacji amylazy i hialuronidazy przez zastosowanie błon substratowych. Przedstawiono lokalizację amylazy w trzustce i śliniance przyusznej szczura oraz lokalizację hialuronidazy w jądrze szczura.

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STUDIES ON THE CRABTREE EFFECT

II. FACTORS INHIBITING AND ENHANCING THE CRABTREE EFFECT IN HUMAN NEOPLASTIC HeLa CELLS

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The Crabtree effect, which is a characteristic feature of the metabolism of neoplastic cells, has been the subject of numerous investigations carried out with Ehrlich's ascites tumour cells [17, 12]. The occurrence of this effect in cells derived from tissue cultures, including not only neoplastic cells but also normal cells adapted to growth on glass, was described in a previous communication [13].

The Crabtree effect was considered from the standpoint of the regulation of cellular respiration. According to Chance & Williams [8, 9] the regulation of cellular respiration is associated with oxidative phosphorylation in mitochondria. A deficiency of ADP as phosphate acceptor for oxidative phosphorylation inhibits the transfer of electrons in the respiratory chain of the mitochondria. Chance & Hess [6, 7] observed that after adding glucose to ascites tumour cells suspension the respiration was stimulated for a very brief period of time, lasting about 1 min., and a typical inhibition of oxygen consumption followed. They assumed that the increase of oxygen consumption was due to ADP being released in amounts adequate to stimulate the respiratory chain of the mitochondria. After the stage of stimulation the stores of ATP at the site of phosphorylation of glucose became exhausted. Since they could not be easily replaced by ATP regenerated in mitochondria, the inhibition stage due to the lack of ADP developed.

Obviously, a knowledge of certain factors abolishing or enhancing the Crabtree effect might throw some light on the mechanism of this phenomenon. This paper presents the results of experiments carried out with compounds associated with the first stage of sugar metabolism, i.e. various simple sugars, insulin, sugar phosphates, ATP, and methylene blue which overcomes the Crabtree effect in Ehrlich's ascites

tumour cells [20]. The experiments were carried out with the HeLa line of cells derived from cancer of the uterine cervix. Some experiments were also performed with Ehrlich's ascites tumour cells.

MATERIALS AND METHODS

A description of the medium, technique of cultivation of HeLa cells, preparation of the material for the experiments, and methods of estimation of oxygen consumption, has been given in a previous communication [13]. All the reagents employed as substrates were of analytic grade. The following substrates were studied: glucose ("Motor", Poland), fructose (Pfanstiehl), mannose (B. D. H.), galactose (Merck), ribose, glucose-6-phosphate and fructose-1,6-diphosphate calcium salt (Light). The last one was dissolved in dilute acetic acid, and calcium ions were precipitated by an equivalent amount of sodium oxalate. The solution of fructose-1,6-diphosphate also served for preparing a mixture of phosphoglyceraldehyde and phosphodihydroxyacetone under the influence of aldolase (K. a. K. Laboratories Inc.). To 10 ml. of 30 mM-fructose-1,6-diphosphate solution 0.1 mg. of aldolase was added. Crystalline insulin (Tarchomińskie Zakłady Farmaceutyczne, Poland), crystalline ATP (Light) and methylene blue (B.D.H.), were also used. The final concentration of sugars in the incubation mixture was 10 mM. In the control experiments only Krebs-Ringer phosphate buffer was added instead of substrate.

Determinations of sugar utilization by the suspensions of HeLa cells were made by the method of Somogyi & Nelson [21, 18]. The number of cells in the studied suspensions was determined by counting in a Bürker haemacytometer.

RESULTS

The endogenous respiration of HeLa cells amounted to 10.7 µl. O_2 per 10^6 cells per hr. The effect of different substances on the respiration is shown in Table 1. Only three simple sugars elicited the Crabtree effect, namely glucose, causing a decrease of oxygen consumption to 8.2 µl., fructose (9.2 µl. O_2) and mannose (9.1 µl. O_2). Galactose did not affect endogenous respiration, and ribose even had an activating effect (12.6 µl. O_2). The reason for these differences may lie either in the different rates of phosphorylation of the sugars, or in the different rates of their penetration into the cells.

The rate of penetration of sugar into the cells is influenced, among others, by insulin [19, 22]. The Crabtree effect was increased when the experiments were performed in the presence of insulin and glucose

(Table 1). This effect was also confirmed in the experiments with Ehrlich's ascites tumour cells in which oxygen consumption was diminished from 13.5 to 4.8 µl. when insulin was added together with glucose This marked decrease of oxygen consumption was probably due to an increased rate of supply of glucose to the cells, although phosphorylation of sugar could also play a role.

Table 1

Oxygen consumption by neoplastic HeLa cells with various substrates

HeLa cells were suspended in Krebs-Ringer phosphate buffer, pH 7.4. Substrates dissolved in the same buffer were introduced from the side-arm after 20 min. incubation. The results represent mean values of oxygen consumption in µl. O_2 per 10^6 cells per hr. during the first 20 min. and after 1 and 2 hr. of incubation

Substrate	Number of determinations	Final concn. (mM)	µl. $O_2/10^6$ cells/hr. Time of incubation (min.)		
			0—20	20—60	60—120
Endogenous respiration (control)	16	—	12.7	10.7	8.8
Glucose	16	10		8.2	7.6
Fructose	4	10		9.2	8.2
Mannose	4	10		9.1	8.6
Galactose	4	10		10.5	9.3
Ribose	4	10		12.6	10.8
Glucose + insulin (0.1 unit)	4	10		4.6	5.4
Glucose-6-phosphate	4	10		9.5	8.4
Fructose-1,6-diphosphate	4	10		10.1	8.9
Fructose-1,6-diphosphate + aldolase	4	10		10.4	8.9
ATP	4	1		10.9	10.3
Glucose +ATP	4	10 1		13.5	13.5
Methylene blue	10	6.6×10^{-4}		9.2	9.2
Glucose + methylene blue	10	10 6.6×10^{-4}		18.2	30.9

To ascertain whether the phosphorylation of sugars influences the Crabtree effect, suspensions of HeLa cells were incubated with phosphorylated sugars (Table 1). Phosphate esters did not stimulate the respiration, but neither did they inhibit it like glucose. Inhibition of oxygen consumption by glucose-6-phosphate was much weaker (9.5 µl. O_2) than by glucose itself (8.2 µl. O_2), and fructose-1,6-diphosphate produced almost no change in endogenous respiration (10.1 µl O_2). The mixture of two phosphotrioses formed from fructose-1,6-diphosphate also failed to inhibit oxygen consumption.

For a correct interpretation of these results it was necessary to ascertain whether the phosphate esters of hexoses failed to elicit the Crabtree effect because they were not assimilated by the cells, or because their mode of action was different from that of glucose so that they could not disturb the equilibrium of adenylic nucleotides. Utilization of phosphorylated sugars incubated with cells was therefore determined and compared with the utilization of glucose (Table 2). It may be seen that phosphate esters of hexoses were utilized by HeLa cells even in larger quantities than glucose, without inhibiting oxygen consumption. This indicates that the Crabtree effect is indeed connected with the phosphorylation of sugars, and that its occurrence may be due to the reactions preceding the formation of fructose-1,6-diphosphate in which two ATP are used for one molecule of sugar. So the Crabtree effect would be connected with a local exhaustion of ATP, resulting in diminished production of ADP and lowered oxygen consumption.

Table 2

Utilization of some sugars by HeLa cells

The concentration of sugars in the incubation media was 10 mM

Substrate	Utilization of sugar (μmole/10^6 cells/hr.)
Glucose	0.9 - 1.4
Glucose-6-phosphate	1.5 - 1.9
Fructose-1,6-diphosphate	2.0 - 2.3

In accordance with the hypothesis of Chance & Hess [5] another experiment was performed in the presence of ATP, or ATP with glucose (Table 1). ATP alone did not affect the endogenous respiration of HeLa cells (10.9 µl. O_2), but in the presence of ATP and glucose the consumption of oxygen was markedly increased (13.5 µl. O_2). Not only was the Crabtree effect overcome, but the respiration was activated.

A similar although weaker effect was observed on Ehrlich's ascites tumour cells, particularly when the concentration of ATP was not less than 1 mM per 20×10^6 cells. Smaller concentrations of ATP (1 mM per 40×10^6 cells) did not overcome the Crabtree effect.

It can be supposed that the activation of respiration by ATP and glucose is connected with the release of ADP which activates the respiratory chain in the mitochondria. The electrons from the substrate can by-pass the main electron pathway if they find some other acceptor than oxygen. Methylene blue is such an acceptor causing oxidation of substrates by accepting electrons. If the decrease of respiration in the presence of glucose is connected in some way with the respiratory chain

then methylene blue can be expected to overcome this effect. The results of the experiment in which suspensions of HeLa cells were incubated with methylene blue alone or methylene blue and glucose are shown in Table 1.

Respiration was not stimulated by methylene blue alone, the oxygen consumption being even slightly inhibited (9.2 µl. O_2). On the other hand, methylene blue together with glucose not only overcame the Crabtree effect but even strongly stimulated the respiratory activity (18.2 µl. O_2). This experiment demonstrated that the Crabtree effect is connected not only with the phosphorylation of sugars, but also indirectly with the respiratory chain.

DISCUSSION

The observation indicating that only some of the simple sugars are able to inhibit oxygen consumption in Ehrlich's ascites tumour cells [17, 20] was confirmed on cells derived from tissue cultures. Sugars with a similar fate in the cells, namely glucose, fructose and mannose, elicited the Crabtree effect while galactose and ribose failed to do so. These results supplement the findings of Eagle *et al.* [11] who found that for HeLa cells, besides glucose, only fructose, maltose and mannose can be used as sources of energy for growth and reproduction. Galactose cannot be employed for this purpose, and ribose enhances the growth of only some strains of the HeLa cells.

For several years the Crabtree effect has been studied and variously interpreted. The view that it is connected with a deficiency of inorganic phosphate in the cells may be neglected since phosphate can penetrate into the cells from the medium [23, 15]. The results of the experiments with iodoacetate and 2-deoxyglucose [14, 24] contradict the view that the Crabtree effect is connected with acidification of the intracellular medium [3, 10]. It seems, therefore, that the Crabtree effect originates already during the first stages of sugar metabolism, i.e. the penetration of sugars into the cells or their phosphorylation, and not during the later stages, e.g. the production of lactic acid.

This assumption is supported by the experiments on the influence of insulin on the incidence of the Crabtree effect. The influence of insulin on sugar metabolism can be attributed to three different mechanisms, among which particular importance is attributed to the action of insulin on the permeability of cell membranes and the phosphorylation of sugars [22]. If the Crabtree effect is connected with at least one of these mechanisms, then the increase of the effect in the presence of glucose and insulin becomes clear. The influence of insulin on the intensity of the Crabtree effect has not been investigated so far; several authors [2, 16] have only described an increase of respiration

in some normal tissues in the presence of glucose and insulin. The presented experiments indicate that the Crabtree effect is very strongly enhanced in the presence of insulin. Thus it seems that either the penetration of sugar through the cell walls, or its phosphorylation are decisive for the appearance of the effect.

The importance of phosphorylation for the appearance of the Crabtree effect is shown by the fact that phosphate esters of sugars do not elicit the Crabtree effect. Brin & McKee [4] in experiments on Ehrlich's ascites tumour cells also noted a lack of inhibition of oxygen consumption by phosphate esters of hexoses, which they attributed to the poor assimilation of these esters. The presented experiments, however, show that the sugar esters disappear from the medium where HeLa cells are present. These data suggest that the Crabtree effect is connected with reactions preceding the formation of fructose-1,6-diphosphate and with the phosphorylation of sugars.

This assumption is also supported by the results of experiments on HeLa cells with added glucose and ATP. The addition of ATP alone did not change the endogenous respiration, but ATP together with glucose not only overcame the Crabtree effect but even stimulated the respiration. Brin & McKee [4] in analogous experiments with ascites tumour cells did not notice any influence of ATP on the Crabtree effect. The only possible explanation of this discrepancy is the much greater intensity of the Crabtree effect in ascites tumour cells as compared with HeLa cells, and a more difficult inhibition of the phenomenon. In fact, using greater concentrations of ATP it was possible to abolish the Crabtree effect also in ascites tumour cells.

It is possible that ATP enters into reactions on the surface of cells. By taking part in the phosphorylation of sugars on the cell surface ATP might spare the intracellular stores of ATP; or, ATP might be decomposed by ATPase present in the cell wall into products more easily penetrating into the cell and activating the respiration. Acs *et al.* [1] demonstrated that in neoplastic cells almost all ATPase activity is located in the cell wall. However, since the addition of ATP alone, without glucose, does not change the endogenous respiration, it can be assumed that the activation of respiration depends on the presence of glucose, probably by way of phosphorylation. Inclusion of exogenous ATP into the reaction of phosphorylation of glucose could also spare the intracellular stores of adenylic nucleotides besides releasing certain amounts of ADP for the activation of respiration.

The experiments of Chance & Hess [5, 6, 7] on the Crabtree effect in ascites tumour cells and their conclusions are of particular interest. According to these writers the first brief stage of stimulation of respiration is a result of phosphorylation of glucose with liberation of ADP,

which, on penetrating into mitochondria, activates the respiratory chain. The affinity of ATP to mitochondria, according to this view, is so great that glycolysis can not cope with it. The stage of inhibition which follows the phase of stimulation is attributed to the retention of ATP in the mitochondria and to a diminished supply of ADP to the mitochondria for further oxidative phosphorylation. The supply of ADP to mitochondria must be balanced by the transfer of ATP from the mitochondria to the cytoplasm where the phosphorylation of sugars takes place. When the restoration of ATP to the cytoplasm is outbalanced by the flow of ADP into the mitochondria, the addition of exogenous ATP may restore the balance.

The fact that the Crabtree effect is overcome by the addition of ATP to the medium supports the view of Chance & Hess and also the assumption that the appearance of the Crabtree effect is connected with the phosphorylation of sugars. Nevertheless, this is not the only mechanism initiating the Crabtree effect. Some of the data, e.g. from the experiments with methylene blue, seem to indicate that the starting point of the effect may lie in the respiratory chain. The same can be inferred from experiments by authors who studied the agents uncoupling the oxidative phosphorylation [14, 5, 6].

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SUMMARY

The Crabtree effect was studied on human neoplastic HeLa cells from tissue cultures. Besides glucose, fructose and mannose elicited this effect. No influence of galactose and ribose was observed. Insulin enhanced the effect of glucose. Glucose-6-phosphate, fructose-1,6-diphosphate and a mixture of phosphotrioses were without effect. Phosphate esters of hexoses were utilized by the cells.

ATP alone had no effect on endogenous respiration but together with glucose markedly increased the oxygen consumption. Methylene blue alone inhibited, but in the presence of glucose markedly enhanced the respiration.

It is concluded that the Crabtree effect is initiated by the penetration of sugar into the cell and its phosphorylation. This stage is connected with the respiratory chain through the adenylic nucleotides.

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BADANIA NAD EFEKTEM CRABTREE

II. CZYNNIKI HAMUJĄCE I WZMACNIAJĄCE EFEKT CRABTREE W KOMÓRKACH NOWOTWORU LUDZKIEGO HeLa

Streszczenie

Badano efekt Crabtree w komórkach nowotworu ludzkiego HeLa, pochodzących z hodowli tkankowej. Zużycie tlenu hamowała glikoza, fruktoza i mannoza; galaktoza i ryboza nie miały wpływu. Insulina podana wraz z glikozą znacznie zwiększała efekt Crabtree. Glikozo-6-fosforan, fruktozo-1,6-dwufosforan i mieszanina fosfotrioz nie wywierały wpływu na oddychanie, mimo że były zużywane przez komórki.

ATP podany sam nie zmieniał oddychania endogennego komórek, podany wraz z glikozą zwiększał zużycie tlenu. Błękit metylenowy hamował oddychanie, lecz w obecności glikozy bardzo znacznie zwiększał zużycie tlenu.

Przeprowadzone doświadczenia wskazują, że momentem zapoczątkowującym efekt Crabtree jest wniknięcie cukru do komórki i jego fosforylacja. Etap ten wiąże się z łańcuchem oddechowym poprzez nukleotydy adenilowe.

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A COMPARISON OF CHEMICALLY AND ENZYMICALLY PREPARED *Se*-ADENOSYLSELENOMETHIONINE

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In 1957 Mudd & Cantoni [17] found that during incubation of DL-selenomethionine with the enzyme activating methionine, adenosine triphosphoric acid and pyrophosphatase, a selenium analogue of active methionine is probably formed, namely (-)*Se*-adenosyl-L-selenomethionine.

However, the enzymic synthesis of active selenomethionine and the demonstration of its activity in transmethylation [2, 17] are not sufficient proofs of its structure. To confirm the chemical structure of active selenomethionine a method of chemical synthesis of this compound was developed [27]. In this paper some of the physico-chemical properties and the activity of the chemical preparation as a donor of methyl groups, were compared with the preparation obtained by the enzymic method according to Mudd & Cantoni [17]. Confirmation of the structure of active selenomethionine as an analogue of active methionine [4] is of considerable interest in studies on the metabolism of biologically active selenium analogues of sulphur amino acids and on the role of selenium as trace element in living organisms [22, 24, 25].

METHODS

Se-*adenosyl-5'*-DL-*selenomethionine*. This compound was synthesized by an adaptation of the method of chemical preparation of active methionine [27]. The product was stored at -30°.

Enzymic synthesis of active selenomethionine. The method of Mudd & Cantoni [17] was used. DL-Selenomethionine obtained according to Painter [19] was incubated for 3 hr. at 37° with the enzyme activating methionine, and ATP. Active selenomethionine was isolated as reineckate, purified according to De la Haba & Cantoni [7], and after lyophilization stored at -30°.

The enzyme activating methionine was obtained from fresh yeast (*Saccharomyces cerevisiae*) and purified according to Mudd & Cantoni [18]. Its activity was measured after Cantoni & Durell [5] by estimating at 260 mμ the amount of active methionine formed from L-methionine.

Estimation of adenine and pentose. In preparations of active selenomethionine dissolved in tris buffer, pH 5, the extinction was measured at 260 mμ. Molar extinction of adenine was assumed to be 16×10^3. Pentose was estimated by the method of Mejbaum [13] as modified by Waldvogel & Schlenk [29] employing an aqueous solution containing 0.065 μmole of active selenomethionine, and making the colorimetric measurements at 660 mμ. The amount of pentose was calculated from a standard curve for D-ribose (La Roche Co.).

Estimation of selenium and nitrogen. Selenium was estimated by the method of Gortner & Lewis [10] with codeine sulphate. Samples of active selenomethionine were digested with conc. H_2SO_4 (free of selenium) and after reaction with codeine sulphate examined colorimetrically at 570 mμ. The selenium content was calculated from a calibrated curve plotted for selenium (Merck) solutions in concentrations from 0.05 to 0.6 mg. Se/100 ml. Nitrogen was assayed by the Kjeldahl micro-method according to Ma & Znazaga [11].

Oxidation with HIO_4 and reaction with Schiff's reagent. A solution of HIO_4 was prepared by dissolving 0.5 g. of paraperiodic acid (H_5IO_6) in 100 ml. H_2O [26]. To 2 ml. of HIO_4 solution one drop of conc. HNO_3 was added, followed by one drop of aqueous solution of active selenomethionine. The mixture was agitated during 10 - 15 sec., and 1 - 2 drops of 3% aqueous solution of $AgNO_3$ were then added. Formation of a white precipitate of $AgIO_3$ indicated the oxidation of active selenomethionine; $AgIO_4$ is readily soluble in HNO_3, whereas the solubility of $AgIO_3$ is negligible. The oxidation product of active selenomethionine gave a positive reaction with Schiff's reagent [30].

R_F values. Chromatographic analysis of the preparations of active selenomethionine (approx. 6 μg.) was performed on Whatman no. 1 filter paper by the ascending technique. The chromatograms were developed according to Parks [20] in a mixture of ethanol - acetic acid - - water (65 : 1 : 34, by vol.) at room temperature, during 16 - 18 hr.; after drying at 40 - 50°, the spots were localized with 0.3% solution of ninhydrin in anhydrous acetone.

Paper electrophoresis. A sample of an aqueous solution containing 10 μmoles of active selenomethionine was applied in the middle of a strip of Whatman no. 4 filter paper. Electrophoresis was carried out according to Markham & Smith [12] employing acetate buffer, pH 7.0, and a current of 200 V during 3 hr. The electrophorograms were stained with 0.3% solution of ninhydrin in anhydrous acetone. By developing

the electrophorograms after different intervals of time, the electrophoretic mobility of the preparation was determined.

Acid and alkaline hydrolysis and identification of the products of decomposition. Acid hydrolysis of active selenomethionine (10 μmoles) was carried out in 20 ml. of 0.1 N-HCl at 100°, under reflux over 2 hr. The hydrolysate was then concentrated under reduced pressure (12 mm. Hg) until nearly dry; the residue was treated with 2 ml. water and concentrated again. The residue was dissolved in 2 ml. of 10% aqueous solution of isopropanol containing 2 - 3 drops of conc. HCl. Portions of 10 μl. were chromatographed as before. The chromatograms were stained with an acetone solution of $Cu(NO_3)_2$ according to Bode [1].

The hydrolysis with 0.1 N-NaOH was carried out in conditions analogous to acid hydrolysis except that the concentrated hydrolysate was neutralized with solid versenic acid (B.D.H.) to complex the sodium ions, the presence of which is unfavourable for chromatographic separation.

Identification of the products of hydrolytic decomposition of active selenomethionine was carried out by comparing their R_F values with those of parallelly chromatographed standards of adenine (B.D.H.), DL-homoserine (Schwartz Lab. Chem.), and DL-selenomethionine (prepared chemically). Adenine was identified on the chromatograms according to Gerlach & Döring [9], and amino acids with ninhydrin.

Absorption spectrum in ultraviolet light. The spectrum of active selenomethionine was determined in a Unicam SP-500 spectrophotometer using a 6.0×10^{-5} M solution in universal tris buffer, pH 7.0 [6].

Transmethylation activity. The activity of active selenomethionine as a donor of methyl groups was determined by means of the enzymic reaction in which nicotinamide was the acceptor. *S*-Adenosylmethionine : nicotinamide methyltransferase was isolated from rat liver and purified according to Cantoni [3]. The extract of this enzyme contained 27.2 mg. protein per 1 ml. of 0.015 M-tris buffer, pH 7.4.

The reaction mixture consisted of 2.6 μmoles of $MgCl_2$ (chemically pure), 0.2 ml. of enzymic extract, 1.04 - 0.72 μmoles of active selenomethionine, and 1.3 μmoles of nicotinamide. The volume of the mixture was brought to 1 ml. with 0.015 M-tris buffer, pH 7. After 30 min. incubation at 38° the reaction was stopped by adding 1.5 ml. of 7% solution of $HClO_4$. The mixture was centrifuged and the amount of unreacted active selenomethionine in the supernatant was determined. Samples of the solution (1 - 1.5 ml) were applied on a Dowex 50×8 H^+ (100 - 200 mesh, Dow Chem. Co.) column, 7 mm. in diameter, containing a 3 cm. layer of resin treated previously according to Stein [28]. The column was washed at 0° with 2 N-H_2SO_4 until the extinction of the

eluate at 256 mμ fell below 0.01. Under these conditions *Se*-adenosylselenohomocysteine, among others, was removed from the column. Active selenomethionine was next eluted with 6 N-H_2SO_4. Like active methionine [23], the selenium analogue was eluted almost at once. The extinction of the effluent at 256 mμ was checked; and the fall of extinction below 0.02 indicated the end of elution. In the 6 N-H_2SO_4 fraction the concentration of active selenomethionine was determined at 260 mμ (E_M 16 × 10^3).

RESULTS AND DISCUSSION

The supposition of Mudd & Cantoni [17] that a selenium analogue of active methionine is formed enzymically from selenomethionine and ATP, was confirmed.

Table 1

Characteristics of Se-adenosylselenomethionine obtained by chemical or enzymic synthesis

	Preparation	
	Chemical	Enzymic
Selenium (%)	17.59	17.64
Total nitrogen (%)	18.58	18.69
Adenine to pentose ratio	1.09	1.07
Oxidation with HIO_4 and reaction with Schiff's reagent	positive	positive
Mobility in paper electrophoresis, pH 7 ($cm.hr.^{-1}/v.cm.^{-1}$)	0.4	0.4
Products of acid hydrolysis	adenosine homoserine	adenosine homoserine
Products of alkaline hydrolysis	adenine homoserine selenomethionine	adenine homoserine selenomethionine
Maximum absorption in UV	260 mμ	260 mμ

The data presented in Table 1 show that the enzymic preparation of active selenomethionine and chemically synthesized *Se*-adenosyl-5′-selenomethionine possess identical properties. Within the limits of error of the methods used, assays of selenium and nitrogen gave results coinciding with the theoretical content. The molar ratio of adenine to pentose (1 : 1) constitutes further confirmation of the structure of the enzymically obtained active selenomethionine. Both preparations gave positive reactions with HIO_4 and Schiff's reagent. As a result of oxidation with HIO_4 the carbonyl group was obviously formed at the sensitive

point in the molecule (C-5′ and the center of the selenonium configuration), since the oxidation product reacted with Schiff's reagent which is selective for carbonyl groups. This permits to conclude that in the molecules of both preparations of active selenomethionine the amino acid is identically bound with C-5′ of the nucleoside part.

Paper chromatographic analysis showed that the enzymic and chemical preparations of active selenomethionine possess identical R_F values. They also have the same electrophoretic mobility (0.4 cm.hr.$^{-1}$/v.cm.$^{-1}$). The rate of migration of active selenomethionine toward the cathode indicates strong electrophilic properties of this compound.

Further proof confirming the chemical structure of active selenomethionine as an analogue of active methionine was obtained by identification of the products of acid and alkaline hydrolysis of both preparations. Paper chromatography of the hydrolysates showed in both cases the same products of decomposition; the acid hydrolysate contained adenine and homoserine, and the alkaline hydrolysate adenine, homoserine and selenomethionine. According to data from the literature [14, 21] under analogous conditions of hydrolysis active methionine gives the same products, with the addition of *S*-methylthioadenosine, in the acid hydrolysate. Because of the lack of a standard of *Se*-methylselenoadenosine this compound was not identified. It was further found that the chemically and enzymically obtained active selenomethionine have identical absorption spectra in UV light with a maximum at 260 mμ. The spectrum did not differ from that of active methionine analysed under the same conditions.

Table 2

Transmethylation activity of enzymic and chemical preparations of active selenomethionine

The incubation mixture contained methyltransferase prepared from rat liver, active selenomethionine as donor, and nicotinamide as acceptor of methyl groups. Technical details are described in Methods. The values are means of three parallel determinations

Active selenomethionine			Activity of chemical preparation (% of activity of enzymic preparation)
Added (μmoles)	Utilized (μmoles)		
	Enzymic preparation	Chemical preparation	
1.037	0.392	0.202	51.5
0.905	0.373	0.179	47.9
0.875	0.553	0.288	52.1
0.812	0.651	0.329	50.5
0.716	0.707	0.347	49.1

According to Mudd & Cantoni [17] selenomethionine, like methionine, is a donor of methyl groups. The transmethylating activity of both preparations is shown in Table 2. The enzymically prepared active selenomethionine was two times more active as a donor of methyl groups than the chemical product. It seems very probable that the methyltransferase of nicotinamide is selective for only one form of diastereoisomeric active selenomethionine, namely the one which is formed enzymically. From the chemically obtained racemate probably only one optical antipoda (L-form) is involved in the transmethylation. These suggestions are supported by the results of studies on the transmethylating activity of different isomers of active methionine [8, 15, 16] which showed that only the isomer (-)*S*-adenosyl-L-methionine is a donor of methyl groups in animals, plants and microorganisms.

SUMMARY

Enzymically and chemically obtained preparations of active selenomethionine were compared. Content of selenium, nitrogen, ribose and adenine were found to be identical. The R_F value in paper chromatography, the mobility in paper electrophoresis, the ultraviolet spectrum, and the products of acid and alkaline hydrolysis were the same.

During enzymic methylation of nicotinamide the enzymic preparation was two times more active as a donor of methyl groups than the chemical product. This is explained as being due to the specificity of methyltransferase for the L-form of active selenomethionine.

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PORÓWNANIE SYNTETYCZNEJ *Se*-ADENOZYLOSELENOMETIONINY ZE ZWIĄZKIEM OTRZYMANYM ENZYMATYCZNIE

Streszczenie

Porównano preparat aktywnej selenometioniny otrzymanej enzymatycznie z preparatem syntetyzowanym chemicznie. Stwierdzono taką samą zawartość selenu, azotu całkowitego, rybozy i adeniny. Wartości R_F w chromatografii bibułowej, ruchliwość w elektroforezie bibułowej, widmo spektralne w ultrafiolecie i produkty hydrolizy kwaśnej oraz zasadowej były identyczne.

Natomiast w reakcji enzymatycznej metylacji amidu kwasu nikotynowego preparat otrzymany enzymatycznie był 2 razy bardziej aktywny jako dawca grupy metylowej od preparatu otrzymanego chemicznie. Tłumaczy się to specyficznością metylotransferazy dla L-formy aktywnej selenometioniny.

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STUDIES ON LUPIN PROTEINS

V. C-TERMINAL AMINO ACIDS IN CONGLUTIN β

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In the previous report [12] it has been shown that in the conglutin β the glutamic acids are the *N*-terminal amino acids, and some analogy in the structure of all the polypeptide chains of conglutin β has been pointed out. This paper deals with the determinations of C-terminal amino acids by means of the carboxypeptidase [5, 4] and hydrazinolysis [1, 8] methods.

MATERIAL AND METHODS

Protein. The same preparation of conglutin β isolated from the seeds of *Lupinus angustifolius*, in which *N*-terminal amino acids had been studied [12], was used. To confirm the homogeneity of the preparation, besides the previously reported paper electrophoresis in veronal buffer, pH 8.6, additional paper electrophoresis in acetate buffer, pH 4.7, and phosphate buffer, pH 7.0, and immunoelectrophoresis [Scheidegger, 9] were performed. The anti-conglutin β serum was obtained from rabbits injected intramuscularly with increasing doses of 1% conglutin β solution. The injections were made during a fortnight, every second day with one week's pause.

The results of all the tests indicated that the studied protein is homogeneous (Fig. 1, 2, 3).

Reagents. Carboxypeptidase crystallized 6 times was received as an aqueous suspension from Prof. Dr. J. Pliwa, Prague. Small amounts of free amino acids, especially serine and glycine, were found in the preparation. This could be due to partial autolysis which had probably occurred during transport.

Anhydrous hydrazine was distilled twice over NaOH, heated for 3 hr. with BaO under reflux and redistilled. Benzaldehyde was freshly

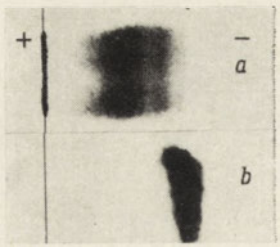

Fig. 1. Paper electrophoresis of conglutin β. (*a*), Crude preparation, (*b*), pure preparation. Acetate buffer, I 0.25, pH 4.7, 10 v/cm, 6 hr.

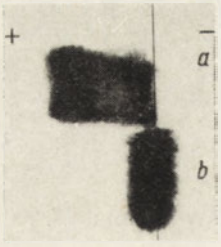

Fig. 2. Paper electrophoresis of conglutin β. (*a*), Crude preparation, (*b*), pure preparation. Phosphate buffer 0.05 M, pH 7.0, 10 v/cm., 6 hr.

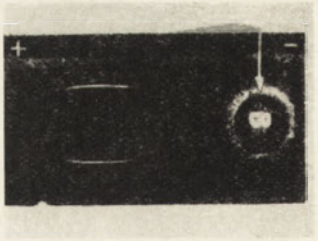

Fig. 3. Immunoelectrophorogram of conglutin β. Phosphate buffer 0.05 M, pH 7, 10 v/cm., 6 hr.

distilled under reduced pressure. Fluoro-2,4-dinitrobenzene was L. Light, England; amino acids, La Roche, Switzerland; other reagents, Biuro Obrotu Odczynnikami, Poland.

Amino acids liberated by carboxypeptidase

Conglutin β was dissolved in 25 ml. of $NaHCO_3$ and NaOH mixture (10 ml. water, 1 ml. 1% $NaHCO_3$ and 3 ml. 0.1 N-NaOH, adjusted to pH 8.0 with 0.1 N-HCl). The enzyme was dissolved at 0-4° in the same mixture. The amounts of incubated conglutin β and carboxypeptidase, time and temperature of the incubation in individual experiments are presented in Table 1. For amino acids analysis 5-ml. samples were taken and deproteinized with 10% (v/v) trichloroacetic acid (TCA). TCA was removed from the filtrate by extraction with ether and the aqueous layer was concentrated under reduced pressure to about 0.1 ml.

Acidic and basic amino acids were identified by high voltage paper electrophoresis in pyridine buffer, pH 5.85 [Masłowski, 7]. The neutral

Table 1

Conditions of hydrolysis of conglutin β by carboxypeptidase

Conglutin β (mg.)	Enzyme (mg.)	Temp.	Time of incubation				
					(hours)		
102.6	1.6	25°	1	2	3	4	7
177.1	1.6	40°	1.5	5.4	—	—	—
					(minutes)		
219.0	0.8	35°	10	20	40	70	120

amino acids after elution from the electrophorogram were separated two-dimensionally (Fig. 5) by a combination of electrophoresis in acetate buffer, pH 2.0 [7] and paper chromatography in butanol - acetic acid - - water (4 : 1 : 5, by vol.). Neutral amino acids eluted from the electrophorogram in pyridine buffer, were also hydrolysed for 3 hr. in 2 N-HCl at 120°. The acid was evaporated under vacuum and the hydrolysate submitted to electrophoresis.

Quantitative analysis

Solution of 208.17 mg. of conglutin β (25 ml.) was incubated with 1.6 mg. of carboxypeptidase at 35°. After 1, 2, 3, 4 and 7 hr., respectively, 5-ml. samples were taken and deproteinized with TCA.

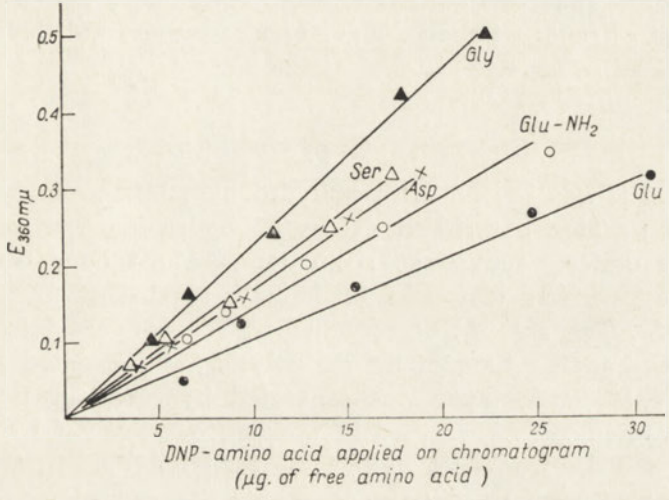

Fig. 4. Standard curves for amino acids determination by fluorodinitrobenzene [6]. Standard solutions of DNP-amino acids were chromatographed on paper, eluted with 10 ml. of 1% $NaHCO_3$ and the extinction measured at 360 mμ

Standard functions equations:

Asp,	$E = c \times 0.0168 - 0.003$	$c/E = \text{const.} \pm 1.31\%$
Glu,	$E = c \times 0.010 + 0.0058$	$c/E = \text{const.} \pm 14.63\%$
Gly,	$E = c \times 0.0226 + 0.0026$	$c/E = \text{const.} \pm 3.47\%$
Glu-NH_2,	$E = c \times 0.0123 + 0.0312$	$c/E = \text{const.} \pm 2.18\%$
Ser,	$E = c \times 0.0176 + 0.0029$	$c/E = \text{const.} \pm 6.01\%$

E, Extinction of a 2 cm. thick layer; c, concentration of DNP-amino acids (μg. per 10 ml.) calculated as free amino acids.

Amino acids were determined as reported by Levy [6]. 3 ml. of the filtrate was neutralized with 1 N-NaOH; then 1 ml. $NaHCO_3$ (8.4 g. in 100 ml. water) and 1 ml. fluorodinitrobenzene (1.88 g. in 50 ml. ethanol)

were added. The mixture was shaken for 2 hr. at 40°, then acidified with 1 N-HCl, and the dinitrophenyl compounds (DNP-compounds) were exhaustively extracted with ether. Ether was then evaporated under vacuum and the excess of fluorodinitrobenzene and dinitrophenol were removed by vacuum-sublimation technique (1 hr., at 60°, 0.01 mm. Hg). The residue was dissolved in 5 ml. of acetone.

Two-dimensional chromatography was applied to the DNP-amino acids solution (2 ml. samples). Ascending technique in 1% ammonia saturated butanol was applied for 16 hr. [2] in the first run, and in the second, descending run 1.5 M-phosphate buffer, pH 6.0, for 14 hr. [6]. The chromatogram was then dried, the coloured spots of DNP-amino acids were cut out and eluted with 10 ml. of 1% NaOH. The extinction of the extract was measured at 360 mμ in 2 cm. cuvettes with a Zeiss' spectrophotometer. The content of amino acids was calculated according to the standard functions equation based on standard amino acids solutions treated as reported above. The standard curves and their equations are presented in Fig. 4.

Hydrazinolysis of conglutin β

Conglutin β was heated with anhydrous hydrazine in sealed tubes on a boiling water bath. The excess of hydrazine was removed by evaporation under vacuum at room temperature over conc. H_2SO_4. The dry residue was dissolved in water and shaken for 2 hr. with benzaldehyde. The amount of protein and reagents used in individual experiments, and the time of hydrazinolysis are presented in Table 2.

Next the aldehyde layer was separated by centrifugation and the free amino acids were examined in the aqueous layer. In experiments no. 1, 2, 3 the dinitrophenol method as reported by Niu & Fraenkel-Conrat [8] was used. In other experiments the amino acids were examined by electrophoresis in pyridine buffer [7] directly after separation from hydrazides.

Table 2

Hydrazinolysis of conglutin β

Expt. no.	Protein (mg.)	Hydrazine (ml.)	Time (hr.)	Water used for dissolving the dry residue (ml.)	Aldehyde used for removing hydrazides (ml.)
1	64.3	0.5	6	1	0.5
2	167.4	1.0	10	2	1.0
3	31.1	0.5	22	1	0.5
4	29.1	1.0	30	1	0.5
5	32.9	1.0	15	1	0.5
6	18.9	0.5	6	1	0.5
7	306.1	5.0	15	5	2.5

The peptides from the hydrazinolysate separated by paper electrophoresis (Fig. 7) were eluted, purified again by electrophoresis and hydrolysed with 6 N-HCl in sealed tubes at 100° for 24 hr. The acidic and basic amino acids of hydrolysates were examined by paper electrophoresis in pyridine buffer, pH 5.85. The neutral amino acids after elution from the electrophorogram were separated by chromatography on Whatman no. 1 paper in butanol - acetic acid - water (4 : 1 : 5, v/v) for about 16 hr., using the descending technique.

To prove that the fraction *h* (Fig. 7) is not formed from arginine during heating with hydrazine, 30 mg. of arginine was heated with 1 ml. of hydrazine for 12 hr. at 100°. The excess of hydrazine was evaporated, the residue dissolved in water, shaked with benzaldehyde, and the aqueous solution submitted to paper electrophoresis.

RESULTS

After 2 hr. incubation of conglutin β with carboxypeptidase the following amino acids were found: aspartic acid, glutamic acid, glutamine, small amounts of serine and glycine, and traces of valine, leucine and threonine (Fig. 5). After 10 min. incubation the amounts of glycine

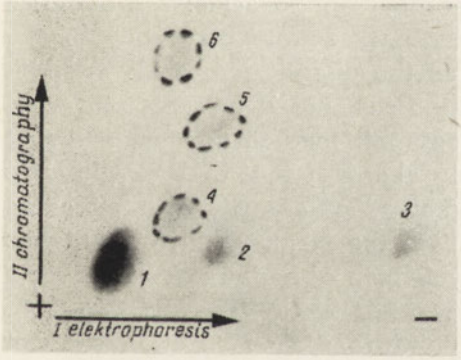

Fig. 5. Electrophoresis and paper chromatography of neutral amino acids liberated from conglutin β incubated with carboxypeptidase. (*I*), Electrophoresis in acetate buffer [7], pH 2.0, 100 v/cm., 165 min. (*II*), Ascending paper chromatography in butanol - acetic acid - water (4 : 1 : 5, by vol.). (*1*), Glutamine, (*2*), serine, (*3*), glycine, (*4*), threonine, (*5*), valine, (*6*), leucine

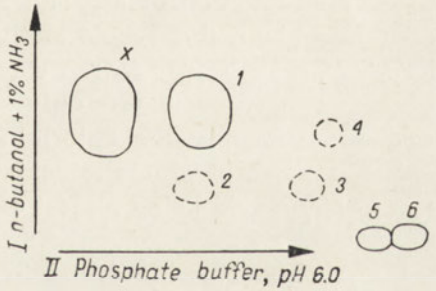

Fig. 6. Chromatogram of the DNP--derivatives of the hydrazinolysis products of conglutin β. (*I*), Ascending run in *n*-butanol - 1% NH_3; (*II*), descending run in 1.5 M-phosphate buffer, pH 6. (*1*), DNP-OH, (*2*), DNP--glycine, (*3*), DNP-serine, (*4*), DNP--threonine, (*5*), DNP-glutamic acid, (*6*), DNP-aspartic acid, (*X*), mixture of DNP-peptides

and serine were larger than those of the other amino acids. A small amount of arginine appeared additionally after 7 hr. incubation. The results of quantitative analysis are presented in Table 3.

The hydrazinolysate of conglutin β after treatment with fluorodinitrobenzene contained aspartic acid, DNP-glutamic acid, and dinitrophenol, very small amounts of DNP-glycine, DNP-serine and DNP-threonine, and a rather large amount of a substance with an R_F not corresponding to any amino acids of conglutin β (Fig. 6).

The electrophoresis carried out directly on the hydrazinolysate of conglutin β revealed small amounts of glutamic and aspartic acids and the presence of 8 peptide fractions (Fig. 7). The same was stated when conglutin β was heated with hydrazine for 6, 15 or 30 hr. and when the hydrazinolysate was shaken twice for 2 hr. with benzaldehyde. The approximate content of amino acids in the polypeptide fraction obtained by hydrazinolysis of conglutin β is presented in Table 4.

In the hydrolysates of all the peptide fractions (except fraction a) an alkaline substance with a higher mobility than ornithine was found. In alkaline or neutral medium this substance (Fig. 8, spot z) was decomposed to 3-aminopiperidon-2 and ornithine (Fig. 8, c). Both these substances were found as the products of hydrazinolysis of arginine [3].

DISCUSSION

The results obtained by the carboxypeptidase method suggest the presence in conglutin β of four C-terminal amino acids: 2 glutamine, 1 aspartic acid and 1 glutamic acid. Some doubts may concern the C-terminal position of aspartic acid, as the quantitative analysis revealed only about 0.75 µmole of this amino acid per 1 mole protein. This, however, may be due to the chromatographic method applied. The spot of DNP-aspartic acid was only slightly ahead of that of DNP-glutamic acid and probably the determined amounts of aspartic acid were too low and those of glutamic acid too high, the mean value for glutamic and aspartic acid being almost exactly 1 mole of amino acid per 1 mole protein.

Glycine and serine found in the mixture after incubation can not be considered as C-terminal amino acids; they resulted probably from autolysis of carboxypeptidase. After 10 min. incubation the amount of those amino acids was larger than that of any other amino acid but did not increase with the time of incubation. The preparation of carboxypeptidase was found to contain glycine and serine, but the amount of enzyme in our possession was not sufficient for quantitative analysis of these two amino acids.

Hydrazinolysis confirmed the presence of C-terminal aspartic and glutamic acids. Only these two amino acids were found in the conglutin β hydrazinolysate together with several peptides (glutamine formed with hydrazine a hydrazide which has been removed by shaking with benzaldehyde).

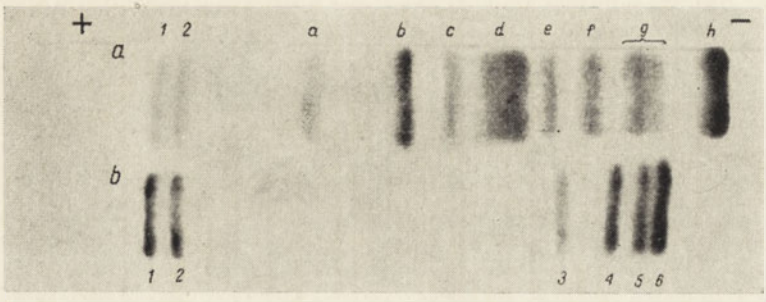

Fig. 7. Paper electrophoresis in pyridine buffer, pH 5.85, 80 v/cm., 90 min. (*a*), Hydrazinolysate of conglutin *β*, (*b*), standard mixture of amino acids. (*1*), Aspartic acid, (*2*), glutamic acid, (*3*), histidine, (*4*), arginine, (*5*), lysine, (*6*), ornithine, (*a* - *h*), peptide fractions

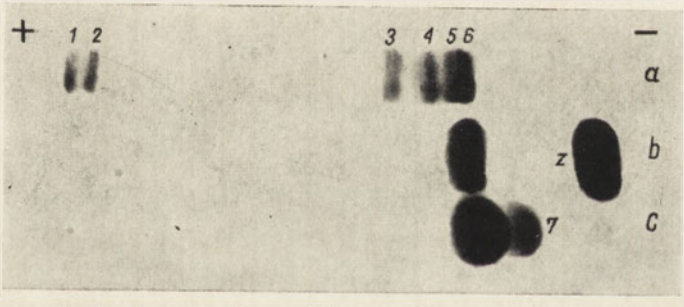

Fig. 8. Paper electrophoresis in pyridine buffer, pH 5.85, 80 v/cm., 45 min. (*a*), Standard mixture of amino acids; (*b*), acid hydrolysate of the peptide fraction *h* (Fig. 7) of hydrazinolysate of conglutin *β*; (*c*), decomposition products of the spot *z* in neutral medium. (*1*), Aspartic acid, (*2*), glutamic acid, (*3*), histidine, (*4*), arginine, (*5*), lysine, (*6*), ornithine, (*7*), 3-aminopiperidon-2, (*z*), one of the products of hydrolysis of peptide *h*

Table 3

Amino acids found after incubation of conglutin β with carboxypeptidase

For extinction, mean values of 2 parallel samples, ± 2.4% are given. The amounts of amino acids were calculated according to standard functions equations (Fig. 4).

Amino acid	Incubation time (hr.)					Incubation time (hr.)					Incubation time (hr.)				
	1	2	3	4	7	1	2	3	4	7	1	2	3	4	7
	Extinction					g./mole protein					mole/mole protein				
Aspartic acid	0.059	0.100	0.082	0.090	0.080	67.20	111.40	92.02	109.23	89.85	0.50	0.84	0.69	0.82	0.68
Glutamic acid	0.072	0.082	0.098	0.098	0.106	120.10	138.12	167.73	167.73	181.68	0.82	0.94	1.14	1.14	1.23
Glutamine	0.168	0.229	0.224	0.211	0.230	201.79	291.82	284.39	265.19	293.27	1.38	2.00	1.95	1.81	2.01
Glycine	0.057	0.053	0.055	0.065	0.055	44.28	40.40	42.02	51.62	42.02	0.59	0.54	0.56	0.69	0.56
Serine	0.044	0.049	0.036	0.053	0.059	42.21	47.27	34.06	51.44	37.14	0.40	0.45	0.32	0.49	0.35

The quantitative analysis of C-terminal residues seems to suggest that the molecule of conglutin β consists of four polypeptide chains. In the previous report [12] we had assumed, considering the analysis of *N*-terminal glutamic acid, that a molecule of conglutin β consists of three polypeptide chains. The values of 3 glutamic acid residues per 1 mole of conglutin β were based on experimental 70% decomposition of DNP-glutamic acid during 18 hr. hydrolysis. As the DNP-protein was hydrolysed for 24 hr., the previously obtained results were now calculated again taking into account the 75% decomposition of DNP--glutamic acid, occurring during 24 hr. hydrolysis. These new calculations indicated the existence of four *N*-terminal glutamic acid per one mole of protein, i.e. four polypeptide chains. Thus the values of both *N*-terminal and *C*-terminal amino acids suggest the four-chains structure of conglutin β molecule, and this concordance seems to eliminate the presence of branched polypeptide chains.

Table 4

Amino acids of the peptide fractions of conglutin β hydrazinolysate

The peptide fractions, separated by electrophoresis (Fig. 7) were hydrolysed for 24 hr. at 100° in 6 N-HCl. In the hydrolysate acidic and basic amino acids were separated by electrophoresis, and neutral amino acids by paper chromatography. For details see Methods

Fraction	Amino acids found
a	Glutamic acid, histidine, glycine and/or serine, small amounts of valine and leucine
b	Glutamic acid, histidine, glycine and/or serine, aspartic acid, ornithine, threonine, small amounts of valine and leucine
c	Glutamic acid, glycine and/or serine, arginine, aspartic acid, alanine, leucine
d	Histidine, arginine, glycine and/or serine, threonine, valine
e	Arginine (?), histidine (?)
f and *g*	Lysine, ornithine, glycine and/or serine, small amounts of threonine, valine, leucine
h	Ornithine

This conclusion disagrees with a very interesting supposition on the structure of globular proteins, presented by Segal, Dronberger--Schiff & Kalaidjew [10]. According to these authors, the polypeptide chains in the globular proteins make a closed loop, and if amino acids with free amine group are determined, this may be considered as a proof of the existence of lateral branches due to the presence of diaminomonocarboxylic and monoaminodicarboxylic amino acids. Moreover, the authors consider the methods used in determinations of the C--terminal amino acids as not reliable. Therefore in their assumption

these data were not taken into account. Close accordance of the number of the *N*-terminal and *C*-terminal amino acids of conglutin β obtained in our experiments suggests that the hypothesis of Segal *et al.* [10] may not apply to all globular proteins, and that more experimental data should be obtained.

As it already has been mentioned, several peptide fractions were obtained besides *C*-terminal amino acids after the hydrazinolysis of conglutin β. Those peptides consisted mostly of basic amino acids (Table 4). It is in accordance with the interesting studies of Wallen & Sjöholm [11] that the basic peptides are highly resistant to anhydrous hydrazine. Prolonged hydrazinolysis (30 hr.) did not cause any evident splitting of the basic peptides. No differences were stated between the electrophorograms performed after 6 and 30 hr. of hydrazinolysis.

After the hydrolysis of all the peptide fractions (except fraction *a*) a strongly basic substance with a mobility greater than that of ornithine was found. This substance, eluted with water from the electrophorogram, was decomposed to 3-aminopiperidon-2 and ornithine, so it consisted of at least 1 molecule of ornithine and 1 molecule of 3-aminopiperidon-2. Fraction *h* of conglutin β was also of great interest. After hydrolysis only ornithine and the above mentioned basic substance were found. As this substance consisted of 2 molecules of ornithine (hydrazinolysis of the protein causes lactamization of ornithine) it may be presumed that fraction *h* is composed of at least 3 molecules of this amino acid. When arginine was treated with hydrazine no condensation was observed, and only ornithine and 3-aminopiperidon-2 were found. Therefore fraction *h* can not be considered as an artifact due to the effect of hydrazine. Thus it seems that fraction *h* was formed from an already present product of arginine condensation, and the existence in conglutin β of amino acid sequences: Arg-Arg-Arg, may be assumed.

The formation of 3-aminopiperidon-2 from arginine treated with hydrazine indicated that this compound may occur in hydrazinolysates of proteins with *C*-terminal arginine. Niu & Fraenkel-Conrat [8] studying the recovery of amino acids after 10 hr. hydrazinolysis found that the loss of most of the amino acids was 40 - 60%. The authors suggested that these losses mainly arise during the separation of amino acids from hydrazides. It seems, however, that they may be also due to a reaction similar to the observed ornithine lactamization. Such reaction may occur also with other amino acids under conditions favourable for dehydration.

The authors express their gratitude to Prof. Dr. J. Pliwa from the Institute of Organic Chemistry and Biochemistry of the Czechoslovakian Academy of Science, for the gift of carboxypeptidase.

SUMMARY

In conglutin β four C-terminal amino acids were found by the carboxypeptidase method: 2 moles glutamine, 1 mole glutamic acid and 1 mole aspartic acid per 1 mole protein; this suggests the presence of 4 peptide chains. The hydrazinolysis of conglutin β confirmed the presence of C-terminal aspartic and glutamic acids, and 8 peptide fractions were also found. They contained mainly basic amino acids; one of the peptides consisted probably of 3 ornithine. Therefore the presence in conglutin β of Arg-Arg-Arg sequences is suggested.

Ornithine and 3-aminopiperidon-2 were found when arginine was heated with hydrazine.

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Z BADAŃ NAD BIAŁKIEM ŁUBINOWYM

V. C-KOŃCOWE AMINOKWASY KONGLUTYNY β

Streszczenie

Przy pomocy karboksypeptydazy oznaczono, że w konglutynie β pozycje C-końcowe zajmują: glutamina (2 mole), kwas glutaminowy (1 mol) i kwas asparaginowy (1 mol na mol białka), co sugeruje 4-łańcuchową budowę badanego białka. Obecność C-końcowych kwasów asparaginowego i glutaminowego wykazano także na drodze hydrazynolizy konglutyny β. W hydrazynolizacie stwierdzono również 8 frakcji peptydowych, złożonych głównie z aminokwasów zasadowych. Jeden z peptydów zbudowany był prawdopodobnie z 3 reszt ornityny; na tej podstawie można przypuszczać, że w konglutynie β znajdują się sekwencje Arg-Arg-Arg.

Zauważono, że arginina ogrzewana z hydrazyną tworzy obok ornityny 3-aminopiperydon-2.

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INFLUENCE OF γ-IRRADIATION ON LIVER REGENERATION IN NORMAL AND STARVED RATS

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Exposure to ionizing radiations provokes a delay in liver regeneration and, in particular, retards the initiation of DNA synthesis and mitotic division which normally take place following the loss of an appreciable portion of the liver cells [19, 10, 18]. The nature of the damage resulting from irradiation is relatively independent of the type of radiation, any differences being largely quantitative, i.e. the dose necessary to evoke similar effects is dependent on the type of radiation employed. The most pronounced biological effects are produced by fast neutrons, followed by X-rays and γ-irradiation [21].

With a view to obtaining some further information about the mechanisms involved, we have made some attempts to influence liver regeneration in the rat following γ-irradiation. Rats, which were to be submitted to irradiation and hepatectomy, were first starved for 24 hr., following which they were fed normally.

During starvation numerous organic substances are either exhausted, or transported from the liver to other organs, while the activity of a number of enzymes is modified [15, 17]. Upon renewal of feeding following hepatectomy, the weight of the animal increases rapidly as compared to normally fed animals which exhibit a decrease in body weight during the initial stages of regeneration. The liver of starved rats consequently differs from that of normally fed animals not only during the period of irradiation, but also during that of regeneration.

In the present investigation only the liver area was irradiated. In addition an examination was made of the modifications resulting during regeneration following irradiation of other parts of the body, such as the head or limbs, with a view to obtaining some information on the formation and possible role of humoral factors resulting from irradiation. Quantitative determinations were also made of the rate

of regeneration of DNA, RNA, protein and liver mass. During the early stages of regeneration, the initiation of mitosis and the mitotic count were also examined.

MATERIALS AND METHODS

The rats employed, of a white race cultivated in this laboratory, were predominantly males weighing from 170 to 200 g. The animals were enclosed in a narrow plastic container which prevented any appreciable movement. The container was orientated with respect to the source so that the abdomen was directly exposed to the latter. A circular area of diameter 38 mm., corresponding to the location of the liver, was irradiated. The remainder of the body was shielded by means of 10 cm. lead, which transmitted doses of the order of 1 - 2 r. For irradiation of other areas, the same shield and opening were used. The irradiation source was a 50 curie cobalt source, elsewhere described [20]. The animals were submitted to a dose of 1340 r. in the space of 20 min. and were then immediately operated on as described by Higgins & Anderson [7], involving removal of the two main lobes. Animals starved for 24 hr., prior to irradiation and operation, were given water *ad libitum*, and were supplied with food immediately following operation. The radiation dose applied was first determined by a series of trial experiments and was such that it provoked a marked inhibition of regeneration, but did not produce any visible symptoms of radiation sickness such as diarrhea or enlargement of the entrails which resulted from the use of higher doses. The control groups, comprising non-irradiated and normally fed rats, always included an equal number of animals.

The increase in liver weight was determined by weighing, it being accepted that 67% was removed by hepatectomy. DNA was determined by the diphenylamine method [5], following isolation of the nucleic acids by trichloroacetic acid (TCA) without prior extraction of lipids [16]. RNA was determined in the same fraction by means of the orcinol reaction [12]. In some experiments, acid-soluble ribose compounds were estimated from the difference between (a), determinations of RNA in the nucleic acids extracted without prior washing with TCA, hence without removal of acid-soluble compounds; and (b), determinations of RNA extracted from tissue which was initially subjected to several washings with cold TCA. Protein was estimated by means of the biuret reaction according to Lowry *et al.* [11]. Mitotic counts were made following staining of 6 μ tissue sections with haematoxylin and eosin; the mitotic index was taken as the number of mitoses counted in ten fields distributed over the section, enlarged 500 times under the microscope.

RESULTS

As for X-irradiation, γ-irradiation provoked an inhibition of the first phase of mitosis normally appearing about 26 hr. following hepatectomy. The first mitoses were noted about 36 hr. following operation, but in number considerably less than that noted during the period of increased cell division following hepatectomy of non-irradiated animals (Fig. 1). The quantitative regeneration of several liver constituents were examined only after about 72 hr. since, according to Myers [13], this is the period at which the quantitative differences in regeneration rate resulting from irradiation are most pronounced.

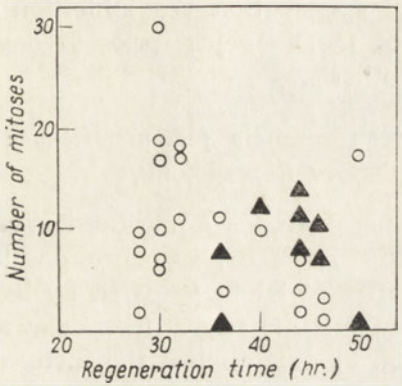

Fig. 1. Effect of irradiation on the appearance of mitosis in regenerating liver. The ordinate scale gives the number of mitoses counted in 10 fields under the microscope; each point represents results for one animal: (○), number of mitoses in normal, regenerating liver; (▲), number of mitoses in irradiated regenerating liver

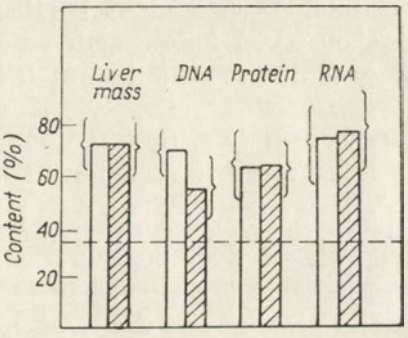

Fig. 2. Influence of irradiation on regeneration of several liver constituents. Each column represents the percentage content of a given liver constituent after 72 hr. regeneration; (□), control; (▨) irradiated animals. Results are means for 6 control, and 12 irradiated, animals. Brackets represent mean statistical deviation. The broken line represents the observed level of measured constituents in the remainder of the liver following hepatectomy

As can be seen from Fig. 2 the rate of regeneration of liver mass, RNA and protein in irradiated animals is unchanged; whereas that of DNA is retarded, in agreement with the known sensitivity of DNA synthesis to irradiation. Rather curious is the observed lack of any radiation effect on the modification of the rate of increase of protein and RNA during regeneration. It has previously been observed [13] that X-irradiation provokes, in addition to a decrease in total DNA during regeneration, a similar, although quantitatively lower, decrease in the rate of growth of protein and RNA. It is, of course, possible that there

exists a small difference, not detectable because of variations between animals.

The curve for initiation of mitosis during regeneration, prior to and following irradiation is very similar to that of Holmes [8] for DNA synthesis under identical conditions, following X-irradiation, with the exception that it appears somewhat earlier. The initiation, and attainment of the maximum rate, of DNA synthesis appear to occur several hours ahead of the corresponding values for cell division. Apparently, following irradiation, the initiation of DNA synthesis, following the period of inhibition, does not attain the same level as for non-irradiated cells. Since initiation of cell division requires the prior doubling of the cellular DNA content [3], it would appear that the inhibition of mitosis observed following irradiation of the liver just prior to hepatectomy is due to inhibition of DNA synthesis.

Differences in liver regeneration between animals fed normally and those starved for 24 hours before hepatectomy

Starvation for 24 hr. prior to operation provokes a marked increase in the rate of liver regeneration. Following 72 hr. regeneration, the largest increase is observed in the quantity of RNA, followed by liver mass and protein; and the least, if indeed there is any difference at all, in the rate of regeneration of DNA (Fig. 3). Nonetheless the increased accumulation of the various constituents of the liver is probably not linked uniquely with the rate of tissue regeneration, i. e. with the increase in number of liver cells, but rather with the accumulation of various constituents in the liver during regeneration. Starvation of the rats for 24 hr. induces an approximately 9% decrease in body weight, and, as appears from the calculations of liver weight removed during operation, the percentage decrease for the liver alone is even greater. It consequently appears that some of the liver substance is either consumed during starvation or is removed to other organs. With renewal of feeding there results an increased displacement of organic compounds to the liver, which is reflected by the remarkable increase in the substances studied, during the regeneration period, in animals starved prior to regeneration. During regeneration in normally fed animals, the increase in liver weight is similar to that for the rate of increase in DNA. This is not so for animals previously starved. The rate of accumulation of particular constituents also varies. As may be seen from the results in Fig. 3 the most marked "mobility" is exhibited by RNA, followed by protein; DNA is hardly affected, if at all. This is particularly clear for shorter regeneration periods, e.g. up to 40 hr. after hepatectomy, where the liver mass of starved animals increases twice as much as for those normally fed; whereas there is no difference

in increase of DNA and protein, but an almost 4-fold increase in RNA, with respect to normally fed rats. Furthermore the increased regeneration rate does not parallel the increased number of mitoses, at least during the initial period.

Of the various constituents studied, the acid-soluble compounds, which are RNA derivatives, are present in liver tissue in appreciable quantities. The average content of these compounds, determined by the orcinol reaction and calculated in terms of equivalent RNA in 1 g. of fresh liver tissue removed during operation is about 11 ± 3 mg. in normally-fed animals; this is to be compared with the equivalent value

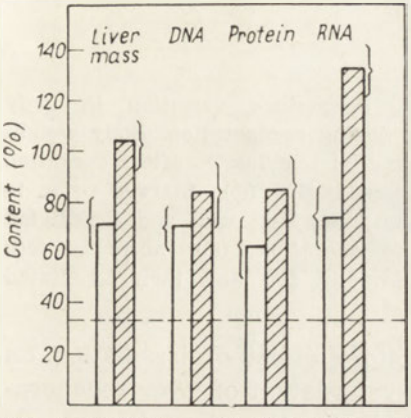

Fig. 3. Influence of starvation prior to operation on the rate of liver regeneration after 72 hr. Percentage content of various constituents in: (□), control animals; (▨), animals starved prior to operation. Results are means for 6 animals in each group. Brackets represent mean statistical deviation. Interrupted line represents the observed content of various liver constituents in controls immediately after operation

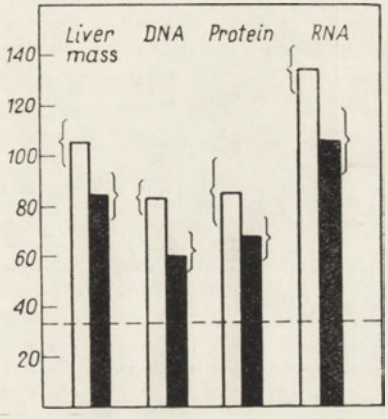

Fig. 4. Influence of irradiation on rate of regeneration of liver constituents for animals starved prior to operation, expressed in percentage content after 72 hr. regeneration: (□), animals starved prior to operation; (■), animals starved and irradiated prior to operation. Means for 6 control, and 12 irradiated, animals. Brackets give mean statistical deviation. Interrupted line represents the observed level of liver constituents immediately after operation

for RNA, which is 8.5 ± 1.3 mg./g. tissue. It should consequently be noted that the relative quantity of these compounds is markedly reduced in starved animals and equals, on the average, 2.5 ± 0.7 mg./g. fresh tissue. Since the orcinol method reveals only purine derivatives, we are dealing in the foregoing largely with changes in concentration of adenine derivatives such as AMP, ADP and ATP, which are most predominant in this fraction [9].

Influence of γ-irradiation on course of regeneration in starved animals

In Fig. 4 are exhibited the contents of the individual substances, following 72 hr. regeneration, in the liver of rats starved prior to operation, with respect to animals starved and irradiated prior to operation. The Figure shows clearly the inhibitory effect of irradiation for each of the liver constituents examined, and that this appears to be closely linked to the general decrease in weight of the animal. Fig. 5 presents the percentage variation in body weight for all cases examined. The larger difference in body weight provoked by irradiation in starved

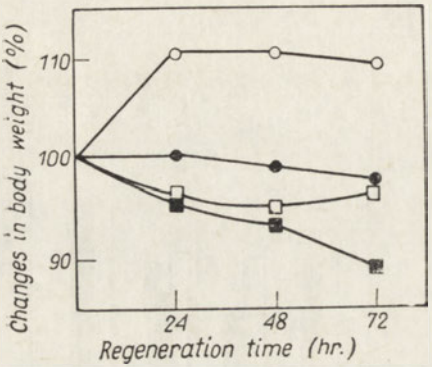

Fig. 5. Percentage variation in body weight during regeneration. Body weight of animal immediately after operation was taken as 100. (○), Starved prior to operation; (●), starved and irradiated prior to operation; (□), normally fed; (■), normally fed and then irradiated

animals corresponds to the greater inhibitory effect of irradiation on the rate of liver regeneration. Hence the retardation of liver regeneration following irradiation would appear to be the consequence, not only of the damaging effect of irradiation on DNA synthesis and cell division, which is of primary importance in the early phase of regeneration, but also of the reduction in body weight and of the state of exhaustion of the animal. A comparison of the results exhibited in Fig. 2 and 4 demonstrates that the contents of the individual constituents of animal starved prior to operation and irradiation are not, with the exception of the DNA content, below the level of the corresponding constituents in operated animals which have not been irradiated following normal feeding. This is in agreement with the differences resulting from variations in body weight between corresponding animals.

Effect of irradiation of other areas of body on course of liver regeneration

In order to obtain some information as to how radiation exerts harmful effects on liver regeneration and, in particular, on the retardation of mitosis, which is a rather sensitive test of the changes provoked by irradiation, some experiments were performed in which either the head or limbs were irradiated, following which the rats were operated

and the process of mitosis followed in the regenerating liver. It was, in fact, found that irradiation of other organs provokes mitotic disturbances during the early stages of regeneration, although differing somewhat from those following irradiation of the liver (Fig. 6). The period of retardation of mitosis is much shorter, about 4 hr.; subsequently there is an increased mitotic rate which prevails for some time; about 36 hr. following regeneration the number of observed mitoses is fairly high and exceeds twice that observed in a normally regenerating liver.

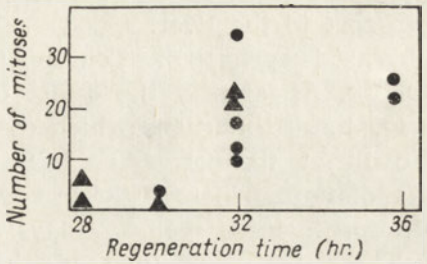

Fig. 6. Mitosis in regenerating liver following irradiation of: (●), head; (▲), limbs

However, since no simultaneous measurements were made of the rate of DNA synthesis, it is not possible to state whether the foregoing is the result of modifications in DNA synthesis or due to some direct effect on the mechanism of mitosis. The production of an effect of this type of such short duration on cellular division is very likely due to the formation of some toxic factors during irradiation. It is, on the other hand, not known whether the observed smaller effect of radiation under these conditions is due to a diminished participation of some toxic factors on the observed modifications in regenerating liver, or to the lack of any damage to the structure of the liver cells and their resultant greater detoxification ability.

DISCUSSION

The regeneration ability of the liver in some mammals is very pronounced. Because of its varied and important function in animal organisms, the liver must be rapidly reformed to permit the normal functioning of the organism. Hence even fairly high radiation doses provoke only a retardation of regeneration resulting from a lag in DNA synthesis and cellular division. Possibly this is linked to the known radiation resistance of the liver. Although cellular division necessarily requires a doubling of DNA content, a fact which renders difficult attempts to examine these phenomena individually, conditions are known under which radiation does not affect DNA synthesis while retarding the appearance of cellular mitosis [14, 4], suggesting that each of these processes is in fact individually affected by irradiation.

With the exception of DNA, radiation exerts little inhibitory effect on the accumulation of these during the later phase of regeneration, viz. after 72 hr. But this effect is more pronounced in animals starved prior to operation, due to the influence of irradiation on the general condition of the animals, which is reflected in their decreased body weight, as compared to non-irradiated animals. From the measurements of the rate of regeneration of various liver constituents in rats starved prior to irradiation, it appears that, with the exception of DNA, which apparently must be synthesized *de novo*, their growth must result from transport to the liver.

What is perhaps most curious is the duration of the radiation induced damage. Apparently the effect of irradiation is pronounced not only under those conditions which compel the cell to undergo division, but also during the period the cell is capable of counteracting the effects of radiation. Albert [1] observed abnormal mitosis during liver regeneration in mice even 257 days following irradiation.

The experiments involving irradiation of other body organs point to the participation of humoral factors in provoking disturbances during cellular division. Whether these are due to the inhibition of DNA synthesis, or to the direct effect of some radiation formed toxins on mitosis, remains unanswered. The observed shorter inhibition period of mitosis, in these instances, suggests a decreased damaging effect due to humoral factors. It must, however, be recalled that about 28 hr. elapsed from the time of irradiation to the time of appearance of the first visible changes in the regenerating liver. Studies on parabiotic rats, amongst others, likewise point to the involvement of toxic factors resulting from irradiation; irradiation of one of the animals produces similar effects in the non-irradiated partner [22, 6]. Observations have also been made on the effect of simultaneous irradiation of other body organs on the number of abnormal mitoses during liver regeneration in mice [2]. However, the experiments of Myers [14] negate the importance of toxic factors on the retardation of liver regeneration following X-irradiation; following shielding of the caudate liver lobe, he irradiated the anterior lobule of the right liver lobe and found that only in the irradiated lobe was there a lower DNA content and a diminished number of liver cells following 96 hr. regeneration. These experiments indicate rather that direct damage of the cells by irradiation is much less readily repaired by the organism.

SUMMARY

The liver region of rats was submitted to a radiation dose of 1430 r. and, following hepatectomy, a study was made of the effect of irradiation, during early stages of regeneration, on cellular division and,

following 72 hr., on the overall values of liver weight and contents of DNA, RNA and protein. Irradiation was found to retard the appearance of mitosis in regenerating tissue and to result in a decreased rate of regeneration of DNA.

Rats were starved for 24 hr., then irradiated as above and operated, following which they were normally fed. Under these conditions liver regeneration was more rapid; furthermore there was more pronounced retardation of the regeneration of various constituents under the influence of irradiation, which appeared to be linked with the greater variations in body weight between non-irradiated and irradiated animals.

If only the head, or the limbs, were irradiated, there resulted a temporary retardation of mitosis in the operated liver, but this was of short duration and, following the period of inhibition, was succeeded by a period of intense division.

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WPŁYW PROMIENIOWANIA γ NA REGENERACJĘ WĄTROBY U SZCZURÓW NORMALNYCH I GŁODZONYCH

Streszczenie

Naświetlano okolice wątroby szczura dawką 1430 r. promieni γ i po wycięciu 2/3 części wątroby badano wpływ naświetlania we wczesnych okresach regeneracji na mitozę, a po 72 godz. na masę wątroby, zawartość DNA, RNA oraz białka. Stwierdzono, że promienie γ powodują opóźnienie występowania mitoz w regenerującej tkance oraz wolniejszą regenerację DNA.

U szczurów operowanych po 24-godzinnym poście, którym podano pokarm zaraz po operacji, regeneracja przebiegała szybciej. Napromieniowanie takich zwierząt przed operacją powodowało zahamowanie regeneracji poszczególnych składników, co wydaje się być związane z różnicą wagi zwierząt napromieniowanych i nienaświetlanych.

Napromieniowanie tylko głowy zwierzęcia lub tylko nóg powodowało w pozostałej części wątroby przejściowe, krótkotrwałe zahamowanie mitoz, po którym następował okres wzmożonych podziałów.

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DEUTERON AND γ-IRRADIATION OF DRIED PREPARATIONS OF LYSOZYME AND RIBONUCLEASE

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The target theory, initially developed by Lea [8] for estimation of the dimensions of radiation-sensitive biologically active macromolecules, has been widely applied to measurements of molecular weights of enzymes or, more correctly, the effective size in molecular weights units of that portion of the molecule responsible for enzymic activity. The results of different observers are, however, not always readily comparable, because of the differences in experimental conditions employed, such as type of irradiation, different dose-rates, etc. Furthermore, on the basis of reported results, doubts may be entertained with regard to general validity of the application of the target theory to calculations of molecular size [10, 11].

In the following we shall describe the results of some measurements on the inactivation, by means of deuteron and γ-radiation, of dried films of lysozyme and ribonuclease under identical experimental conditions, achieved by the formation of films containing a mixture of both enzymes. The choice of these two enzymes was dictated by the following considerations: (a) their chemically and physically determined molecular weights are very close to each other and known with reasonable accuracy, that of ribonuclease being 13 700 [5] and of lysozyme 14 100 [6] [1];(b) each molecule consists of a single polypeptide chain, the secondary structure of which is maintained largely by several disulfide linkages; (c) the amino acid sequences are now reasonably well known [5, 6]; and the ability to remove a number of terminal amino acids without impairment of enzymic activity [7] suggests that it may

[1] At the time this study was initiated, the molecular weight of lysozyme was generally accepted as 15 000 [4]. However, following completion of this work, an improved amino acid analysis [6] necessitated revision of the foregoing to 14 100, a value supported by independent measurements of small-angle X-ray scattering [9] and sedimentation constants [15].

eventually be possible in this way to arrive at some estimate of the magnitude of the active enzymic sites for each enzyme.

Some findings have already been reported on the inactivation, by means of ionizing radiations, of dried preparations of lysozyme [12] and ribonuclease [3]. We shall revert to these in the Discussion, below.

For calculations of molecular weights we employ, in what follows, the $D_{37\%}$ dose in its usual connotation [8]. If $D_{37\%}$ is the dose necessary to score an average of one hit per enzyme particle, then the number of active enzyme molecules, n, remaining after a dose D is

$$n = n_0 \times e^{-D/D_0}$$

where n_0 is the initial number of active enzyme molecules. For an applied dose $D = D_0$,

$$n = n_0 \times e^{-D/D_0} = n_0 \times 0.368$$

$$\text{i.e.} \frac{n}{n_0} = 0.37 = 37\%$$

so that $D_{37\%}$ is the dose which reduces the survival to 37%.

The active cross section S is given by

$$\ln \frac{n}{n_0} = -SD_{37\%}$$

and the molecular weight by

$$MW = \frac{4}{3}\pi \times \left(\frac{S}{\pi}\right)^{\frac{3}{2}} \times d \times N_0$$

where d is the specific gravity of the enzyme and N_0 Avogadro's number.

MATERIALS AND METHODS

Lysozyme was prepared according to Alderton & Fevold [1], recrystallized several times in the isoelectric form and dialysed exhaustively against distilled water to remove salt. Protein concentration was determined spectrophotometrically [13]. Ribonuclease was a commercial crystalline preparation for which we are indebted to Dr. L. Ledoux. Its absorption spectrum exhibited a ratio of maximum to minimum extinction of 1.8, as compared to 2.1 for a sample of Armour ribonuclease [cf. ref. 14], but the specific activity was identical with that of the Armour sample.

The enzymes were irradiated in the form of films deposited on circular or rectangular microscope cover glasses, or on aluminium foil (see below). The films were formed by spreading 50 µl. of an enzyme solution (4.5 mg./ml.) on about 2.5 cm.^{2} of glass or aluminium surface

and drying at 60° in the presence of P_2O_5. Under these conditions the activity of either enzyme was fully retained even after storage for one year at room temperature. When a mixture of both enzymes was to be irradiated, the original solutions were made up to twice the concentration and the films formed in the same way as control samples.

Following irradiation the films were dissolved out into 1 ml. water and enzymic activities measured according to methods described elsewhere for lysozyme based on the rate of bacterial lysis of *Micrococcus lysodeicticus* [13], and for ribonuclease utilizing the metachromasia of yeast ribonucleic acid with a basic dye, methylene blue [14]. The specific activity of each enzyme was unaffected by the presence of the other, at the concentrations employed, within the limits of error of the determination of enzyme activity, about 3%.

Irradiation techniques

Deuteron irradiations were carried out in the cyclotron of the Institute of Nuclear Research in Kraków, the technique used being similar to that described by Pollard & Forro [10]. Ten samples, each deposited on an 18-mm. diameter microscope cover glass (or 18-mm. diameter aluminium foil, see below), were distributed close to and around the circumference of a circular plate which could be rotated so that it was possible to irradiate 10 samples in turn during one run. The incident beam had a current strength of 3×10^{-8} amps. and an initial energy of 4.1 MeV. Lower energies were obtained by interposition of thin aluminium foils supported on the shutter directly in front of the appropriate sample.

The deuteron dose falling on the sample was obtained from measurements of the current to an equal area on the metal supporting plate, for those instances where the samples were deposited on glass. For those series where samples were deposited on discs of aluminium foil and irradiated in the same manner, measurements of current were made on each sample by direct connection of the integrator to the metal supporting plate. The results obtained in this way were in reasonably good agreement with those obtained by irradiation of samples on cover glasses.

Two sources were used for γ-irradiation. One of these was the 4 000 curie cobalt closed source at the Institute of General Chemistry, Warszawa. Twelve samples, deposited on 18-mm. diameter microscope cover glasses, as above, were located around the circumference of a 90-mm. diameter plastic plate. The plate containing the samples was placed in a 10-cm. diameter Petri dish which was covered and sealed with paraffin, and which contained a vial of P_2O_5 to eliminate moisture.

The entire assembly was appropriately positioned in the source for irradiation such that all samples were located on a circumference over which the dose was uniform. Radiation doses were obtained directly from the periods of exposure (8 to 100 hr.). The dose-rate under these conditions was 2.75×10^5 r./hr.

Some samples were also irradiated by means of a specially constructed open cobalt source of 48 curies [16]. The enzyme films, deposited on rectangular microscope cover glasses, were mounted parallel to each other at various distances below the source on a "scaffold" made of sheet metal. The latter was located inside a metal cylinder containing a receptacle with P_2O_5 and fitted with a stopcock, so that the pressure in the interior could be reduced to several mm. Hg. Irradiation times were rather lengthy with this small source, in some instances as much as 400 hr. for almost complete inactivation. For both cobalt sources, dose measurements were based on the Fricke dosimeter, with G value of 16.

RESULTS

Deuteron irradiation

Typical results for deuteron irradiations are presented graphically in Fig. 1. Since measurements of current intensity were considered to be more reliable when aluminium foil was employed for supporting the enzymes films, only the results of these measurements were used in preparing the curves of Fig. 1. The appropriate data are also set forth in Table 1.

From Fig. 1c, and from the data in Table 1, it will be observed that the survival curve for lysozyme alone is steeper than when it is mixed with ribonuclease.

Table 1

$D_{37\%}$ doses and cross sections for deuteron irradiation of lysozyme and ribonuclease, irradiated separately and as a mixture

Incident energy (MeV)	Irrad. conditions	$D_{37\%} \times 10^{13}$ D/cm²		$D_{37\%}$ Ribonuclease / Lysozyme	Cross section ratio
		Ribonuclease	Lysozyme		
4.1	alone	—	2.7	1.0	1.0
4.1	alone	2.7	—		
1.1	alone	—	1.45	1.0	1.0
1.1	alone	1.44	—		
4.1	mixture	2.7	3.27	0.82	1.2
3.2	mixture	1.85	2.5	0.74	1.35
2.1	mixture	1.65	2.3	0.71	1.40
1.1	mixture	1.44	2.16	0.67	1.50

The ratio of the physically determined molecular weights of ribonuclease to lysozyme being 0.97, it might be expected on the basis of target theory that the ratio of their active cross sections would approximate to this figure, assuming, of course, that the active cross sections are proportional to molecular weight. From the $D_{37\%}$ doses for each enzyme irradiated alone, it will be seen (Table 1) that the calculated

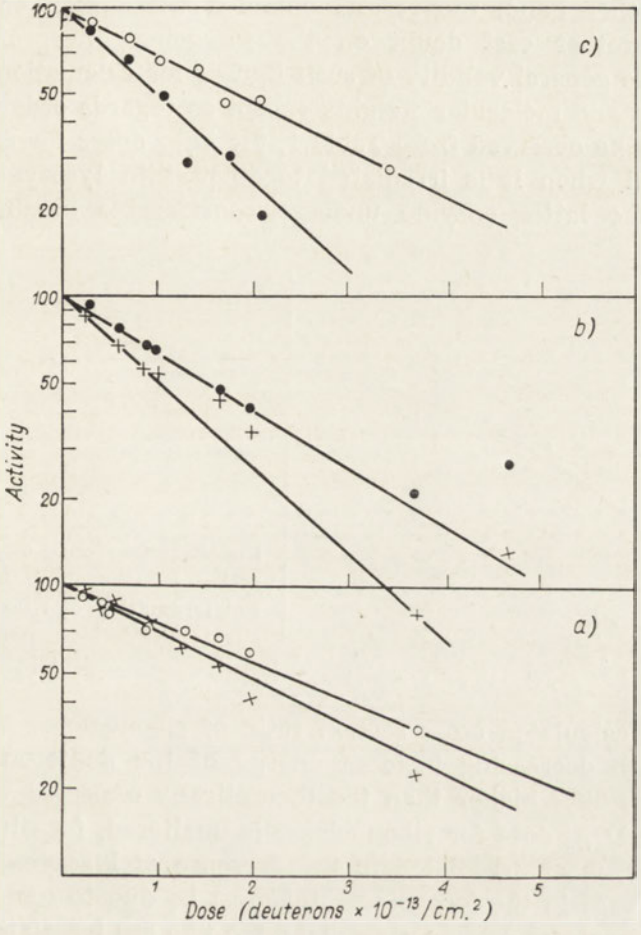

Fig. 1. Deuteron irradiation of films of lysozyme and ribonuclease; (×), ribonuclease at incident energies of 1.1 and 4.1 MeV; (●), lysozyme at 1.1 MeV; (○), lysozyme at 4.1 MeV. Curves *a* and *b* are for mixtures of the two enzymes in equal concentrations by weight. Curve *c* is for lysozyme irradiated alone. Each point is the mean of three independent measurements

cross sections of both enzymes are identical. Since survival curves for the two enzymes irradiated singly were measured for only two values of incident deuteron beam energy, we cannot directly apply the LET (Linear Energy Transfer) method of Pollard *et al.* [10] to calculations

of active cross sections and molecular weights. However, the very close agreement between the $D_{37\%}$ values for the two enzymes at 1.1 MeV and 4.1 MeV suggests that the method is applicable. It might consequently be concluded, even in the absence of absolute measurements of active cross sections, that ribonuclease possesses the same active cross section as lysozyme.

On the other hand, the results obtained when both enzymes are irradiated together cast doubt on the foregoing interpretation and, indeed, on the general validity of such figures for calculations of active cross sections and molecular weights except as regards order of magnitude. As will be observed from Table 1, the $D_{37\%}$ doses for ribonuclease are unaltered when it is irradiated together with lysozyme; but the values for the latter enzyme undergo considerable modification. As

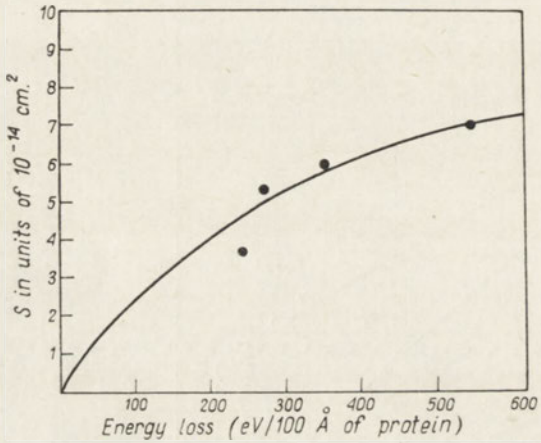

Fig. 2. Dependance of experimental active cross section (S) for deuteron irradiation of ribonuclease versus energy loss per 100 Å of protein

a result the calculated cross section ratio of ribonuclease to lysozyme increases with decreasing incident energy of the deuteron beam and at 1.1 MeV is 50% higher than the theoretically expected value.

Since the $D_{37\%}$ dose for ribonuclease is unaltered, for different incident energies, when irradiated in the presence of lysozyme, it follows that the increase in the foregoing ratio must be due to some protective effect of ribonuclease on lysozyme when the two are irradiated together.

Hence, although lysozyme and ribonuclease exhibit the same $D_{37\%}$ dose when irradiated individually at different incident energies, it is rather difficult to state with certainty whether the ratio of their active cross sections, when they are irradiated together, is really representative of the ratio of their "sensitive volumes" or whether it is merely the result of their mutual interaction. It appears more logical to assume that at 4.1 MeV there is only a smaller protective effect of ribonuclease on lysozyme, about 20%.

In view of the fact that the $D_{37\%}$ doses for ribonuclease are identical

when it is irradiated alone, or in combination with lysozyme, it seems reasonable to apply the LET method [10] to the data in the lower half of Table 1 to a calculation of "molecular target size". From a curve for the dependance of S (active cross section) on energy decrease per 100 A protein (Fig. 2), the extrapolated value of S was calculated to be 0.7×10^{-14} cm^2. The resultant calculated molecular weight is 11 000. The data for lysozyme, irradiated in combination with ribonuclease, permit a similar molecular weight calculation, which comes to 6 400, but it is doubtful whether any significance can be attached to this value in view of the modifications of the $D_{37\%}$ values with incident deuteron beam energy in the presence of ribonuclease.

γ-Irradiations

These were carried out in such a way that each plastic plate contained 4 samples of each enzyme and 4 samples of a mixture of both. The dose survival curves are presented in Fig. 3. Each point therefore

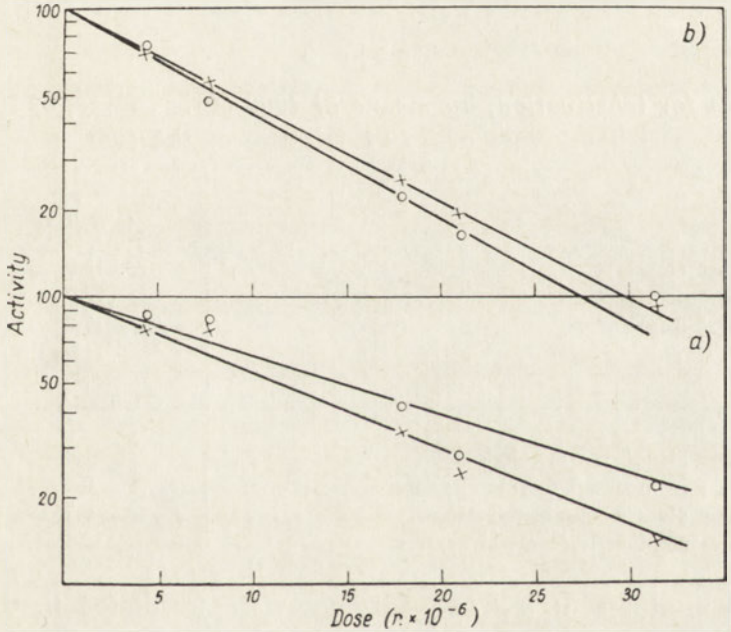

Fig. 3. Results for γ-irradiation of lysozyme and ribonuclease (4000 c source): (×), ribonuclease; (○), lysozyme. (*a*), Ribonuclease and lysozyme irradiated as a mixture; (*b*), ribonuclease and lysozyme irradiated separately. Each point is result of four independent determinations

represents the results of four independent measurements, the variation of which from the mean did not exceed 5%. In a second series of experiments, where all samples consisted of a mixture of equal con-

centrations of both enzymes, each point is the mean of 10 measurements with maximum variations from the mean of 5%. It is, perhaps, worth drawing attention to the fact that, at low residual activities, the points could not be fitted to the curves but were displaced vertically to higher values. This was also true to some extent in the case of deuteron irradiations.

The results shown in Table 2 demonstrate that irradiation of the samples in air is accompanied by some indirect effects, as a consequence of which both enzymes mutually protect each other when they are irradiated together. If the percentage protection is expressed in terms of the reduction in $D_{37\%}$ doses when the enzymes are irradiated together, as compared to irradiation singly, then from Table 2 it follows that ribonuclease is protected in the mixture to the extent of 24% and lysozyme 44%. It is because of this mutual protective effect that the ratio of the $D_{37\%}$ values for the two enzymes is about 0.8 when they are irradiated together. When they are irradiated individually this ratio is about 1.08.

Table 2

$D_{37\%}$ doses for inactivation, by means of 4000 curie source, of lysozyme, ribonuclease and 1 : 1 mixture of the two

	$D_{37\%}$ (r. $\times 10^{-6}$)
RNase alone	13.0
Lysozyme alone	12.0
RNase in mixture	17.2
Lysozyme in mixture	21.6
	Ratio
RNase alone/lysozyme alone	1.08
RNase in mixture/lysozyme in mixture	0.8
RNase alone/RNase in mixture	0.76
Lysozyme alone/lysozyme in mixture	0.56

When mixtures of the two enzymes were irradiated in the small open source, the survival curves were not linear, but exhibited a gradual "break" at about 40% residual activity, the curves then showing a tendency to flatten out for very high doses. Since, for the highest doses used under these conditions, about 6×10^5 r./hr., there remains some doubt as to the accuracy of our dosimetry and the positioning of the samples with respect to the source, these have been omitted from the survival curves, representative ones of which are exhibited in Fig. 4. Note in particular that when ribonuclease and lysozyme are pre-

sent in equal concentrations, their rates of inactivation are almost identical (Fig. 4a); whereas when the concentration of lysozyme is increased with respect to that of ribonuclease, the rates differ very markedly (Fig. 4b), that for lysozyme remaining relatively unaltered, whereas that for ribonuclease is considerably enhanced. This dependance on relative concentrations of the two enzymes testifies to the presence of indirect effects. The results of these experiments differ from those

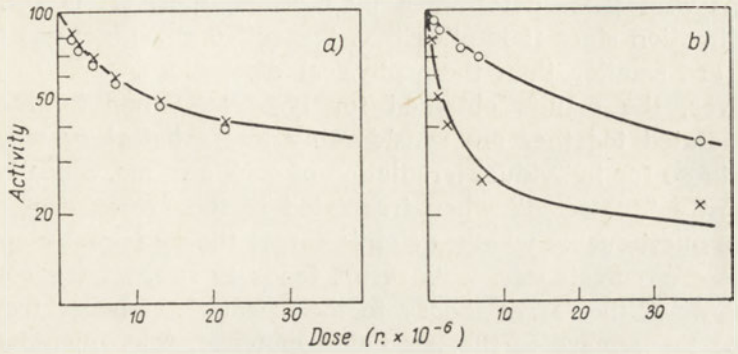

Fig. 4. Results for γ-irradiation with 48 c open source: (×), ribonuclease; (○), lysozyme. (*a*), Ribonuclease and lysozyme irradiated as a mixture of two enzymes in equal concentrations by weight, 250 μg. each on target; (*b*), ribonuclease and lysozyme irradiated together in unequal concentrations; ribonuclease 40 μg. and lysozyme 440 μg. on target

obtained with the large source principally in that samples submitted to different doses were irradiated at different distances, but for identical periods of time, from the source; hence the dose-rate is different for each point on the survival curve. The actual variation in dose-rate for the points represented in Fig. 4b was from 3×10^3 r./hr. to 6×10^4 r./hr. It is therefore possible that the non-linear form of the survival curves is due, at least in part, to a dose-rate effect.

DISCUSSION

The principal objective of the foregoing investigation was to compare the relative active cross sections of two enzymes, the molecular weights of which have been reasonably well established, under the most favourable possible conditions. Such undoubtedly was the case for the deuteron irradiations, where the only major possible source of error when both enzymes were irradiated together was that involving the measurements of enzyme activities.

For those experiments where the enzymes were individually irradiated, the calculated ratio of cross sections for the two enzymes, 1.0,

is in good agreement with that obtained from the ratio of the molecular weights, 0.97, if we bear in mind that the values for measurement of enzyme activities were susceptible to an average error of less than 5%. Since the $D_{37\%}$ doses are calculated from a number of points for each enzyme, they should not be in error by an amount greater than this, so that the maximum possible error in the $D_{37\%}$ ratio of the two enzymes should not exceed 10%, and is probably less than this. The calculated molecular weights for the two enzymes, 11 000, are in reasonably good agreement with those determined by other methods. It is also in the correct direction since it implies that the active cross sections for both enzymes are smaller than their physical dimensions.

However, the results obtained for lysozyme when both enzymes were irradiated together are considerably less satisfactory when compared to those for individual irradiation of each enzyme. Since lysozyme is apparently "protected" when irradiated in the presence of ribonuclease, it is questionable whether simple target theory is really applicable under these circumstances. This result tends to support the contention of opponents of the target theory to the effect that energy transfer of some type takes place in the irradiated material, thus invalidating the concept of "one-hit one-target" [8].

It should be recalled that the target theory implicitly assumes that the energy dissipation of ionizing radiations in proteins is similar to that in gases, viz. 110 eV/primary ionization [8]. For deuteron irradiation it was assumed by Pollard [10, 11] that with an incident energy of 1.1 MeV the distribution of ionizing particles was such that one ionization provoked the inactivation of one enzyme molecule. However, in specific cases based on the dependance of molecular active cross section on the energy dissipated in 100 A protein (LET method) lower values have been applied, e.g. for pepsin 60 eV/p.i. and for trypsin 80 eV/p.i. in place of the standard value of 110 eV/p.i. [11]. Application of these corrected values to calculations of the "molecular target sizes" for pepsin and trypsin, subjected to deuteron irradiation under identical conditions, gave values of 39 000 and 30 600 respectively; the ratio of the molecular target sizes is therefore 1.27, as compared to a ratio of 1.75 (35 000 to 20 000 [4]) for their physicochemically determined molecular weights. Since we have no other independent method for estimation of the ratio of molecular target size to molecular weight, it is difficult to assess the validity of the foregoing modified values of energy dissipation to calculations of the molecular target sizes for pepsin and trypsin.

For more densely ionizing radiations, e.g. α-radiation, it is essential to introduce corrections for the effect of δ-rays. There is, however, some disagreement as to the magnitude of this correction, the values

used by Deering [3] differing from that of Lea [8] on the basis of a comparison of data obtained by bombardment with deuterons and α-radiation. For the bombardment of trypsin with "stripped nuclei" (light atomic ions) it was found necessary to correct the experimental values of energy dissipation per primary ionization by a factor of 15 in order to obtain results in agreement with the physical molecular weight.

It is consequently of interest that in the present investigation, where we have been dealing with two enzymes of almost identical molecular weight, the calculated target sizes are almost identical when each enzyme is irradiated separately either with deuterons or with γ-rays at a high and constant dose-rate. It is only when the two enzymes are irradiated together that marked discrepancies make their appearance. This is perhaps not entirely unexpected when γ-irradiation was employed, in view of the presence of oxygen as well as some moisture (the use of P_2O_5 even under moderate vacuum will not remove appreciable quantities of adsorbed water), both of which may markedly contribute to indirect effects. The presence of such indirect effects is most clearly attested to by the marked modification in rate of inactivation of a mixture of the two enzymes when their relative concentrations are altered (Fig. 4). But even with the deuteron irradiations, conducted under conditions of high vacuum, anomalies appear when both enzymes are irradiated together. While "energy transfer" has been invoked to explain anomalies which have been reported under such conditions of irradiation, it must be borne in mind that even a high vacuum will not necessarily remove all of the "bound" water of a macromolecule and this may certainly contribute to some "indirect" action.

Somewhat curious is the fact that our calculated "molecular target size" for ribonuclease is much lower than that obtained by Deering [3] who irradiated the enzyme with deuterons at the same incident energy and obtained a value for the active cross section twice that of ours and a corresponding molecular weight of 30 000. Although his method for measurement of enzyme activity differed from ours, and involved the spectral estimation of acid-soluble hydrolysed RNA, it has previously been shown that the two methods give similar results [14]. There consequently remains only the possibility of a discrepancy in dose measurements.

Our results for the γ-irradiation of lysozyme are likewise not in full agreement with those of Shalek & Gillespie [12]. With the 4000 c closed source, our $D_{37\%}$ dose is 12×10^6 r. in air as compared to their value of 30×10^6 r. in oxygen. The principal difference in conditions applied was that their dose-rate was 7-fold higher than ours. It is quite possible that this difference in applied dose-rate in the presence of oxygen may in whole or in part explain the discrepancy in results.

Attention is drawn once more, in this connection, to the fact that under the conditions in which the survival curves were obtained with the use of the small source in the present work, there appears to be a net dose-rate effect. It should be recalled that target theory is applicable only when the survival curve is exponential and its slope independent of dose-rate [8]. There are, however, few instances where the influence of dose-rate has been examined, particularly in the case of enzymes.

An examination of the various results obtained during the past ten years on calculation of molecular target sizes by means of ionizing radiations shows that most of the values are higher than the size calculated from the known molecular weights, whereas the converse should be true. It is consequently not surprising that interest in this field has been waning. It appears to us that a comparison of target sizes for similar enzymes under identical irradiation conditions may prove more profitable than attempting to measure absolute values of target sizes.

We should like to express our thanks to Prof. Dr. H. Niewodniczański for making available the cyclotron of the Institute of Nuclear Research in Kraków; to Mgr. M. Słapa for assistance with the deuteron irradiations; and to Doc. St. Ciborowski of the Institute of General Chemistry, Warszawa, for permission to use the 4000 c cobalt source and Mgr. Z. Przybyłowicz for carrying out irradiations with this source.

SUMMARY

Lysozyme and ribonuclease, two enzymes with similar structure and almost identical molecular weights, have been irradiated in the dry state with deuterons and γ-rays. Individual irradiation of each enzyme either with deuterons or γ-rays, under identical conditions, shows that their active cross sections are almost identical; and from the deuteron irradiations their molecular weights are calculated as 11 000, so that their active cross sections are about 20% less than those calculated directly from the accepted molecular weights. However, if both enzymes are irradiated together, in the form of a mixture, anomalies make their appearance in the form of a mutual protection of each enzyme by the other. This effect is particularly pronounced with γ-irradiation and under conditions where some air and moisture are present; but it is also present, although to a smaller extent, with deuteron irradiation under conditions of high vacuum. In addition there is some evidence for a dose-rate effect with γ-irradiation in the presence of air. The results are discussed in relation to the general validity of application of the target theory to absolute calculations of active cross sections and molecular weights. The data are also compared and discussed in relation to those of other observers.

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NAŚWIETLANIE DEUTERONAMI I PROMIENIAMI γ SUCHYCH PREPARATÓW LIZOZYMU I RYBONUKLEAZY

Streszczenie

Dwa enzymy, lizozym i rybonukleazę, posiadające podobną strukturę i prawie taki sam ciężar cząsteczkowy, naświetlano w stanie suchym deuteronami i promieniami γ. Naświetlanie każdego z tych enzymów osobno w identycznych warunkach wykazało, że powierzchnie ich przekrojów czynnych są prawie identyczne. Ciężar cząsteczkowy obliczony z wyników naświetlania deuteronami wynosił 11 000; wskazywałoby to, że przekrój czynny jest o około 20% mniejszy niż przekrój obliczony z ogólnie przyjętego ciężaru cząsteczkowego. Jednakże przy naświetlaniu mieszaniny obu enzymów zachodzą pewne odchylenia spowodowane wzajemnym wpływem ochronnym jednego enzymu na drugi. Wpływ ten występuje zwłaszcza przy naświetlaniu promieniami γ w obecności resztek powietrza i wilgoci, a w mniejszym stopniu przy działaniu deuteronami w wysokiej próżni. Ponadto uzyskano dane o wpływie szybkości dawkowania promieniowania w obecności powietrza na szybkość inaktywacji enzymów.

W świetle uzyskanych wyników przedyskutowano ogólną przydatność teorii „tarczy" do obliczania bezwzględnych wartości przekrojów czynnych i ciężaru cząsteczkowego oraz przeprowadzono porównanie z danymi uzyskanymi przez innych badaczy.

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THE CATHODIC PROTEINS IN HOG KIDNEY MITOCHONDRIA

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In a preliminary communication [10] it has been reported that with sulphosalicylic acid some proteins could be extracted from animal tissues, which in paper electrophoresis at pH 8.6 migrated toward the cathode. These protein fractions have not been observed by workers using different extracting media, i. e. water alkalized to pH 7.8 (Caravaglios & Franzini [3]), sodium chloride solution (Dallam [4]), veronal buffer, pH 8.6 (Pellegrino & Toreigliani [9]), sucrose solution (Adjutantis [1]), 0.15 M-potassium chloride (Belajewa [2]). It was only with diluted hydrochloric or sulphuric acid that Dallam [4] and Wolfe & Mc Ilwain [11] extracted from cell nuclei proteins having the properties of histones and migrating toward the cathode.

The purpose of the present study was to find out whether the cathodic, sulphosalicylic acid-soluble proteins originated from nuclei or from other cell particles.

MATERIALS AND METHODS

Cell fractions. Hog kidneys were removed about 10 min. after the animal had been killed, and were transported in ice to the laboratory. Then the capsule, fat and renal pelvis were discarded, and the tissue was homogenized with 10 vol. of 0.25 M-sucrose solution in a Warring blendor for 2 min. The homogenate was filtered through 2 layers of gauze, and centrifuged at 0° to separate cell particles. The nuclei were sedimented at 800 *g* after 10 min., the heavy mitochondria at 10 000 *g*, 10 min., and light mitochondria at 25 000 *g*, 10 min. The remaining supernatant which contained cytoplasm and microsomes was called the cytoplasmic fraction. The sedimented subcellular fractions were washed twice (the nuclei sometimes 5 times) with 0.25 M-sucrose solution. The washings were discarded, except in experiments in which the proteins were extracted from washings. In those experiments the nuclei obtained from 2 hog kidneys were washed with about 300 ml.

of sucrose solution. After washing the solution was centrifuged at 25 000 *g* and the supernatant used again for washing the nuclei. This procedure was repeated twice.

Extraction with sulphosalicylic acid and sulphuric acid. All manipulations were carried out at 0 - 10°. The total kidney homogenate and the subcellular fractions were extracted with 0.2 N-sulphosalicylic acid (SSA). The amount of the extracted protein did not increase when higher concentrations of the acid were used. The extraction was carried out as follows. To the homogenate and the cytoplasmic fraction appropriate amounts of 4 N-SSA were added; the nuclei and mitochondria were first suspended in 4 vol. of water and next 5 vol. of 0.4 N-SSA was added. Then the mixtures were homogenized for 3 min. and after 30 min. the extracts were centrifuged for 10 min. at 1500 *g*, the supernatant filtered through Whatman no. 3 paper, and the proteins present in the solution concentrated.

Histones were extracted from nuclei with 0.2 N-H_2SO_4 [11] by homogenizing the nuclei with the acid in an ice-cooled Potter apparatus for 10 min. The solution was kept for 1 hr. in the refrigerator, homogenized again and centrifuged at 800 *g*. The supernatant was filtered and adjusted to pH 6.5 with sodium hydroxide solution. The "neutral precipitate" formed was discarded, and the clear filtrate containing protein was concentrated.

Concentration of extracted proteins. This was done according to the Mejbaum-Katzenellenbogen tannin-caffeine method [7]. It was found that from the SSA extracts the proteins could be completely precipitated already at the ratio of protein to tannin 1 : 0.5, if the solutions were brought to pH between 3 and 7 (Fig. 1). At the ratios of protein

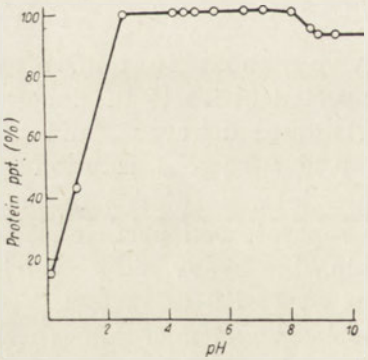

Fig. 1. The effect of pH on the precipitation with tannin of proteins soluble in sulphosalicylic acid. To 1.8 ml. of buffer [7] at different pH, 0.1 ml. of protein extracted with sulphosalicylic acid (150 μg.) and 0.1 ml. of 0.075% tannin solution were added. Half an hour later tannin-protein precipitate was centrifuged off and the protein was determined in the supernatant by the tannin turbidimetric method [6]. The protein to tannin ratio was 1 : 0.5

to tannin 1 : 1, or 1 : 2 the precipitation occurred at an even wider range of pH. Therefore the SSA extracts were brought to pH 4 - 6, and the proteins were precipitated with the two-fold amount of tannin.

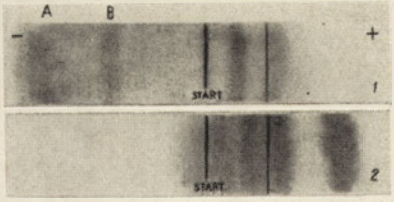

Fig. 2. Paper electrophorograms of (*1*), proteins extracted with sulphosalicylic acid from hog kidney homogenate, and (*2*), whole hog serum. (A) and (B), protein cathodic fractions

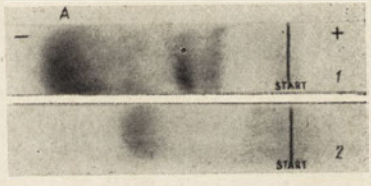

Fig. 3. Paper electrophorograms of hog kidney nuclei proteins, (*1*), extracted with 0.2 N-sulphosalicylic acid (300 μg. protein), and (*2*), extracted with 0.2 N-H_2SO_4 (150 μg. protein)

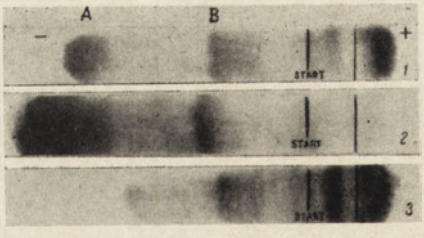

Fig. 4. The electrophoretic separation of proteins extracted with sulphosalicylic acid from (*1*), nuclei-free homogenate; (*2*), mitochondria (fraction 800 - 25 000 *g*); (*3*), cytoplasmic fraction

The regeneration of proteins with caffeine was carried out as indicated by Mejbaum-Katzenellenbogen at the ratio of protein to caffeine 1 : 2 [7]. The obtained concentrated solution contained 2 - 4% proteins.

Paper electrophoresis. The concentrated protein solutions were submitted to electrophoresis on Whatman no. 1 paper (3 × 28 cm. strips) in veronal buffer, pH 8.6, *I* 0.05 [8]. About 10 - 50 µl. of the solution, corresponding to 0.5 - 1 mg. of protein, was placed 2 cm. from the centre toward the cathode on strips moistened with buffer. The voltage of 200 v was used, and the separation lasted 6 - 7 hr. Then the strips were dried at 105° for 15 min. and stained with bromophenol blue. The individual fractions were cut out, eluted with 0.1 N-NaOH, and their quantities calculated from the extinction at 590 mµ on the assumption that the concentration of protein is directly proportional to the amount of dye combined with the protein [8].

Determination of proteins. This was done with the tannin turbidimetric method according to Mejbaum-Katzenellenbogen [6], except for electrophoretic fractions as described above.

Reagents. Tannin and caffeine were products of Cefarm, Warszawa; sulphosalicylic acid, Unia, Warszawa.

RESULTS

Proteins extracted from whole kidney homogenate with sulphosalicylic acid were separated by paper electrophoresis at pH 8.6 into several fractions, some of which (20 - 30% of protein) migrated toward the cathode (Fig. 2).

Table 1

The effect of washing on the amount of protein extracted with sulphosalicylic acid from hog kidney nuclei

The isolated nuclei were washed with 500 ml. portions of 0.25 M-sucrose solution

Nuclei	Extracted protein (mg./100 g. nuclei)
Non washed	100 - 150
Washed 2 times	30 - 50
Washed 5 times	6 - 13

Electrophorograms of proteins extracted from isolated nuclei with SSA and with H_2SO_4 indicated that the SSA-soluble proteins were not histones (Fig. 3), and it could be seen that they were the same as the fractions present in the whole homogenate. The amounts of protein extracted with SSA from nuclei were much smaller than those found in the whole homogenate (5 - 10%). Moreover, when the nuclei were

washed several times with sucrose solution, the amount of SSA-extractable proteins decreased about 10 times (Table 1).

This allowed to assume that the cathodic protein fraction did not originate from the nuclei but from a contamination with other subcellular particles or even intact cells which could be washed out with sucrose solution.

When the protein extracts of other subcellular fractions were examined it was found that the protein moving fastest toward the cathode (marked *A* on the Figures) occurred only in the mitochondria, whereas it did not occur in the cytoplasmic fraction (Fig. 4). The anodic fractions did not occur in the mitochondria but were found only in the nuclei-free homogenate and in the supernatant after centrifugation at 25 000 *g*.

Table 2

The amount of total protein and of fraction A *extracted with sulphosalicylic acid from cellular fractions*

The cathodic fraction A (fastest moving) is given as percentage of total extracted protein. The figures represent the mean from two experiments

Cellular fraction	Protein (mg./1 g. dry wt.)	Fraction *A* (%)
Nuclei washed 2 times	0.12	60
Nuclei-free homogenate	3.8	35
Mitochondria sedimented at 800 - 25 000 *g*	3.2	68
Cytoplasmic fraction (supernatant at 25 000 *g*)	1.1	0.03

The data in Table 2 complete the results presented in Fig. 4; it can be seen that proteins soluble in SSA were extracted from nuclei in quantities about 20 times smaller than from mitochondria.

DISCUSSION

The obtained results show that the fastest moving cathodic fraction *A* could be extracted with sulphosalicylic acid from the mitochondria, but was absent in the supernatant after the kidney homogenate had been centrifuged at 25 000 *g*. This supernatant consisted of microsomes and cytoplasm. It can be assumed that a part of the microsomes was spun down together with light mitochondria, but the majority of microsomes did not sediment. Therefore, the fact that in the supernatant at 25 000 *g* the fraction *A* was not observed shows that either this fraction is not present in the microsomes or cannot be extracted from them with SSA.

The results presented in Table 2 show that fraction A forms about 30% of the total proteins extractable with SSA from the nuclei-free homogenate, and about 60% of proteins extractable from mitochondria. These figures are approximate, first because the separation by means of paper electrophoresis is not very accurate, secondly because the calculations were based on the assumption that different protein fractions uniformly absorb bromophenol blue, and this is not always true. The determination of the dry weight of cellular fractions carries also a certain error, as a correction for the content of sucrose should be introduced. The presence of the cathodic fraction in the SSA extracts of nuclei is probably due to an admixture of mitochondria or of intact cells, or to the extraction of histones.

The demonstration of cathodic proteins in mitochondria makes it possible to carry on further work on their isolation and the determination of their amino acidic composition.

SUMMARY

Proteins migrating toward the cathode at pH 8.6 were found in hog kidney mitochondria. These proteins separated into several fractions; the fastest moving fraction amounted to 60%.

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BIAŁKA KATODOWE MITOCHONDRIÓW NERKI WIEPRZA

Streszczenie

W mitochondriach nerki wieprza stwierdzono białka wędrujące do katody przy pH 8,6. Białka te dzieliły się na kilka frakcji, z których najszybciej wędrująca do katody stanowiła 60%.

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