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MARIA SARNECKA-KELLER

THE PEPTIDE STRUCTURE OF PROTEIC ACIDS

Department of Physiological Chemistry, Medical School, Kraków
Head of the Department: Prof. Dr. B. Skarżyński

In the biochemical literature the term oxyproteic acids is used to designate a group of compounds which were first described by Bądzyński & Gottlieb [8] as physiological components of urine, but which have not been clearly defined. Although a great deal of interest was accorded to these compounds between the years 1897 - 1926, the work done did not succeed in elucidating their chemical structure. None of the investigators was able to produce evidence of their polypeptide nature, or to separate the mixture of these substances.

Since earlier investigations, as well as personal preliminary experiments indicated that proteic acids are indeed mixtures of peptides, a study was undertaken and this problem, seemingly not a new one, was found to be connected strictly with a number of current problems in the field of biochemistry.

Nomenclature of the studied compounds. The name "oxyproteic acids" really pertains only to a fraction of the compounds investigated in this paper, although the term is employed by some writers to designate all the substances which can be isolated from urine in the form of alcohol-insoluble barium salts. Since this mixture has been named by Gawiński [31] proteic acids, I have adopted for the studied compounds a uniform nomenclature which is based on the original assumptions of the first investigators to study this problem.

Proteic acids

(Mercuric salts insoluble in water; barium salts soluble in water, but insoluble in alcohol)

Alloxyproteic acids (Lead salts insoluble in water)	Antoxyproteic acids (Mercuric salts insoluble in acid media)	Oxyproteic acids (Mercuric salts insoluble in weakly alkaline media)

Methods of isolation of proteic acids from urine. In 1897 Bądzyński & Gottlieb [8] reported a method for isolating from urine a mixture of antoxy- and oxyproteic acids (according to the authors, oxyproteic acid). Sulphates were partially precipitated from concentrated and acidified urine by means of ethyl alcohol, and then the rest, together with phosphates, by a solution of $Ba(OH)_2$. The excess of Ba^{2+} ions in the filtrate was precipitated by passing CO_2, the solution was concentrated and poured into an excess of ethyl alcohol. The obtained sediment contained chiefly barium salts of proteic acids. The authors then transformed them into mercuric salts by adding $Hg(NO_3)_2$ in an alkaline medium. The sediment of mercuric salts was decomposed with H_2S; HgS formed was separated; H_2S was removed by aeration, and the nitric acid formed, by means of $Pb(OH)_2$. The excess of Pb^{2+} ions was removed with sulphuric acid, and barium salts of antoxy- and oxyproteic acids were precipitated with $Ba(OH)_2$ in the presence of ethyl alcohol from the remaining concentrated solution.

In 1902 Bądzyński & Panek [9] reported a method for isolating alloxyproteic acids from precipitates of mercuric salts of proteic acids obtained by a method similar to the one described above. Three years later Bądzyński, Dąbrowski & Panek [7] elaborated a method for obtaining all three fractions of proteic acids from urine. Phosphates were separated by means of $Ca(OH)_2$, sulphates with $Ba(OH)_2$, and the excess of Ba^{2+} and Ca^{2+} ions was then precipitated with CO_2. The greater part of NaCl and urea was removed by repeated concentration and recrystallization at a low temperature, and barium salts of proteic acids were precipitated from the residue with an alcohol - ether mixture. The salts were then dissolved in water and lead acetate was added to precipitate the alloxyproteic acids in the form of plumbous salts. The remaining Pb^{2+} ions were removed from the filtrate by adding a solution of Na_2CO_3; after neutralization with acetic acid the solution was concentrated, acidified with acetic acid and mercuric acetate was added. Mercuric salts of antoxyproteic acids were then precipitated. By mild alkalinization of the filtrate a precipitate of mercuric salts of oxyproteic acids was obtained. The three fractions of proteic acids were then purified by repeated transformation into free acids and precipitation of insoluble salts.

This method of isolation of proteic acids fractions described by Bądzyński, Dąbrowski & Panek was subsequently used with only slight modifications designed to remove other nitrogenous substances. It was necessary for proteic acids determination, since their content in urine was expressed in percentage of total urinary nitrogen. Modifications of this type were introduced by Ginsberg [33], Gawiński [31], Sassa [48], Fürth [30], and Weissberg [52].

Other simplified methods of isolating the proteic acids [e. g. 22, 42] did not ensure the removal of accompanying substances. In the method of Freund & Sittenberger-Kraft [29] the colloids were first removed from urine with a solution of $CuSO_4$ in alkaline medium before precipitating the proteic acids.

Properties of proteic acids and studies of their chemical structure. Bądzyński *et al.* [7] considered the different fractions of proteic acids to be chemically homogeneous compounds with the following composition:

	Antoxyproteic acids	Oxyproteic acids	Alloxyproteic acids
C	43.21%	39.62%	41.33%
H	4.91%	5.64%	5.70%
N	24.40%	18.08%	13.55%
S	0.61%	1.12%	2.19%
O	26.33%	35.54%	37.23%

According to Bądzyński *et al.* the antoxyproteic acids form sodium and potassium salts soluble in water, and in alcohol give thick, viscid emulsions. Calcium, barium, cadmium and silver salts are soluble in water, but insoluble in alcohol, barium and silver salts being the least soluble. Mercuric antoxyproteinates are insoluble in water. On boiling with alkalis the antoxyproteic acids split off part of their sulphur as sulphide ion. Protein reactions, such as biuret and Millon's reaction, are negative, but reaction with Ehrlich's diazo reagent is positive.

The properties of oxyproteic acids salts are similar to those of antoxyproteic acids salts with the exception of their mercuric salts being soluble in acid medium. The oxyproteic acids give a weakly positive Millon's reaction, and a negative one with Ehrlich's diazo reagent.

The alloxyproteic acids form sodium, potassium, barium, calcium, cadmium and mercuric as well as silver salts with properties similar to the salts of the previously mentioned compounds, but less soluble in water and alcohol. Lead salts are insoluble in water.

All three groups of proteic acids are distinctly acidic, as shown by the high barium content in their salts. These compounds cannot be obtained in crystalline form, and do not dialyze at all, or only with great difficulty [6].

From their physical properties and the results of elementary analysis Bądzyński concluded that these substances are high-molecular products of protein decomposition; however, he did not identify them with the polypeptides found in urine by Abderhalden & Pregl [1].

A more detailed study was carried out by Browiński & Dąbrowski [16]. They estimated the free amino groups in all three fractions of proteic acids before and after acid hydrolysis. The results pointed to a polypep-

tide nature of proteic acids, since acid hydrolysis gave an increase in the free amino groups. The results of Browiński & Dąbrowski were confirmed by Glagolew [34], who also demonstrated the presence of cystine and arginine in hydrolyzates of oxyproteic and alloxyproteic acids.

Edlbacher studied these compounds in 1922 - 1925 [22 - 26]; he confirmed the chemical nature of antoxyproteic acids; he concluded, however, that oxyproteic acids are loose combinations of urea with other compounds and he considered oxyproteic acids to be identical with so-called "ureine" found in urine by Moore [43] in 1903. Freund & Sittenberger-Kraft [29] confirmed that proteic acids are derivatives of urea. However, their preparation displayed complete absence of sulphur, and its products of decomposition contained an unidentified nitrogen--containing organic acid.

In 1926 Giedroyć [32] analysed a preparation of oxyproteic acids obtained 10 years previously by Dąbrowski, as well as proteic acids isolated from urine by the method of Gawiński. Employing urease and xanthydrol, he found no urea in either preparation, before or after acid hydrolysis. Giedroyć demonstrated the errors in the procedure of Edlbacher, which consisted chiefly in incomplete removal of urea; moreover, Cl^- ions remaining in the preparation, together with Hg^{2+} ions formed with urea an insoluble compound which was precipitated together with mercuric oxyproteinates.

In 1937 Lustig & Tüchler [42] came to the conclusion that the composition and properties of proteic acids differ essentially from those of proteins and their degradation products.

Determination of proteic acids in urine and its clinical significance. When reporting the discovery of proteic acids Bądzyński also discussed the diagnostic value of their determination [8]. The amounts of proteic acids in normal urine reported by different authors vary widely. Gawiński [31] states that the nitrogen of proteic acids constitutes 4.5 - 6.8% of the total urinary nitrogen. Similar values, 4.3 - 4.7%, are given by Sassa [48]. Fürth [30] reported 3.15%, Weissberg [52] 3.56% and Lustig & Tüchler [42] only 1.5%. However, all the authors found a marked increase of proteic acids in certain pathological conditions, such as tumours and functional liver disorders. On the basis of a large clinical material, Lustig & Tüchler even concluded, that provided tuberculosis and liver disease could be excluded, determinations of proteic acids in urine constitute a valuable aid in the diagnosis of malignant tumours.

A relationship has also been found between the sulphur of proteic acids and the neutral sulphur in urine. According to Gawiński the sulphur of proteic acids constitutes 91.3% of the neutral sulphur. According to Weiss [51] it is 94.8%, and varies parallelly with the amount of proteic

acids excreted in urine. The results of Gawiński and Weiss were confirmed in 1937 by Lefèvre & Rangier [39].

Ehrlich's diazo reaction used in analysis of urine, has been explained by an increased content of proteic acids. According to Edlbacher [24] the substance conjugating with diazotized sulphanilic acid is histidine, contained chiefly in the antoxyproteic acid fraction.

Studies performed in 1897 - 1937 have not elucidated the chemical structure of proteic acids. Since later no investigations of these compounds have been conducted, a re-examination of this problem with new methods was undertaken.

The presented results concern the general properties of proteic acids, methods of their isolation and estimation, and the properties of individual peptides which were separated from mixtures of these compounds.

EXPERIMENTAL

Isolation of proteic acids; general properties, and methods of estimation

Isolation. With the method of Bądzyński *et al.* [7] it was not possible to obtain proteic acids free from inorganic salts and urea, both of which obscure the chromatographic patterns. To obtain a higher degree of purity of proteic acids, a modification of Gawiński [31] was used, consisting in preliminary precipitation of salts from concentrated urine with alcohol. However, urea was not extracted with alcohol in Sohxlet apparatus to avoid the effect of prolonged heating. The precipitate of barium salts of proteic acids was washed several times with absolute alcohol, and then purified by dissolving three times in water and by precipitation with a mixture of absolute alcohol and ether (2 : 1, v/v). Barium salts of proteic acids obtained in this way were sufficiently pure to allow electrophoretic and chromatographic separation. On chromatograms with Ehrlich's reaction only traces of urea were found.

Urine was albumin and sugar-free, which was tested with sulphosalicylic acid, and Nylander's reagent, respectively. 250 ml. of urine from the amount excreted during 24 hrs. was acidified with acetic acid to pH 5.5 and concentrated *in vacuo* at 40° to ca. 15 ml. After cooling, the formed sediment was removed by centrifuging. The clear solution was then acidified with dilute H_2SO_4 until the colour of Congo red indicator paper was changed, and then 50 ml. absolute alcohol was added. The sediment was centrifuged and washed twice with 10 ml. 75% alcohol. To the pooled alcoholic solution diluted three-fold with water, saturated solution of $Ba(OH)_2$ was added dropwise, with continuous mixing, to slight excess. After 10 min. the precipitate of barium phosphate and

sulphate was centrifuged, and excess of barium ions in the solution was removed by means of CO_2. The precipitate of barium carbonate was centrifuged, and the solution was concentrated *in vacuo* at 40° to approx. 15 ml. The greater part of NaCl and urea was removed by repeated concentration and crystallization. The barium salts of proteic acids were precipitated from the remaining solution with a mixture of absolute alcohol and ether (2 : 1, v/v). The precipitate was washed five times with absolute alcohol, then purified by dissolving three times in water and by precipitation with the alcohol - ether mixture, and dried *in vacuo* over P_2O_5.

The amounts of proteic acids barium salts obtained from three normal urines are shown in Table 1.

Table 1

Amounts of proteic acids in normal urines

Urine	24-hr. volume (ml.)	Ba salts mixture of proteic acids	
		(mg./250 ml.)	(g./24 hr.)
BG	780	350	1.092
MS	1260	175	0.882
JN	1340	225	1.206

Properties. All three precipitates of mixtures of barium salts of proteic acids obtained from normal urines (BG, MS, JN) and other used in the presented work were hygroscopic powders of a more or less brown colour, readily soluble in water giving yellow coloured solutions.

Mixtures BG, MS, JN were dissolved in water to give solutions containing 25 mg. per ml. and the following reactions were carried out with each of the three solutions.

The murexide reaction was negative, indicating the absence of uric acid. Ehrlich's diazo reaction gave an intensively red colour, and the xanthoproteic reaction was also positive; Millon's reaction, however, was only slightly positive. Boiling with sodium hydroxide followed by addition of lead acetate gave an abundant sediment of PbS in mixture BG; the remaining two mixtures gave visible, but much smaller sediment. All three mixtures gave a positive biuret reaction, and also positive reactions with ninhydrin and isatin. The Molisch reaction, as well as the reaction with anthrone, were positive.

In order to ascertain whether chromatographic separation of the peptides in the form of their barium salts is possible, and whether all three mixtures contain the same peptides, a preliminary chromatographic analysis was made. Circular chromatography on Whatman No. 1 filter paper (discs 20 cm. in diameter) with lateral application of the samples

was employed. Amounts of 20 µl. of the solutions were applied, corresponding to 500 µg. of the dry barium salts of proteic acids. The chromatograms were developed in a system of *n*-butanol - acetic acid - water (144 : 13 : 43).

The chromatographic patterns of all three mixtures were analogous. With the solvent used, the R_F values of the investigated peptides lie in the range between 0 and 0.31. Peptides were detected with ninhydrin, isatin, and Pauly's reagent, the latter showing high sensitivity especially for peptides containing histidine.

Barium ions were detected by spraying with 2.5% solution of potassium chromate; after washing out the excess of chromate with water, the chromatograms were immersed in 0.05 N-$AgNO_3$. A reddish-brown spot was located between R_F 0.12 and 0.23, and the remaining surface of the chromatograms occupied by peptides was free from barium ions. It means that in the acidic solvent the barium salts of peptides were split off and were separated as free acids.

Ehrlich's *p*-dimethylaminobenzaldehyde reagent gave a weak yellow spot at the position of urea ($R_F = 0.47$), and a bright yellow reaction in the area occupied by peptides. Jaffe's picric acid reaction for creatinine was negative; negative reactions were also obtained with the aniline-phthalate reagent and with anthrone dissolved in sulphuric acid. When parts of the chromatograms were sprayed with $FeCl_3$ solution, and with a mixture of 1% solutions of $K_3Fe(CN)_6$ and $FeCl_3$, only the latter gave a blue reaction at $R_F = 0.18$.

The behaviour of the proteic acids during dialysis against H_2O was also investigated; 1 ml. of each of the three mixtures of solutions was dialyzed in "Visking" bags against 20 ml. H_2O, which was changed 3 times during 48 hrs. at 4°. After dialysis the contents of the bags were evaporated to dryness *in vacuo* at 40°, and the residues were dissolved in 0.5 ml. H_2O each. 20 µl. of the solution was subjected to chromatographic analysis as above. No colour reactions were obtained with ninhydrin, isatin or Pauly's reagent, showing that all the peptides had passed freely through the membrane, in contrast to Bądzyński's findings.

A preliminary identification of the amino acids in hydrolyzates of proteic acids was carried out; 25 mg. of mixture BG was hydrolyzed in 6 N-HCl for 24 hr. at 110°.

The comparison of chromatograms obtained before and after hydrolysis showed a translocation of almost all the original spots and the appearance of new ones corresponding to free amino acids. It demonstrated the peptide nature of almost all the spots giving positive reactions with ninhydrin, isatin, or Pauly's reagent on the chromatograms of mixtures of proteic acids.

Hydrolyzates of mixture BG were found to contain large amounts of histidine, glycine, glutamic acid, and proline. Cystine + cysteine, aspartic

acid, alanine, valine, leucine, and tyrosine were present in smaller amounts. The results of the analysis give only a general idea of the amino acid composition, since it was impossible on circular chromatograms to detect amino acids present in much smaller amounts than the first four above mentioned.

Determination of proteic acids. The amount of proteic acids was expressed as percentage of nitrogen in relation to total urinary nitrogen. Nitrogen was determined by the micro-Kjeldahl method. Samples of 0.3 ml. of 24-hr. urine were used to determine total nitrogen. To determine the proteic acids nitrogen barium salts sediment obtained by the procedure described above from 200 ml. of the 24-hr. urine was used. The sediment was dissolved in 20 ml. H_2O and 2 ml. samples, corresponding to 20 ml. of original urine were used. In the remaining solution the estimation of peptides was made. The results obtained with normal and pathological urines are shown in Table 2.

Table 2

Percentage of the proteic acids nitrogen in the total nitrogen of normal and pathological urines

Urine	24-hr. volume (ml.)	Total N (mg./24 hr.)	Proteic acids N	
			(mg./24 hr.)	(% of the total N)
Normal				
LK	1395	14 500	98.6	0.68
ZS	410	6 950	88.0	1.27
MS	1100	15 050	138.0	0.91
JN	1340	12 000	101.5	0.84
DN	1070	10 180	93.5	0.92
BG	865	11 200	136.6	1.22
Pathological				
AK leukaemia	1260	12 300	248.5	2.02
CC leukaemia	1340	13 400	292.0	2.18
EK leukaemia	1500	11 550	210.0	1.82
SL hepatic cirrhosis	1100	10 420	147.0	1.41
JS hepatic cirrhosis	370	5 800	91.5	1.57
SO hepatic cirrhosis	1090	7 750	102.0	1.32

The chromatographic analysis disclosed the presence of considerable amounts of peptides in the mixtures of proteic acids. An attempt was therefore made to express the proteic acid content as peptides in per cent of the total amount of compounds in urine reacting with the biuret reagent.

Estimation of peptides in the urine and in the proteic acid fraction was carried out by the method of Balikov, Lozano & Castello [4, 5] with

the exception that the readings were made at 570 mμ and not at 405 mμ. The relative peptide content was calculated from a glutathione standard curve.

The determinations were carried out as follows. 10 ml. urine or 5 ml. of solution of barium salts of proteic acids was shaken 5 min. with permutite. After centrifugation, 3 ml. of the supernatant was mixed with 5 ml. H_2O, 1 ml. 10% sodium tungstate, and 1 ml. 2/3 N-H_2SO_4. After 5 min. the sediment was removed by centrifugation; 5 ml. of the clear supernatant was mixed with 5 ml. of biuret reagent, left for 20 min. and again centrifuged for 5 min. Then, 30 min. after adding the biuret reagent, the extinction was determined at 570 mμ with the Hilger spectrophotometer. A blank sample was prepared in the same way with 3 ml. water in place of the examined solution. Results obtained in normal and pathological urines are shown in Table 3.

Table 3

Percentage of the proteic acids peptides in total peptides of normal and pathological urines

The amount of peptides is expressed in mg. of glutathione used as standard

Urine	24-hr. volume (ml.)	Total peptides (mg./24 hr.)	Proteic acids peptides	
			(mg./24 hr.)	(% of the total peptides)
Normal				
ZS	410	1850	351	19.0
MS	1100	2770	446	16.1
JN	1340	2640	483	18.3
DN	1070	3140	640	16.9
BG	865	3220	546	20.9
Pathological				
AK leukaemia	1260	5040	712	14.7
CC leukaemia	1340	5170	649	12.5
EK leukaemia	1500	3450	500	14.5
SL hepatic cirrhosis	1100	4590	678	14.8
JS hepatic cirrhosis	370	3080	437	14.2
SO hepatic cirrhosis	1090	3720	618	16.6

Results of the characterization of proteic acids. By employing the method of Gawiński for isolating barium salts of proteic acids from urine and further purification of the sediments by extraction with absolute alcohol, followed by repeated dissolution and precipitation with alcohol-ether, preparations were obtained suitable for chromatographic separation. Preparations from three normal urines were found to contain chro-

matographically similar substances, both in respect to their R_F values and the colour reactions with various reagents.

None of the mixtures of barium salts of proteic acids contained any uric acid or creatinine, and only traces of urea were found. The different mixtures revealed similar properties, were dialyzable, gave colour reactions with ninhydrin and isatin, and positive reactions for certain amino acids, such as Ehrlich's diazo, xanthoproteic, and Millon's reactions. They also reacted with Molisch's reagent and anthrone. The biuret reaction showed the presence of peptides. During chromatography in an acid solvent the Ba^{2+} ions were separated from proteic acids.

Acid hydrolysis of one of the isolated mixtures disclosed a large content of histidine, glycine, glutamic acid and proline, and smaller amounts of cysteine or cystine, aspartic acid, alanine, valine, leucine and tyrosine.

Determinations of nitrogen gave values which were much lower than those reported in literature. The nitrogen content, expressed as percentage of total urinary nitrogen, ranged from 0.68 to 1.27%.

Peptides precipitated from urine in the conditions used for isolating proteic acids were, in normal urines, 16.1 to 20.9% of the total of biuret-positive substances excreted by the kidneys.

Proteic acids isolated from six pathological urines (hepatic cirrhosis, leukaemia) compared with those from normal urines showed different contents of nitrogen and peptides.

Separation of proteic acids into alloxy-, antoxy-, and oxyproteic acids and characteristics of the different fractions

Bądzyński *et al.* [7] fractionated the proteic acids into three groups of compounds which they designated antoxy-, alloxy-, and oxyproteic acids. A large part of earlier work on the proteic acids was concerned with these fractions, but the results were often highly controversial. It was therefore decided to separate the proteic acids fractions according to the method of Bądzyński, to study some of their properties and components.

The alloxy-, antoxy-, and oxyproteic acids were obtained from 500 mg. of barium salts of proteic acids precipitation from normal urine BG, and in the second case from 3 g. isolated from urine JP.

Separation of the alloxy-, antoxy-, and oxyproteic acids. To separate the different fractions the method of Bądzyński *et al.* [7] was used. The sediment of barium salts of proteic acids was dissolved in water, 25% solution of basic lead acetate was added until precipitation was complete and the insoluble plumbous salts of the alloxyproteic acids were centrifuged. A saturated Na_2CO_3 solution was added to the supernatant and

the precipitate of $PbCO_3$ was removed by centrifugation. The remaining solution was neutralized with acetic acid, concentrated *in vacuo* at 40°, brought to pH 6 with acetic acid and then a 20% solution of mercuric acetate was added; the precipitate contains, according to Bądzyński, mercuric salts of antoxyproteic acids. The remaining solution was extracted three times with ether and after mild alkalinization with Na_2CO_3 a precipitate of mercuric salts of oxyproteic acids was obtained.

To transform the mercuric salts of antoxy- and oxyproteic acids into barium salts, the precipitates were washed with water until the filtrate gave negative reaction for Cl^- ions and then decomposed with hydrogen sulphide; the HgS was removed by centrifuging and H_2S by aeration. Saturated $Ba(OH)_2$ was added to slight excess; surplus of Ba^{2+} ions was removed with CO_2 and the solution was concentrated *in vacuo* at 40°. The barium salts of the corresponding acids were then precipitated with a mixture of absolute alcohol and ether (2 : 1, v/v).

The barium salts of the alloxyproteic acids were obtained according to Bądzyński. The plumbous salts were washed with a small volume of water, and 0.1% solution of oxalic acid was added. After centrifugation the supernatant was discarded and concentrated oxalic acid was added to the sediment, followed by centrifugation. After adding to the supernatant the concentrated solution of $Ba(OH)_2$ the further procedure was the same as that with which the barium salts of antoxy- and oxyproteic acids were obtained.

All three fractions of proteic acids gave positive xanthoproteic and biuret reactions. The reactions with ninhydrin and isatin were positive and approximately equal for all three fractions and the initial mixture. The alloxy-, as well as antoxy- and oxyproteic acids contained sulphur splitting off in alkaline medium in the form of sulphide ion, gave weak, barely visible reactions with Millon's reagent, and positive reactions with Molisch's reagent and anthrone. Only Ehrlich's diazo reaction, in accordance with Bądzyński's findings, was strongly positive with the antoxyproteic acid fraction, although the alloxy- and oxyproteic acids also reacted weakly with this reagent.

Chromatographic separation of the peptides. Analysis of the peptide composition of the different fractions of proteic acids was carried out with a combination of ascending and descending chromatography. Whatman No. 1 filter paper in 4 × 37 cm. strips was employed. In order to obtain good separation of the peptides, maximum development was first carried out by the descending technique in a solvent composed of *n*-butanol - glacial acetic acid - water (144:13:43). Neutral red, applied in alcoholic solution together with the peptides, was used as indicator of maximum development. In this solvent system the R_F of neutral red is somewhat greater than that of the fastest peptide. Development was

therefore finished as soon as the dye was washed out from the chromatogram. The chromatograms were then dried and developed in the same direction with the ascending technique, with the same solvent system as before. In this way separation of the peptides was achieved on a maximum distance and during rechromatography the peptide spots were concentrated on a smaller area. Peptides were located with 0.5% ninhydrin dissolved in acetone, 0.3% isatin in acetone, and Pauly's reagent.

The chromatograms showed similarity of many peptide spots in all three fractions of the proteic acids, differences being rather quantitative than qualitative. This was shown especially in the amount of histidine-containing peptides, large amounts of which were observed particularly in antoxyproteic acids. Urea was not found in any of the fractions of proteic acids.

Electrophoretic separation of peptides. Peptides in the mixtures of barium salts of alloxy-, antoxy-, and oxyproteic acids were separated by means of high-voltage electrophoresis into fractions of acidic, neutral and basic peptides. Separation was carried out in an apparatus of the Wieland & Pfleiderer type [54], employing a pyridine buffer, pH 6.0. This buffer was prepared by mixing 2.5 ml. glacial acetic acid with 25 ml. freshly distilled pyridine and making up the volume to 250 ml. with water. Strips of Whatman No. 1 filter paper, 59 × 5 cm., were employed, on which 0.02 ml. of the solution of barium salts of proteic acids in buffer was applied, corresponding to 2.5 mg. of dry weight of the preparation. Electrophoresis was conducted for 45 min. at 1800 v. Peptides were localized as in paper chromatography with ninhydrin, isatin, and Pauly's reagent.

It was found that both the total mixture of barium salts of proteic acids and the barium salts of alloxy-, antoxy-, and oxyproteic acids in these conditions were separated into four fractions (Fig. 1).

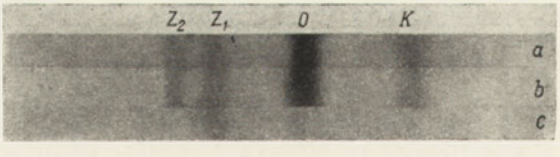

Fig. 1. Electrophoretic separation of peptides contained in the mixture of proteic acids. Stained with (*a*), ninhydrin; (*b*), isatin (*c*), Pauly's reagent

K — fraction of acidic peptides giving practically negative reaction with Pauly's reagent, and a green colour with isatin.

O — fraction of neutral peptides, giving positive reaction with Pauly's reagent and a violet colour with isatin.

Z_1 — fraction of basic peptides with slower mobility, giving very strong reaction with Pauly's reagent, and a gray colour with isatin.

Z_2 — fraction of basic peptides, somewhat faster, not reacting with Pauly's reagent, and giving a gray colour with isatin.

All four electrophoretic fractions of peptides reacted positively with the aniline-phthalate reagent.

It was noted that the urinary pigment which accompanies the proteic acids migrates together with fraction *K*, but occupies greater area than the acidic peptide spot detected with ninhydrin or isatin. Ehrlich's reaction with *p*-dimethylaminobenzaldehyde gave a light yellow spot of urea, lying in the area of the *K* peptide fraction. Spots corresponding to other peptide fractions did not react with this reagent. The Ba^{2+} ions detected with potassium chromate, as previously described, gave spots lying very near the end of the filter paper strip connected with the negative pole. Hence, after electrophoretic separation the peptide fractions were composed of free peptides.

Comparing the intensity of the spots with ninhydrin in electropherograms of alloxy-, antoxy-, and oxyproteic acids. differences in the amount of fractions *K*, *O*, Z_1 and Z_2 were noted.

Estimation of peptides in various fractions after electrophoretic separation. For estimation of peptides in electrophoretic fractions the biuret method was not suitable because the amounts were below the sensitivity of this method. Hence, the ninhydrin method was used, although it was realized that only approximate values could be obtained, in view of the different sensitivity of the ninhydrin reaction to different peptides. The procedure was based on the method of Kay, Harris & Entenman [36] for amino acids estimation on chromatograms. The ninhydrin positive spots were eluted and the extinction was determined in the Coleman spectrophotometer at 575 mμ.

From the results presented in Table 4 it can be concluded that in the mixture of proteic acids the neutral peptides predominate, the acidic peptides occur in smaller and the basic peptides in the smallest amounts. After separation into alloxy-, antoxy-, and oxyproteic acids the amount of acidic peptides is increased in all three fractions of proteic acids, and basic peptides containing histidine are most abundant in the antoxyproteic acids.

The method of proteic acids fractionation employed by Bądzyński *et al.*, besides being very tedious, is not accurately described and the procedure is associated with considerable loss of material; moreover, analysis of peptide composition of the three fractions showed only quantitative differences. Therefore an attempt was made to elaborate other methods of separation giving better defined fractions.

Separation of peptides in a mixture of proteic acids by means of high-voltage electrophoresis and paper chromatography, and properties of the different peptides

High-voltage paper electrophoresis. Apparatus and buffer of pH 6.0 as previously described were employed. 250 mg. of the mixture of barium salts of proteic acids obtained from the urine I (Table 4) was dissolved in 1.0 ml. pyridine buffer. From this solution 0.1 ml. was applied to the strips 59 × 7.5 cm. of Whatman No. 3MM filter paper. Electrophoresis was carried out during 60 min. at 1650 v.

A part of the electropherogram 1 cm. wide was cut off, and peptides were located with 0.5% acetone solution of ninhydrin. From the remaining strip areas corresponding to the spots were cut out and eluted with water; the eluates were concentrated *in vacuo,* at 37°, and dissolved in an appropriate volume of water and then subjected to chromatographic analysis. The volume of solution was chosen on the basis of the previously determined amounts of the different electrophoretic fractions, with the object of obtaining approximately equivalent concentrations of the peptides. Fraction *K* was dissolved in 0.60 ml., fraction *O* in 2.50 ml., Z_1 in 0.55 ml., and Z_2 in 0.45 ml. of water.

Table 4

Quantitative ratio of electrophoretic fractions of peptides

The amounts of peptides are expressed in terms of extinction of ninhydrin positive eluates. Peptides: *K*, acidic; *O*, neutral; Z_1, basic; Z_2, basic

Urine I	*K*	*O*	Z_1	Z_2
Proteic acids	1.30:	5.50:	1.22:	1.00
Alloxyproteic acids	6.60:	6.28:	0.50:	1.00
Antoxyproteic acids	9.80:	13.6 :	7.80:	1.00
Oxyproteic acids	6.50:	19.1 :	1.30:	1.00
Urine II				
Proteic acids	4.35:	7.00:	1.82:	1.00
Alloxyproteic acids	3.75:	3.71:	0.76:	1.00
Antoxyproteic acids	10.26:	13.25:	4.05:	1.00
Oxyproteic acids	3.75:	7.48:	0.66:	1.00

Chromatographic separation of the peptides. The paper chromatographic method described on p. 399 was employed for the separation of the different fractions of peptides eluted from the electropherograms; the volumes applied to chromatography were 20 µl. of each; and after development, a part 1 cm. wide was cut off from each strip and peptides located with ninhydrin solution in acetone.

When determining the location of the peptides in the chromatograms an R_x value was introduced. This value expresses the ratio of the distance of the middle of the peptide spot from its point of application to the distance of the middle of the fastest moving spot from the point of application. The R_x values of different peptides obtained from electrophoretically separated peptides are presented in Table 5.

Table 5

R_x *values of peptides*

Electrophoretic fraction	Peptide	R_x	
		average	limits
Basic, Z_1	Z_1 *I*	0.12	0.12—0.12
	Z_1 *II*	0.50	0.48—0.51
	Z_1 *III*	0.86	0.85—0.87
	Z_1 *IV*	1.00	—
Basic, Z_2	Z_2 *I*	0.34	0.33—0.35
	Z_2 *II*	0.54	0.54—0.54
	Z_2 *III*	0.78	0.78—0.78
	Z_2 *IV*	1.00	—
Neutral, *O*	*O I*	0.14	0.12—0.15
	O II	0.24	0.22—0.26
	O III	0.33	0.31—0.35
	O IV	0.53	0.52—0.54
	O V	0.68	0.67—0.69
	O VI	0.77	0.76—0.77
	O VII	1.00	—
Acidic, *K*	*K I*	0.08	0.08—0.08
	K II	0.23	0.23—0.23
	K III	0.33	0.32—0.33
	K IV	0.44	0.43—0.44
	K V	0.57	0.57—0.57
	K VI	0.71	0.71—0.71
	K VII	1.00	—

From the remaining strip the peptides were eluted with water from the areas corresponding to the stained spots. The eluates obtained from 20 strips were concentrated to dryness *in vacuo* at 37°, and dissolved in 2 ml. H_2O; two 0.2 ml. samples were taken for C-terminal amino acids determination and two 0.4 ml. samples for determinations of N-terminal amino acids. The remaining solution was again concentrated to dryness as before, and the residue dissolved in 2 ml. 6 N-HCl and hydrolyzed. In the case of peptides in which only the amino acid composition was determined, the dry residue after the concentration of the eluate was dissolved directly in 2 ml. 6 N-HCl and subjected to hydrolysis.

Determinations of the amino acid composition of peptides. The solutions in 2 ml. 6 N-HCl were hydrolyzed at 100° for 24 hrs. in tightly stoppered test tubes. Then the hydrolyzate was concentrated to dryness *in vacuo* at 50°, and the residue dissolved in a small volume of water; HCl was removed by concentrating and dissolving in water three times. Finally, the residue was dissolved in 0.2 ml. water and the amino acid composition of the hydrolyzate was determined chromatographically.

Circular chromatography was employed with lateral application of the samples [45], and ascending chromatography. Whatman filter paper No. 1 was used, the diameter of the disks being 20 cm., and the length of the strips 37 cm. Chromatograms were developed in a system of *n*-butanol - acetic acid - water (144 : 13 : 43). The amino acids were identified by comparison with standard mixtures using differential detecting [45] and reagents specific for the various amino acids.

C-terminal amino acids. The method of hydrazinolysis described by Akabori *et al.* [2] was chosen for the determination of C-terminal amino acids in the different peptides eluted from the chromatograms. In spite of the reservations of Locker [41] and Ching I Niu & Fraenkel-Conrat [18] the hydrazinolysis method gave quite satisfactory qualitative results.

Commercial hydrazine hydrate was dehydrated according to Keiichi Kusama [37], and the water content was then determined by iodometric titration according to Kolthoff [38].

For each peptide, hydrazinolysis was carried out with two parallel samples, as follows. Into a test tube 0.2 ml. of the solution of the peptide was added and dried *in vacuo* over concentrated H_2SO_4. Then 0.2 ml. of dehydrated hydrazine was added, the test tube was tightly stoppered, and heated in the oven at 100° for 10 hrs. When hydrazinolysis was completed the excess of hydrazine was removed by drying *in vacuo* over concentrated H_2SO_4 for 24 hrs. To remove the hydrazides, 0.5 ml. H_2O and 0.5 ml. of a mixture of freshly distilled benzaldehyde in benzene (1 : 1, v/v) was added to the dry residue and shaken for 2.5 hrs. The benzene layer was discarded, and the remaining aqueous solution was washed five times with benzene to remove benzaldehyde and centrifuged each time to ensure accurate separation of the layers. The aqueous solution containing free C-terminal amino acids was dried over concentrated H_2SO_4, dissolved in 0.2 ml. H_2O and subjected to chromatographic analysis according to the method previously described.

N-terminal amino acids. N-terminal amino acids were determined as phenylthiohydantoin derivatives according to Edman as modified by Sjöquist [49]. Phenylthiohydantoin derivatives of amino acids used as standards were prepared by the method of Edman [27]. The purity of the preparations was checked chromatographically and by determinations of melting points.

Determinations of N-terminal amino acids were carried out on two parallel samples of each peptide employing 0.4 ml. of peptide solution for each determination. After chromatographic separation the spots of phenylthiohydantoin derivatives of amino acids were located in ultraviolet light.

RESULTS

High-voltage electrophoresis permitted separation from a mixture of barium salts of proteic acids of four peptide fractions: acidic (*K*), neutral (*O*), and two basic fractions (Z_1) and (Z_2). All four fractions gave colour reactions on filter paper with ninhydrin, and somewhat weaker colour reactions with isatin. All the fractions also reacted positively with the aniline-phthalate reagent. Pauly's reaction was most distinct with fraction Z_1, less distinct with fraction *O*, and still weaker with fraction *K*. The second fraction of basic peptides (Z_2) did not react at this concentration with Pauly's reagent.

During electrophoresis complete separation of the Ba^{2+} ions from the proteic acids was achieved.

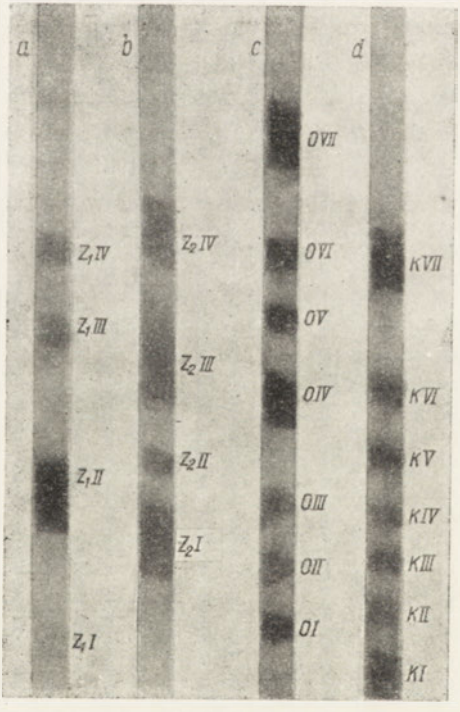

Fig. 2. Paper chromatography of peptides separated from proteic acids by high-voltage electrophoresis. Electrophoretic fractions: (*a*), basic Z_1; (*b*), basic Z_2; (c) neutral *O*; (*d*) acidic *K*. Chromatogram stained with ninhydrin

Fraction Z_1 after chromatographic separation gave four distinct peptide spots, Z_1 *I* to Z_1 *IV* (Fig. 2). Peptide Z_1 *II* was present in the largest amounts. With isatin it gave a dark-violet colour, and an intensive reac-

tion with Pauly's reagent. Peptides Z_1 *III* and Z_1 *IV* were present in much smaller amounts, but also gave positive reactions with Pauly's reagent. Peptide Z_1 *I* occurred in approximately the same amounts as peptides Z_1 *III* and Z_1 *IV*, although in some of the chromatograms a tendency to further separation was noted. In view of this observation, this peptide was not further studied. In peptides Z_1 *III* and Z_1 *IV* the composition of the amino acids was determined, and in peptide Z_1 *II* also the C- and N-terminal amino acids.

Fraction Z_2 also separated in the chromatograms into four peptide spots: Z_2 *I* to Z_2 *IV*. As in fraction Z_1, one of the peptides Z_2 *I* dominated over the remaining ones. It gave a brown-violet colour reaction with isatin, but with Pauly's reagent only a barely visible pink reaction. This peptide was analysed for amino acid composition and both terminal amino acids were determined. Peptides Z_2 *II - IV* were in much smaller amounts and did not react with isatin or Pauly's reagent.

Fraction *O* after chromatographic separation gave seven peptide spots, *O I - O VII* (Fig. 2c). Peptides *O IV* and *O VII* gave the strongest colour reaction with ninhydrin and a pink reaction with isatin. Peptide *O IV* also reacted weakly with Ehrlich's aldehyde reagent and with Pauly's reagent. In both of these peptides the amino acid composition and terminal groups were determined. With isatin peptide *O I* gave a violet reaction, peptide *O V* a pink-orange and peptide *O VI* a pink one. With Pauly's reagent, only peptide *O I* gave a very weak reaction. Peptides *O II* and *O III* were not sufficiently separated in the chromatograms and therefore were not analysed.

Table 6

Amino acid composition of some urinary peptides

Peptide	C_yS+C_ySH	Lys	His	Gly	Arg	Asp	Glu	Ala	Pro	Tyr	Val	Leu	Phe	Unknown
Z_1 II			●●●●	●			●●	●●			●	●		1
Z_1 III		●●	●●	●●		●●	●●	●●	●		●●	●●		1
Z_1 IV		●●	●●	●●		●●●	●●	●●	●●		●●		●●	
Z_2 I		●●●●	●	●●			●●	●			●●	●		1
Z_2 II		●●		●●		●●	●●	●●						
O I	●●		●●	●●		●●	●●	●●	●●					
O IV			●●	●●●		●●●	●●	●	●		●●	●●		
O V	●●	●●			●●			●●●				●●		1
O VI		●●		●		●●	●●●	●●	●●			●●		
O VII	●●	●●		●●			●●	●●	●●					
K II	●●●	●●	●	●●		●●	●●	●●	●●	●●●				
K V	●●		●	●●		●●	●●	●●	●●					
K VI	●●	●●	●	●●		●●	●●	●●	●●					
K VII	●●	●●	●	●	●●	●	●●	●●	●					

● *Traces*, ●● *Moderate amount*, ●●● *Large amount*, ●●●● *The main component of the peptide*

Fraction *K* separated in the chromatograms into peptides *K I* to *K VII*. The amounts of each of the peptides estimated on the basis of the intensity of the colour reaction with ninhydrin were found to be similar. Peptides *K I*, *K III* and *K IV* were not analysed; peptide *K I* was accompanied by a yellow pigment which resulted in the tailing of the spot up to the point of application, and peptides *K III* and *K IV* were not satisfactorily separated. In the remaining peptides, i.e. *K II*, *K V*, *K VI* and *K VII*, the amino acid composition and terminal groups were determined. All the peptides gave a greenish-gray colour with isatin; only peptides *K II* and *K V* reacted, although very weakly with Pauly's reagent.

Table 6 shows the amino acid composition of 14 peptides isolated from the mixture of barium salts of proteic acids. The approximate content of amino acids was assessed only on the basis of the intensity of the colour reaction with ninhydrin and isatin on the chromatograms.

Table 7

Terminal amino acids of peptides

Details in the text

Peptide	N-terminal amino acid	C-terminal amino acid
Z_1 *II*	—	histidine
Z_2 *I*	—	lysine
K II	alanine	glycine
K V	alanine	glycine
K VI	glycine	glycine
K VII	glycine	glycine
O IV	glycine	glycine
O VII	glycine	alanine

The spots listed in the table as unknown concern substances which gave colour reactions with both ninhydrin and isatin, but could not be ascribed to any of the known amino acids occurring in proteins.

Determinations of N-terminal and C-terminal amino acids in 8 peptides gave results presented in Table 7.

DISCUSSION

In the present paper it was demonstrated that the so-called proteic acids of Bądzyński are mixtures of peptides which can be isolated from urine in the form of barium salts. They constitute a fairly homogeneous group; at any rate, the composition of the mixtures isolated in the same conditions is practically identical. Since the fractionation according to Bądzyński *et al.* [7] is associated with considerable loss of material, and the

obtained fractions cannot be well characterized, this method of separating of proteic acids was abandoned. The adopted high-voltage electrophoresis separated proteic acids into 4 different peptide fractions. By separating these fractions by means of paper chromatography it was possible to show that the mixture of proteic acids contains at least 14 different peptides and 8 compounds which are probably also peptides.

Little is known about peptides occurring in urine. Apart from hippuric acid, no peptides have been isolated and analysed. The presence of bound amino acids in the normal and pathological urine has been reported frequently. Their amounts and general characters have been determined only on the basis of increase of amino acids after acid hydrolysis. Stein [50] after acid hydrolysis of 24-hr. urine found the greatest increase of glycine, glutamic acid, aspartic acid, histidine and proline; a smaller increase of serine, lysine, tyrosine, cystine with cysteine, threonine, alanine, valine, phenylalanine, and leucine; arginine, isoleucine and methylhistidine were present only in traces. Large amounts of glycine, glutamic acid, aspartic acid and histidine after hydrolysis of urine have also been reported by Dunn *et al.* [21], who used a microbiologic test. In addition to the mentioned amino acids, Müting [44] found large amounts of methionine, which was previously reported also by Albanese *et al.* [3].

Similar results were obtained after acid hydrolysis of isolated proteic acids peptides. Out of 14 peptides which were analysed, alanine was present in all cases, glycine, histidine, aspartic acid and proline in 13, cysteine or cystine in 7, leucine in 6, and valine in 5; only two peptides contained arginine, and one tyrosine and phenylalanine. In comparison with the other peptides, the proteic acids are characterized by a comparatively large content of alanine, and absence of serine and threonine. Methionine, found in urinary hydrolyzates by Müting, was not present in our material. The large amounts of methionine found by this investigator (335 - 365 mg. per 24 hr.), however, have not been confirmed by other authors.

Some detailed analyses were made of the small-molecular peptides occurring in urine. These, however, cannot be compared with proteic acids peptides which are composed at least of five different amino acids. These peptides can be compared only with the peptides isolated from urine by Hanson & Fittkau [35]. They are, however, different. Hanson & Fittkau did not find any histidine in any of 17 isolated peptides, whereas 10 of the proteic acids peptides were found to contain it. Moreover, one of the peptides was composed almost exclusively of histidine, with only traces of other amino acids. Another basic peptide was built almost exclusively of lysine. A similar urinary peptide, but containing only lysine, has already been reported by Westall [53].

In the isolated lysine-peptide (Z_1 *II*), as well as in the histidine-peptide (Z_2 *I*) no terminal amino groups were found. Since both peptides contained free terminal carboxyl groups, excluding cyclic structure, it may be probable that they occur in an acetylated form. The presence of this type of peptides in urine was also assumed by Plaquet *et al.* [46].

Some of the peptides contained sugar since both the whole mixtures of proteic acids barium salts and the electrophoretic fractions gave positive reactions with Molisch's reagent, with anthrone, and with aniline--phthalate. These reactions were seen most clearly in the fractions of acidic and neutral peptides. The possibility of combinations of sugars with the amino acids is supported by the finding of Pollack & Eades [47] of large amounts of glycuronic acid bound with aspartic and glutamic acids in urine. The presence in urine of peptides bound with a sugar component is also postulated by Bode *et al.* [11]. On the other hand, Hanson & Fittkau [35] did not find any sugar in their peptides.

Some of the investigated peptides after hydrolysis displayed on chromatograms some spots reacting with ninhydrin and isatin which could not be identified with the available amino acid standards.

Although the origin of these peptides was not investigated, it is possible that some of them may be identical with the biologically active peptides encountered in tissues and body fluids, probably performing functions of tissue hormones [28]. This view is supported also by some data of Hanson & Fittkau [35].

The proteic acids constitute not more than 20% of the compounds occurring in urine giving a positive biuret reaction (Table 3); most of the components of this mixture belong to the higher polypeptides. Differences between the presented results and those of other authors, which have already been pointed out, probably result from the fact that most of the peptides described by other authors do not belong to the group of proteic acids.

The fact that proteic acids are a group of compounds capable of being accurately characterized and differentiated from other urinary peptides opens a new problem concerning changes in the levels of these compounds in normal and pathological urine, which has already been pointed out by Bądzyński *et al.* [7].

At that time, however, no adequate methods of separation and estimation of the proteic acids mixtures were available; the procedures used were very tedious and virtually not adapted to practical use. The procedure applied in the present work seems to simplify the problem; instead of considering proteic acids as a whole, we are able now to differenciate and characterize the individual fractions of urinary peptides.

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SUMMARY

Compounds included in the mixture isolated from urine by Bądzyński and designated as oxyproteic acids were investigated. Some properties of these compounds were studied and compared with those reported in the literature. The similarity of mixtures of proteic acids isolated from normal urines was demonstrated as well as their peptide nature. It was found that in normal urines the nitrogen of these compounds constitutes 0.68-1.27% of total nitrogen, and the peptides 16-21% of the sum of biuret-positive substances in the urine. Proteic acids isolated from pathological urines displayed quantitative differences pertaining to nitrogen and peptide content.

The peptide composition of proteic acids was studied. The fractionation of proteic acids into alloxy-, antoxy, and oxyproteic acids according to Bądzyński does not permit a separation into definite peptide fractions, and moreover is associated with considerable loss of material. The application of high-voltage electrophoresis and paper chromatography enabled isolation from the mixture of proteic acids of 22 substances giving colour reactions with ninhydrin and isatin. Fourteen of these substances were identified as peptides, and their amino acid composition was determined. The C- and N-terminal amino acids of eight of these peptides were determined.

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STRUKTURA PEPTYDOWA KWASÓW PROTEINOWYCH

Streszczenie

Poddano badaniu związki wchodzące w skład mieszaniny wyizolowanej z moczu przez Bądzyńskiego i nazwanej przez niego kwasami proteinowymi. Określono ogólne własności tych związków i porównano je z własnościami opisywanymi dotychczas w piśmiennictwie. Wykazano powtarzalność składu jakościowego mieszaniny kwasów proteinowych wyizolowanych z moczy prawidłowych, oraz uzasadniono charakter peptydowy tych substancji. Stwierdzono, że w moczach prawidłowych na azot tych związków przypada 0,68-1,27% azotu całkowitego moczu, natomiast peptydy zawarte w mieszaninie kwasów proteinowych stanowią 16-21% ogółu związków w moczu reagujących z odczynnikiem biuretowym. Kwasy proteinowe otrzymane z moczy patologicznych wykazały w porównaniu do moczy normalnych ilościowe różnice w zawartości azotu i peptydów.

Badano skład peptydowy mieszaniny kwasów proteinowych. Wykazano, że podział kwasów proteinowych na frakcje kwasów antoksy, alloksy- i oksyproteinowych wg metody Bądzyńskiego i współpr. nie doprowadza do rozdziału mieszaniny na określone frakcje peptydowe i wiąże się z dużymi stratami rozdzielanego materiału. Zastosowanie elektroforezy wysokonapięciowej i chromatografii bibułowej pozwoliło na wyizolowanie z mieszaniny kwasów proteinowych 22 substancji barwiących się ninhydryną i izatyną. 14 z tych substancji zidentyfikowano jako peptydy i określono ich skład aminokwasowy. Dla 8 peptydów oznaczono N- i C-końcowe aminokwasy.

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R. PAKUŁA, W. WALCZAK and D. SHUGAR

INACTIVATION OF THE STREPTOMYCIN RESISTANCE MARKERS OF THREE SPECIES OF BACTERIA BY IONIZING RADIATION

Departments of Microbiology and Biochemistry, State Institute of Hygiene, Warszawa

Ultraviolet and ionizing radiations have been widely used as tools for the biological inactivation of transforming DNA (T-DNA), largely with a view to investigation of the accompanying physico-chemical modifications. One of the most characteristic features accompanying the radiation induced inactivation of T-DNA is the complex nature of the dose-survival curve, the source of which is rather puzzling and for which no adequate interpretation has yet been advanced. In previous studies on the ultraviolet inactivation (2537 A) of the streptomycin-resistance markers of Haemophilus, pneumococci and streptococci [19], two-stage inactivation curves were obtained, similar to those reported by others for ultraviolet [26, 27, 28, 14, 6] and ionizing [16, 8, 9, 12, 5, 15] radiations. Entirely apart from the theoretical implications of the complex nature of the inactivation curves, their existence introduces particular complications in those instances where target theory is applied to estimate the size of genetic markers in T-DNA under conditions where presumably only the so-called direct effects of radiation are operative.

In the above-mentioned investigation on the ultraviolet inactivation of the streptomycin resistant (*Sr*) markers of the T-DNA's of several organisms, rather marked differences in radiation sensitivity were observed, e.g. the *Sr* marker of streptococcus required more than 100 times the dose necessary for Haemophilus to reduce the activity to a residual value of 1%. If only the initial slopes of the survival curves are compared, the difference is less, but still appreciable [19]. Experiments with model oligo- and polynucleotides have shown that moderate differences in sensitivity to ultraviolet radiation are readily explicable in terms of base composition and base sequences [23, 24], but differences of more than two orders of magnitude, even at low residual activities, are not so readily accounted for.

It consequently appeared of interest to us to examine the sensitivities of the above-mentioned three *Sr* markers to ionizing radiations, the more so in that the mechanism of action of the latter differs fundamentally from that due to ultraviolet light. In particular it was considered desirable to estimate, from target theory, the sizes of the three markers. Such measurements are subject to considerable theoretical uncertainties [15] and we have therefore placed greater emphasis on a comparison of the relative sizes of the three markers, irradiated under identical conditions. It is our feeling that, if target theory is at all applicable [1], such a procedure should remove at least some of the prevailing objections with regard to absolute measurements and we have been applying a similar technique to enzymes (Tramer & Shugar, in preparation).

MATERIALS AND METHODS

The donors used for transformation were the capsuleless *Rd* strain of *Haemophilus influenzae*, the pneumococcal rough strain *R36A* and, for streptococci, the strain *Streptococcus sanguis* type I/II which has previously been shown to give transformations in relatively high yields [18].

The receptor strains were mutants resistant to 2 mg/ml. streptomycin, as follows: strain *Rds* for *Rd;* strain *R36A, Sr* for *R36A;* and the Challis strain of group H haemolytic streptococci for *Streptococcus sanguis* type I/II.

Transforming DNA was prepared from the donor strains according to the method of Marmur (personal communication): a suspension of the cells in versene-saline was lysed by treatment with detergent; protein was then removed by means of the Sevag procedure and contaminating RNA by treatment with ribonuclease, followed once more by deproteinization by the Sevag procedure. In the case of streptococci, however, the washed cells were dissolved by means of the lytic factor from a Streptomyces strain in the presence of 0.1 M-sodium citrate [21]. DNA concentrations were estimated by the method of Burton [4].

The DNA samples were irradiated both in solution and in the form of dried films. The solvent medium was 0.15 M-NaCl. The dried preparations were formed as follows: microscope cover glasses, cut to a size of 10×20 mm., were carefully degreased and washed and 10 µl. of a solution of DNA in 0.15 M-NaCl deposited on each slide; if the slides have been properly cleaned, the entire surface is instantly covered by this volume. They were then dried for 30 min. at 30° in the presence of $CaCl_2$ (and *in vacuo* over P_2O_5 prior to irradiation, see below). Such preparations do not lose more than 25-30% of their transforming activity during a period of 18 - 20 hr. at room temperature.

For direct comparisons of the sizes of the sensitive volumes of the *Sr* markers of the DNA of the different bacterial species, the dried "films" were prepared from solutions containing a 1 : 1 mixture of two DNA samples in a 10% Difco yeast extract. The purpose of the yeast extract was to provide additional protection against the indirect effect of free radicals [15]. Irradiations were performed on mixtures of Haemophilus and streptococcal DNA and on Haemophilus and pneumococcal DNA. A combination of streptococcal and pneumococcal DNA could not be employed because of reciprocal transformation reactions [3, 17]. At the concentrations used (about 1.0 μg./cm.^{2} on the slides), the possibility of inhibition of streptococcal and pneumococcal transformations by *H. infl.* T-DNA is negligible [20]. Transformation of *H. infl.* is only very slightly inhibited by high concentrations of thymus or *E. coli* DNA and it is consequently extremely unlikely that minute concentrations of streptococcal and pneumococcal DNA could exert any effect.

The dried preparations on cover glasses were stacked parallel to each other on a specially constructed "scaffold", two cover glasses being located on each level and subjected to the same dose, the mean for the two being used to estimate % inactivation. After all the samples had been located on the scaffold, the latter was inserted into a metal cylinder which was then closed with a lid containing a rubber gasket. The cylinder contained a receptacle with P_2O_5 and was exhausted to a pressure of a couple of mm. Hg. The roof of the cylinder consisted of a thin layer of aluminium foil.

The irradiation source consisted of a disc of 50 Curies ^{60}Co mounted on a rod which could be displaced vertically; the source was brought down until it made contact with the roof of the cylinder containing the samples. The dose rate at various distances below the source was obtained from a calibration curve prepared by means of a ferrous sulphate dosimeter. However, for those samples almost in contact with the source, there is probably an appreciable error in the calculated dose. Hence in Figs. 3, 4 and 5 the final points, corresponding to the highest doses used, should be regarded with some reserve.

Solutions were irradiated in 5 mm. in diameter test tubes supported at various positions on a plastic stand placed symmetrically with respect to the source. Duplicate samples were irradiated at any given distance from the source. Irradiation periods for solutions were from 20 to 120 min., which corresponds to a maximum of 30×10^3 r., and for dried preparations about 17 hr. and 15×10^6 r., respectively.

Transformations were carried out with competent cultures stored in 10% glycerol at -20° [10]. Following thawing the competent culture was diluted 10-fold in the transformation medium, warmed for 7 - 8 min. at 32°, and to 1.8 ml. of this diluted competent culture was added 0.2 ml. of

suitably diluted transforming factor. The mixture was kept for 30 min. at 32° and 120 min. at 37°, following which samples were seeded on agar plates with streptomycin for scoring of transformants.

RESULTS

Irradiation in aqueous medium

Fig. 1 presents the dose-survival curves for DNA samples irradiated in 0.15 M-NaCl solution at neutral pH. Each point is the mean of at least three independent experiments on duplicate samples. It will be seen that for each DNA sample the survival curve exhibits a break at approximately 1% residual activity.

Of the three different transforming factors, it will be noted that the sensitivity of *H. infl.* DNA is slightly greater for small doses and least for the larger doses. For streptococcal DNA the situation is reversed;

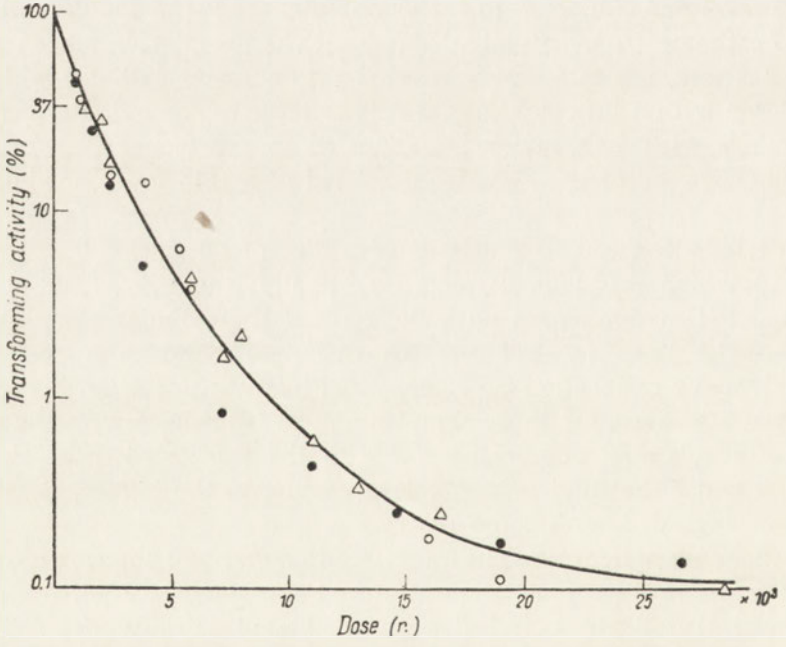

Fig. 1. Dose-survival curves in saline solutions of transforming DNA samples of: (△), *H. influenzae*; (○), Pneumococcus; (●), Streptococcus. Concentration of DNA in irradiated solutions 1 μg./ml.; 0.1 μg./ml. used for transformations

it is most sensitive for small doses and least sensitive for larger doses (not included in the Figure). On the whole, however, the differences between the three transforming factors are not appreciable and attention

is drawn to them only because of the reproducibility of the results. For the DNA concentration used, the $D_{37\%}$ dose is about 900 r. for pneumococcal T-DNA as compared to a value of 750 r. reported by Latarjet *et al.* [15] for the same marker in liquid solution under analogous conditions.

As might be expected the influence of concentration is quite marked. Fig. 2 exhibits the inactivation curves for concentrations of 1, 10 and 100 μg./ml. of streptococcal DNA. Only the initial portions of the survival curves are shown, but the lower segments exhibit the usual break

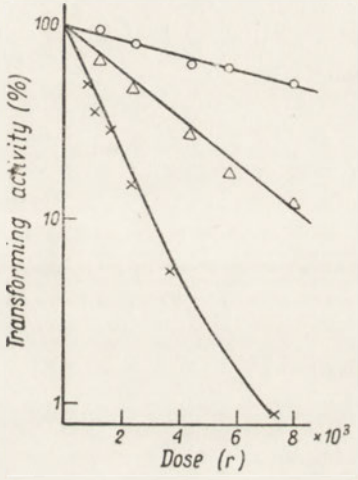

Fig. 2. Dose-survival curves for streptococcal DNA in saline solution at concentrations of: (×), 1 μg./ml.; (△), 10 μg./ml.; (○), 100 μg./ml.; with 0.1 μg./ml. used for transformation

at about 2 - 3% residual activity. The slopes of the curves for 1, 10 and 100 μg./ml. correspond to first-order inactivation rates in the approximate ratio 9:3:1 respectively.

Irradiation of dried preparations

The survival curves for dried DNA films are represented in Fig. 3 and are considerably more complex than the analogous curves obtained by irradiation in aqueous medium. The curves in Fig. 3 may be resolved into three, or even more, straight line components. The initial components, to about 10 - 30% residual activity, are approximately linear, bearing in mind the experimental error in the individual determinations of transforming activity. It should, nonetheless, be emphasized that the breaks shown in the figure for the individual DNA preparations were found to be reproducible in repeated experiments. As for the curves for inactivation in aqueous medium, each point represents the mean of three separate experiments with duplicate samples.

Furthermore, as for DNA in solution, *H. infl.* DNA exhibits initially a higher radiation resistance, while streptococcal DNA is most sensitive

For the lower portions of the curves, at residual activities of about 1 - 3%, the sensitivity of *H. infl.* DNA is increased with respect to that for pneumococci. On the other hand, at very high doses, not represented in the Figure, it becomes more sensitive than streptococcal DNA. Summarizing, it appears that the relative radiation sensitivities for the DNA films are qualitatively similar to those for solutions, but the differences are considerably more marked, quantitatively, for the films.

These differences are appreciably enhanced for DNA films prepared from solutions containing 10% yeast extract, the effect of which is, presumably [8], to reduce even further the influence of any indirect action of the radiation. All of these experiments were conducted on films containing a mixture of two different DNA samples as described above.

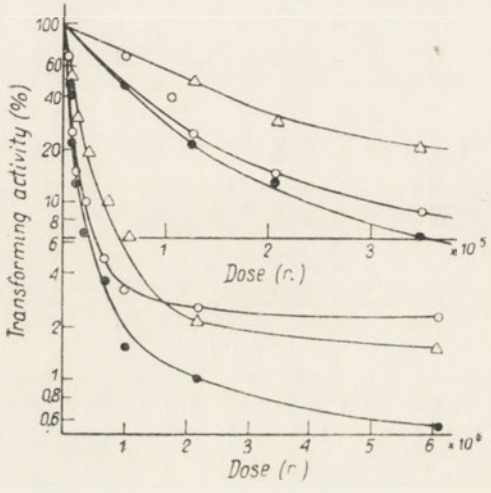

Fig. 3. Dose-survival curves of preparations of transforming DNA dried from saline solutions (2 μg. DNA on cover slip); 0.5 μg./ml. used for transformation. (△), *H. influenzae*; (○), Pneumococcus; (●), Streptococcus

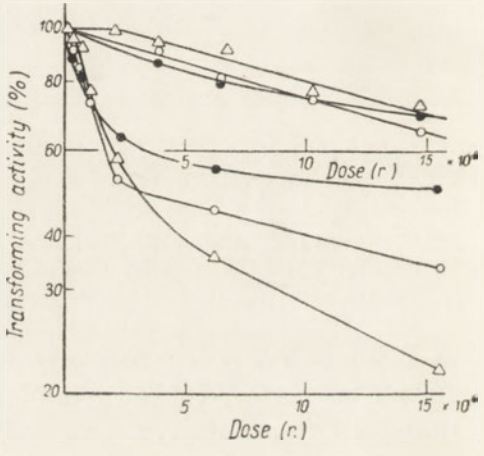

Fig. 4. Dose-survival curves in presence of yeast extract for dried transforming DNA of: (△), *H. influenzae*; (○), Pneumococcus; (●), Streptococcus; with 2 μg. DNA of each on cover glass (see text for details); for transformation 0.5 μg./ml. DNA used

From a comparison of Fig 3 and 4, it will be observed that there is a marked protective effect due to the presence of the yeast extract. This is shown also in Table 1. The figures given in the Table are, of course, only representative and will vary considerably for different residual activities, particularly beyond the points where the curves exhibit pronounced breaks, as can be seen from Fig. 4. To a residual activity of 70%, *H. infl.* DNA is more resistant than streptococcal DNA; below 70% residual activity, the relationship is reversed.

Table 1

Doses, in roentgens, required to reduce activity of transforming DNA films to 50% in presence and absence of yeast extract

T-DNA	Without yeast extract	With 10% yeast extract
H. influenzae	1.2×10^5	2.8×10^6
D. pneumococci	0.6×10^5	2.3×10^6
Streptococci	0.5×10^5	16×10^6

The rather complex form of the survival curves in the presence of yeast extract raises considerable doubts as to the validity of simple target theory for estimation of the size of the units responsible for biological activity. To a rough approximation, each of the curves in Fig. 4 may be regarded as consisting of two components, with the main break occurring for each sample at residual activities well above 37%. If we extrapolate the initial portions of the curves to $1/e$ or 37% residual activity, and apply the usual procedure for calculation of the equivalent molecular weights of those portions of the DNA molecules responsible for transforming activity, we obtain the results shown in Table 2.

Table 2

Calculated Sr marker sizes in T-DNA

Transforming factor	D_{37} (r.)	Mol. wt.
Pneumococcal		
R36A	2.4×10^6	1.2×10^5
H. infl. Rd	3.2×10^6	0.9×10^5
Streptococcal		
Challis	4.1×10^6	0.75×10^5

If, as has been done by others, we use the slopes of the lower components of the curves to calculate marker sizes, the results fall well below the values of 10^5 in molecular weight units for all three DNA samples, a result the validity of which is most improbable.

More significant, however, is the reversal in relative sensitivities of the Challis and *Rd* markers at the higher radiation doses, as well as the pronounced differences in sensitivities between all three T-DNA samples exhibited at the higher radiation doses (Fig. 4). In fact if any doubts existed as to the reality of the differences in sensitivity between the different markers at low radiation doses, these are at once dispelled by the data for the higher doses.

If inactivation of T-DNA in the form of films in yeast extract is really provoked by the so-called direct effect, implying the applicability of target theory, the inactivation curves should be independant of the

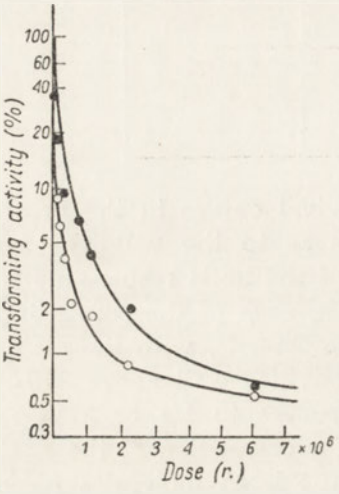

Fig. 5. Influence of concentration on dose-survival curve for dried streptococcal DNA. (O), 0.5 μg. DNA on cover slip; (●), 15 μg. DNA on cover slip. For transformation 0.5 μg./ml. used

concentration of material in the films. Some trials were made in which the DNA concentrations were 0.5 μg. and 15 μg/cm.^{2} (Fig. 5). It will be observed that a definite concentration effect does, in fact, exist.

DISCUSSION

The magnitudes of the various streptomycin markers calculated above from the initial slopes of the inactivation curves obtained in the presence of yeast extract are approximately of the same order of magnitude as that reported by Latarjet *et al.* [15] for *D. pneumococci* DNA irradiated in the presence of 10% yeast extract plus catalase in frozen solution. However, in view of the influence of yeast extract in our experiments on the shapes of the inactivation curves, and of the concentration of DNA on the rate of inactivation, the validity of calculations of marker size from simple target theory remains, in our opinion, at best questionable. Table 3 presents the minimum values for marker sizes calculated by several observers from target theory with ionizing radiations and from quantum yields for inactivation with ultraviolet light (2537 A).

More significant, in our opinion, is the demonstration of differences in apparent size of the markers from different DNA samples. This finding is quite unequivocal, the more so in that the differences, if any, are so slight in aqueous medium where the inactivation process is due entirely to the action of free radicals in the solvent medium.

It is therefore of interest to recall the finding of Marmur & Fluke [16] on a dried preparation of pneumococcal T-DNA with four different markers, the dose-survival curves for all of which were indistinguishable from each other. In view of our own results it seems rather odd that four non-linked markers exhibited no differences in sensitivity and it is consequently of interest that the minimum size for a given marker calculated from their data is considerably higher than that reported by other observers (Table 3). A difference in sensitivity was reported by the foregoing authors for two pairs of linked markers but even this difference was not appreciable, and was most marked at low survival ratios, as for our own results.

Table 3

Calculated minimum size of Sr *markers in pneumococcal transforming DNA from target theory and quantum yields*

	Marker size (mol. wt. units)	Reference
From target theory with ionizing radiations	$1—5\times10^6$	Marmur & Fluke, 1955 [16]
	3×10^5	Guild & Defilippes, 1957 [12]
	$0.7—3\times10^5$	Latarjet *et al.*, 1959 [15]
	2×10^5	Hutchinson & Arena, 1960 [13]
	$\sim10^5$	Present work
From quantum yield at 2537 A	5×10^5	Lerman & Tolmach, 1959 [14]
From quantum yields for model oligonucleotides and changes in absorption spectrum & temp. profile	$\sim10^5$	Shugar, 1960 [23]

There is relatively little new we can add as to the nature and origin of the complexity of the inactivation curves. It seems to us, however, that the validity of all calculations of marker size remain questionable in the absence of a better understanding of the observed deviations from simple target theory. It is consequently not out of place to draw attention to at least a few of the anomalies encountered, not only in the present study, but also by other investigators.

Particularly striking is the effect of yeast extract on the residual activity at which the initial break in the inactivation curve occurs. A similar effect had previously been observed by Guild & Defilippes [12]; when T-DNA was dried from 4% yeast extract or brain-heart infusion broth, the initial break in the curve was raised from about 1% residual activity to between 15 and 30%; their result differs from ours in that the slope of the initial portion of the curve was unaltered. It should also be noted that X-ray inactivation of frozen or dried samples

of TMV-RNA was markedly affected by added substances: in the presence of 2% glutathione there was a two-fold decrease in sensitivity [11]. These findings were obtained under conditions of high vacuum, superior to our own, further testifying to the reality of the effect.

The influence of DNA concentration on the inactivation rate and shape of the curves has apparently not hitherto been reported but may well merit further study, particularly with particle radiations. It should be recalled that irradiation of dry films of DNA with particle radiations has been found to result in cross-linking between molecules [22, 2] and should consequently lead to an increased rate of inactivation with increasing concentration, as observed here (see above). The actual result of an increase in concentration is, however, a reduction in the rate of inactivation (see Fig. 5). Aside from this, previous trials with γ-radiation failed to show evidence of cross-linking by physico-chemical criteria at DNA concentrations similar to those used here [2].

Attention should also be drawn to the observation, amongst others, of Wilson [25] on the influence of added substances on the radiation sensitivity of enzymes in the dry state. When DNase, dried from buffer solution at a given molarity, was irradiated, the inactivation rate was found to vary over a $3^1/_2$-fold range when the pH of the buffer was varied from 4.7 to 7.5. The addition of various other substances was also found to affect the radiation sensitivity and, in particular, the DNase substrate, DNA. Some of the above findings may be questioned because of the unusual nature of the pH-activity curve for DNase presented by Wilson [25], which differs appreciably from the accepted one. But a number of other observers have reported similar effects of varying magnitude, and it has even been demonstrated by Alexander [1] that the presence or absence of air may markedly affect the results. The latter, as well as a number of other investigators, have ascribed some of these effects to energy transfer; this is certainly conceivable in those instances where a dried preparation of an enzyme with its substrate are irradiated. Whether this is the only explanation is still an open question; the possibility of cross-linking should certainly be examined in more detail. The fact remains that, in agreement with Alexander [1], ordinary target theory is certainly not applicable if the rate of inactivation is influenced either by the concentration of the molecule being irradiated or by the presence of other substances (either solid or gas) in the irradiated sample. However, while enzymes may be dried in the form of films from water, T-DNA may not without the presence of at least some salt if its activity is not to be destroyed.

There are several instances where inactivation of nucleic acids by ionizing radiations is apparently exponential over the entire range of the dose-survival curve. Hutchinson & Arena [13] report that the dose-sur-

vival curve for T-DNA in bacterial cells is exponential over several orders of magnitude of residual activity. The inactivation of TMV-RNA is likewise reported to exhibit no break [11] a finding in agreement with that of Englander *et al.* [7], who showed that an ionization in the backbone chain of TMV-RNA will cleave the strand with an efficiency of 100% and that a single ionization anywhere in the molecule is effective in rupturing bonds.

The inactivation of enzymes by ionizing radiations has usually been reported to follow an exponential course. But it must be borne in mind that, whereas transforming activity and virus infectivity can be measured with high sensitivity at very low residual activities, measurements of enzyme activities over a range of concentration of even two orders of magnitude are rather difficult. Enzyme dose-survival curves are consequently usually presented to residual activities of about 10 - 30%. There are, nonetheless, some instances where measurements have been made to low activities and, when this is done, examples appear where breaks do occur (Tramer & Shugar, in preparation). Hence the source of complexity of the inactivation curves for nucleic acids is not unique to the latter, a fact that must be borne in mind in any attempt to elucidate its nature.

Finally attention should be drawn to the experiments of Ellison & Beiser [6] on the UV inactivation of the *Sr* marker of pneumococcal DNA, the dose-survival curve of which showed a break at 40% residual activity. Column fractionation gave a number of fractions which exhibited similar behaviour: viz. an initial segment with a steeper slope and a second segment with the same slope as for the unfractionated DNA. One fraction was, however, isolated which exhibited a dose-survival curve with a single slope parallel to the second segments for all the other fractions, and hence also to the second segment for the unfractionated DNA. No quantitative data are, however, given as to what % of the total activity resided in this fraction which, apparently, was the only one out of several hundred to exhibit such a behaviour. Furthermore it was inactivated only to a residual activity of 20%, so that the absence of a second segment at lower residual activity cannot be considered as established.

SUMMARY

The streptomycin resistance markers of the transforming principles of a streptococcus, pneumococcus and *Haemophilus influenzae* have been inactivated by γ-irradiation in aqueous solution and in the dried state. The three transforming factors exhibited only slight, but detectable differences in sensitivity in aqueous medium. In the dried state these differences were considerably accentuated, particularly in the presence of a protecting substance such as yeast extract. The complex character

of the dose-survival curves for the dried preparations was appreciably enhanced in the presence of yeast extract and the relative sensitivities of the three DNA samples varied with the applied dose. Calculations from simple target theory indicated a minimum molecular weight of about 10^5 for the size of the markers; but the complex nature of the inactivation curves, and their dependance on DNA concentration and on the influence of yeast extract raises serious doubts as to the validity of such calculations. The results are discussed in relation to those of other observers.

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INAKTYWACJA PROMIENIOWANIEM JONIZUJĄCYM MARKERÓW STREPTOMYCYNO-OPORNOŚCI TRZECH GATUNKÓW BAKTERII

Streszczenie

Inaktywowano promieniowaniem γ ze źródła ^{60}Co markery streptomycyno-oporności czynnika transformującego. Poddano badaniu czynniki transformujące paciorkowca, dwoinki zapalenia płuc i szczepu *H. influenzae*, równolegle w roztworze 0,15 M-NaCl oraz w stanie suchym (błonki). W roztworach stwierdzono nieznaczne, lecz uchwytne różnice we wrażliwości czynników z badanych szczepów. W doświadczeniach z błonkami różnice te występowały o wiele wyraźniej, zwłaszcza w obecności substancji osłaniających, jak wyciąg z drożdży. Wyciąg ten potęgował również złożony charakter krzywych ilustrujących inaktywację markerów, przy czym wrażliwość danego DNA w porównaniu z pozostałymi zmieniała się w zależności od stosowanej dawki promieniowania.

Stosując teorię trafień można wyliczyć, że minimalny ciężar molekularny markerów streptomycyno-oporności wynosi około 10^5. Jednakże złożony charakter krzywych inaktywacji, zależność ich przebiegu od stężenia DNA i wpływ wyciągu z drożdży, budzą poważne wątpliwości co do wartości tych obliczeń.

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HALINA SIERAKOWSKA and D. SHUGAR

(with the technical assistance of Halina Szemplińska)

GROSS HISTOCHEMICAL LOCALIZATION OF TISSUE NUCLEASE ENZYMES

Institute of Biochemistry & Biophysics, Polish Academy of Sciences, and Department of Biochemistry, State Institute of Hygiene, Warszawa

Of the relatively few attempts hitherto devoted to the cytochemical localization of enzymes attacking nucleic acids, undoubtedly the most promising is that of Marshall [13] using the antigen-antibody technique. No attempts appear yet to have been made to repeat or extend this technique, presumably because of the fact that it is so arduous.

In a previous publication [16] we presented the details of some trials, using standard cytochemical staining techniques, for nuclease enzymes based on the use of substrates specific for those enzymes attacking RNA, viz. the nucleoside cyclic phosphates. The incubation medium contained, in addition to the substrate, exogenous alkaline phosphatase and calcium ions. Following opening of the cyclic phosphate ring by tissue RNase [1], the resulting 2′(3′)-nucleotide was dephosphorylated by the exogenous phosphatase and the liberated phosphate precipitated with the calcium and finally stained according to the Gomori procedure for alkaline phosphatase [10]. The results were, however, found to be largely artifactual, principally because of the high rate of diffusion of the enzymes during incubation. The same phenomenon, as well as several additional factors [16] were found to prevail and render dubious the techniques of Aronson *et al.* [2] and Alridge *et al.* [1] for acid deoxyribonuclease. The recently reported method of Zugury *et al.* [19], while possibly limiting enzyme diffusion by the use of rapid formalin fixation, most likely suffers from artifacts due to diffusion of the reaction products. In this method, the sections were incubated in a medium containing yeast RNA and a lead

[1] The following abbreviations will be used in this text: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; RNase, ribonuclease; DNase, deoxyribonuclease; PDase, phosphodiesterase; poly-A, poly-adenylic acid; poly-MeU, poly-(methyluridylic acid); APA, apurinic acid; oligo-G, oligo-guanylic acid.

salt, in the pH range 5.5 - 8.5; the enzymatically released nucleotides are presumed to be deposited at the sites of activity as the lead salts, and these are rendered visible by treatment with ammonium sulfide to give black lead sulfide. From our own experience the nucleotide-lead complexes possess a rather high solubility, and at pH 8 the nucleotide must attain a concentration of about 2 mM to form a precipitate with lead at the concentrations normally employed. This effect is even more pronounced at lower pH values where the solubility of the nucleotide-lead complex is considerably higher. It is consequently uncertain, under these conditions, that precipitate formation occurs at sites of enzymatic activity. Finally it should be noted that incubation periods were from 8 to 10 hr. at 38°. In view of our own findings [16], it would be worth while checking the enzymatic activity of sections which have been immersed for such long periods in an incubation medium.

While examining possibilities for circumvention or reduction of enzyme diffusion with the forementioned techniques, we have simultaneously tested the substrate-film method of Daoust and collaborators [6, 7] in which fresh, frozen, tissue sections are applied to a thin film of gelatin containing either RNA [7] or DNA [6] as substrate. Following incubation the gelatin film is stained with a basic dye, toluidine blue; in those regions where the substrate has been attacked by the tissue enzymes, staining is reduced. This technique provides, of course, only gross histochemical localization patterns of the enzymes involved; but, in the absence of more suitable cytochemical procedures, the information it is capable of supplying is of unquestionable value.

As hitherto applied the substrate-film method is lacking in specificity. Its originators assume, for instance, that the localization patterns obtained with DNA correspond to DNase while that obtained with RNA provides RNase localization. Such an assumption is unwarranted because of the variety of nuclease and non-specific phosphodiesterase enzymes (see ref. [16] for bibliography) which attack both types of nucleic acid. It consequently appeared of interest to us to attempt to apply this technique to the differentiation of various types of nuclease enzymes by the use of different substrates. Even this method of approach is, of course, limited by the fact that the substrate-film technique requires relatively high molecular weight substrates which can be retained in the gelatin film; the specificity of such substrates is necessarily limited. Nonetheless, preliminary trials with several types of nucleotide polymers gave results which encouraged us to pursue the matter further.

It might be added, in passing, that preliminary trials have also suggested the feasibility of applying the foregoing technique to the localization of such enzymes as amylase and hyaluronidase; these are now being investigated and will be reported on elsewhere.

EXPERIMENTAL

Substrates

Commercial yeast RNA, sodium salt.

Highly polymerized DNA prepared in this laboratory according to established procedures [8, 17].

Poly-A, prepared with polynucleotide phosphorylase as described by Grunberg-Manago *et al.* [11]. We are indebted to Dr. S. Ochoa for a gift of the enzyme.

Oligo-G, prepared and kindly supplied by Dr. A. M. Michelson. This oligonucleotide, prepared by chemical procedures [15], contains a mixture of 2′,5′ and 3′,5′ internucleotide linkages. It was exhaustively dialyzed against 2 M-NaCl and water and only the dialysis residue used, the mean chain length being approximately 15 residues.

APA was prepared from DNA according to the procedure of Durand & Thomas [9]. This polymer is completely resistant to DNase.

RNA core was prepared as described by Markham & Smith [12]. This polymer has a chain length of 6 - 7 residues [12]. During preliminary trials it was found to diffuse out of the gelatin film during the various operations necessary for staining and was subsequently abandoned. However, a comparison of this polymer with oligo-G is useful in that it suggests that the substrates used must have chain lengths greater than 10 residues for suitable results.

Poly-MeU was prepared with polynucleotide phosphorylase as elsewhere described [18]. This substrate is resistant to pancreatic ribonuclease, but is slowly hydrolyzed by a non-specific PDase present in some mammalian tissues [18]. It gave films which stained well, but was resistant to enzymes contained in spleen and kidney sections and, on the other hand, gave increased staining at sites corresponding to PDase I sites (see below). For this reason no further data on results with this substrate will be given in this paper.

A few remarks are perhaps in order with regard to specificity. Although poly-A is normally regarded as resistant to RNase, it has been shown recently [3], and confirmed in our laboratory, that it is slowly degraded by very high concentrations of RNase. It must consequently be borne in mind that the concentration of a given enzyme in a tissue may be sufficiently high at some sites to exhibit activity at variance with that to be expected from *in vitro* tests. We have also found that high concentrations of DNase will slowly alter the metachromasia of RNA against basic dyes; we have not determined whether this is an enzymatic reaction or simply due to formation of salt linkages between

an acidic polymer and a slightly basic protein but even the latter effect might conceivably influence results obtained with the substrate-film technique.

Preparation of substrate films

Films were prepared as described by Daoust [6] and Daoust & Amano [7]. It was, however, found necessary to bring the solutions of gelatin and substrate to pH 8 prior to forming the films, otherwise the latter exhibited some granularity. This step was unnecessary with RNA.

Not all samples of gelatin were found to be suitable for this purpose. Of several commercial varieties tested, Difco USP proved to be the best. One of the others contained some unknown impurities which apparently stained with toluidine blue and was likewise acted upon by some enzyme system in the tissue sections used, so that the incubated films exhibited some localization due to enzymes other than nucleases, in the outer cortex of the kidney and in splenic red pulp. Using Difco gelatin films without substrate, no reaction could be observed even after 5 hr. incubation with pancreas, spleen and kidney sections.

When preparing films with RNA, 10% gelatin was used with 10% RNA in place of the 5% gelatin recommended by Daoust & Amano [7]. When films were prepared by the draining technique on vertically maintained slides, it was found necessary to keep the temperature of the gelatin substrate mixture only slightly above its melting point; otherwise the substrate flowed more rapidly than the gelatin and the substrate concentration in the film was correspondingly reduced. This applied only to RNA since such high concentrations of the other substrates were not employed. Poly-A and oligo-G were used at a final concentration of 5 mg./ml., APA at 2 mg./ml., DNA at 1 mg./ml. The spreading technique was employed for preparation of the films with all these substrates.

For orientative purposes the various substrate films were exposed to aqueous solutions of pancreatic RNase I, DNase I and snake venom phosphodiesterase. The enzyme solutions were made up to a concentration of 0.1 mg./ml. and pH 7 for RNase and DNase, pH 9 for PDase. For each enzyme, a small drop was deposited on the substrate film and a control drop of the solvent alone alongside. Incubation was then carried out in Petri dishes well saturated with water vapour. APA and oligo-G were unaffected by any of the three enzymes. The DNA substrate film was acted on only by DNase; whereas RNA was easily and completely hydrolyzed by RNase, less so by PDase and to a small extent affected by DNase. Poly-A gave a positive reaction with RNase, a slight positive reaction with PDase and a negative reaction with DNase. Poly-MeU was resistant to all the enzymes used.

Of the enzymes used in the foregoing controls, pancreatic RNase I and DNase I were commercial crystalline preparations (Worthington); PDase was prepared by Mrs. F. Rzendowska, in this laboratory, from Jugoslavian snake venom (*Viper ammodytes*) by salt elution from a cellulose column.

Preparation of sections

Sections were cut at a nominal thickness of 20 μ on a Spencer freezing microtome installed in a cold room. For staining purposes the sections were fixed in 80% ethanol and then stained according to Cason [4], with slight modifications, for kidney sections; and as described by Daoust [6] for spleen sections. For rat kidney the zonal nomenclature employed was that of McFarlane [14].

RESULTS

Before presenting the results for localization with different substrates, a few remarks are in order with respect to the technique itself.

For heat-inactivated controls, 3 mm. thick slices were boiled for 20 min. and sectioned as above. Incubation of such sections, on films containing the various substrates, for 4 hr. gave a negative reaction in all instances with the exception of APA where a diffuse reaction was observed over the entire area of contact. With RNA as substrate, formalin and alcohol fixed sections were likewise negative.

In the published version of the Daoust technique [6] the tissue sections are embedded on the surface of a gelatin block, the latter serving as a support for placing the section in contact with the substrate film as well as for removal of the section following incubation. We have found it extremely convenient to place the sections directly on the substrate film [2], incubation being then carried out in covered Petri dishes saturated with water vapour. Following incubation the sections may be flushed off the film with a stream of water. Adjacent serial sections then served as controls. In our hands this variant proved a more convenient technique.

An additional disadvantage of the original method is the fact that prolonged contact of the substrate film with the unbuffered gelatin block results in some discoloration of the former, with the result that areas of the film which have been in contact with enzyme-free areas of the tissue section are more intensely coloured than areas which have been in contact with the gelatin block surface alone. This phenomenon was observed with all substrates. With RNA as substrate and the gelatin

[2] Since completion of this work we have found that Daoust [5] tested this type of technique, but apparently subsequently abandoned it.

block at a pH below 8, the effect is quite pronounced, but is absent if the gelatin block has been brought to pH greater than 8. With DNA and APA substrates the effect is most pronounced when the pH of the gelatin block is below 6. This phenomenon must therefore be watched for when gelatin embedded tissue sections are employed with substrates which are slowly digested and require prolonged incubation times.

Gelatin-embedding is, on the other hand, essential for an examination of the influence of pH on the reaction. The substrate-film method suffers from the disadvantage that the incubation must be conducted at an ill--defined pH. The pH of the substrate film itself is about 5 and it is difficult to change this pH by immersion, following rinsing after formalin fixation, in buffer at a given pH; only by immersion in buffer at pH 10 is the pH of the substrate film modified, to about pH 8. But following prolonged immersion in alkaline solutions the film irreversibly loses its staining properties.

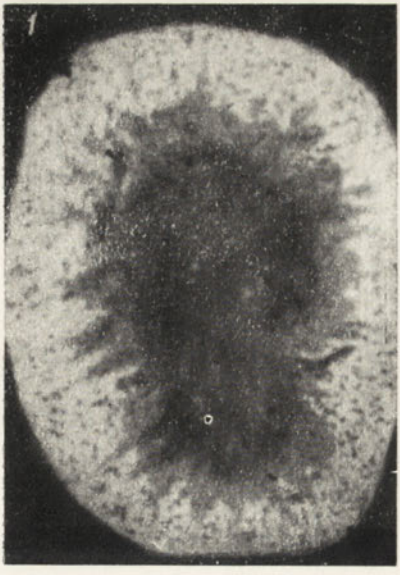
We have been able to demonstrate qualitatively the effect of pH by rough modifications of the pH of the gelatin supporting blocks. Using RNA as substrate and with sections embedded in glycerol-gelatin blocks brought to pH 8.2, the reaction was about 3-4 times more rapid for spleen and kidney sections, respectively, than at pH 5.

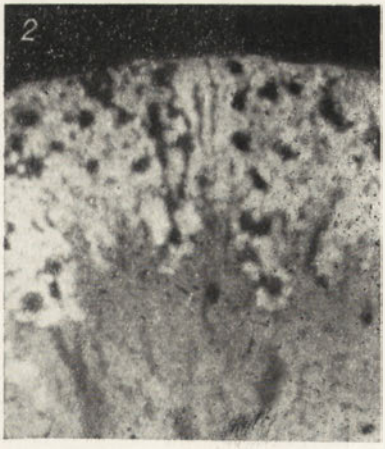
With RNA as substrate, pronounced temperature dependance of the reaction was also observed by incubation at 5°, 20° and 37°, but qualitative observations are the best that can be attained. Higher incubation temperatures can only be used when free sections are deposited directly on the substrate film.

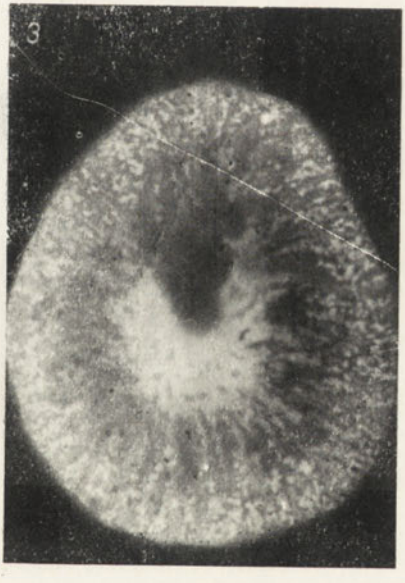
Localization

Before describing the type of localization obtained, it is perhaps necessary to emphasize once more that the results described below were obtained by incubation of free sections on the substrate film. The pH of the film under these conditions is in the neighbourhood of 5 but, where it is in contact with the tissue section, it is most likely that the pH is somewhat higher due to the buffering action of the section. The localizations described below may consequently not correspond fully to those prevailing at the pH of optimum activity of such enzymes as alkaline RNase and DNase. As against this, it should be mentioned that incubation of RNA at different pH values (by means of gelatin-embedded sections, see above) gave different localizations in kidney, which are investigated further.

In kidney the enzyme exhibiting activity towards RNA (Fig. 1 and 2) is concentrated predominantly in the cortical labirynths, mainly in the proximal convolutions; glomeruli remain negative. The inner cortex exhibits less intense activity corresponding probably to the straight por-







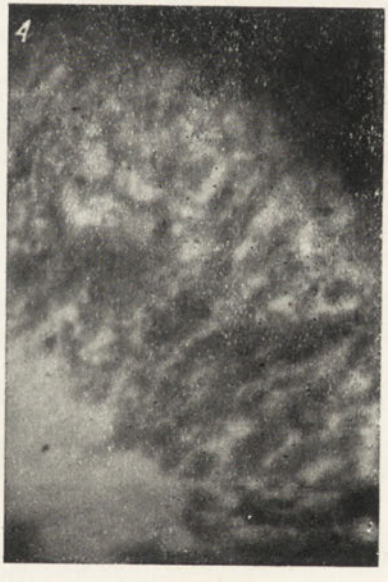
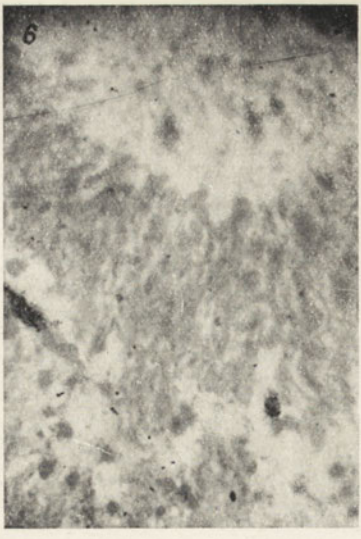


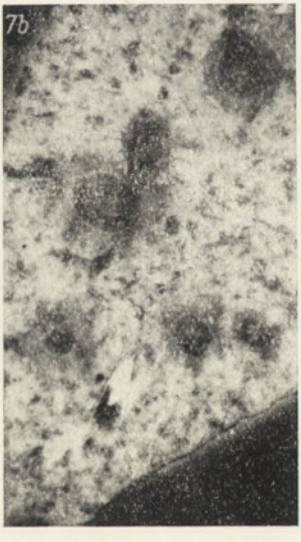
Fig. 1. RNA film exposed to a transverse section of rat kidney for 20 min. at 18° Positive reaction given predominantly by the outer cortex, some also by the inner cortex. Note the negative glomeruli. (x 6).

Fig. 2. Enlarged cut-out from Fig. 1 showing the outer and inner cortex. (x 15)

Fig. 3. DNA film exposed to a transverse section of rat kidney for 2 hr. at 18°. Note positive reaction in outer medulla, and in the loops in the inner cortex. Pronounced activity also in the cortical labirynths with glomeruli negative (x 6)

Fig. 4. Enlarged cut-out from Fig. 3 showing the three reactive zones. (x 15)





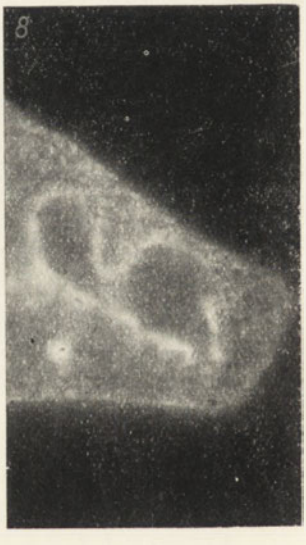


Fig. 5. Poly-A film exposed to a transverse section of rat kidney for 2 hr. at 18°. Note the intense activity in the outer cortex (with glomeruli negative) and in the outer medulla, with intermediate activity in the inner cortex. (x 6)

Fig. 6. Enlarged cut-out from Fig. 5 showing the three reactive zones. (x 15)

Fig. 7a. RNA film exposed to section of rat spleen for 30 min. at 18°. Note the intense activity in the red pulp area immediately surrounding the white pulp. (x 12)

Fig. 7b. Adjacent tissue section stained with toluidine blue. (x 12)

Fig. 8. Poly-A film exposed to rat spleen section for 2 hr. at 18° showing similar localization of enzymatic activity as in case of RNA. (x 12)

tion of the proximal convoluted tubules. Both zones of the medulla are negative.

DNA is hydrolyzed most actively by enzymes located (Fig. 3 and 4) in the outer medulla, in the ascending limbs of the loops of Henle, as well as in the loops of both the outer and inner cortex. Other elements of the cortical labirynths also show some activity. Glomeruli are negative.

Enzymes acting on poly-A are located mainly (Fig. 5 and 6) in the cortical labirynths and in the outer medulla, with the inner cortex showing intermediate activity; glomeruli negative. Activity sites in the outer medulla correspond probably to the broad limbs of the loops of Henle.

In the case of APA and oligo-G much weaker activity is centered exclusively in the cortical labirynths and corresponds to the proximal convolutions. Glomeruli negative.

In the spleen no differences in the localization of enzymes acting on the various substrates were detected. In all cases (Fig. 7 and 8) most intense activity appeared in the red pulp area immediately surrounding the white pulp enveloping the lymphatic nodules. With longer incubation periods the entire red pulp showed activity, whereas the lymphatic nodules remained negative.

DISCUSSION

The outer cortex of the kidney, particularly the proximal convolutions, contain probably both an RNase and a non-specific PDase. This may be concluded from the intense activity exhibited towards RNA, the less intense activity towards poly-A, a substrate digested by both enzymes, although with varying rapidity (see above, section on Substrates), and the weak activity shown towards oligo-G and APA, both of which are resistant to RNase. The outer cortex exhibits also DNase activity.

The inner cortex contains a PDase acting on RNA and poly-A, but with lower activity than that of the outer zone, and exhibiting no activity toward APA and oligo-G, possibly an RNase. This zone also contains elements exhibiting activity toward DNA.

The outer medulla contains enzymatic activity toward DNA and poly-A but not toward the other substrates.

On the basis of these findings it may be presumed that the kidney contains four enzymes exhibiting activity toward the substrates used:
RNase, located in the outer and, at a lower concentration, in the inner cortex, active toward RNA and poly-A.
PDase I, located in the outer cortex, acting unlike snake venom PDase on oligo-G, APA and, possibly, also on the remaining substrates.
PDase II, located in the outer medulla and acting only on poly-A.

DNase, located in the outer and inner cortex, but predominantly in the outer medulla, and hydrolyzing only DNA.

Another conceivable explanation is that the outer medulla and inner cortex contain PDase II which acts on both DNA and poly-A, but not on APA and oligo-G, so that no DNase is involved. It is, however, unlikely that there should be one enzyme exhibiting such wide specificity as to include both DNA and poly-A, and at the same time not attack RNA, APA and oligo-G. Thus the concept of two enzymes, DNase and PDase, attacking DNA and poly-A, respectively, in the outer medulla and inner cortex seems more plausible. The assumption that the cortex contains not only a non-specific PDase, but also RNase, is supported by the extremely rapid hydrolysis of RNA as compared to the other substrates; by the pH-activity effect characteristic of kidney RNases, and by certain differences in the inner cortical pattern obtained with RNA and poly-A.

In case of the spleen, where no variation in the localization of enzymes acting on the various substrates was detected, it is difficult to draw any conclusions as to the specificities of the enzymes involved. However, RNA undergoes hydrolysis at a rate far exceeding that of the other substrates and the pH optimum seems to point to RNase as the principal enzyme responsible for RNA hydrolysis.

The only available comparative data concerning the localization of these enzymes are those furnished by Aronson *et al.* [2] for acid DNase, and according to whom only the cortex of the kidney contains acid DNase, no activity being found in the outer medulla. The discrepancy between this result and ours could conceivably be due to the fact that the method of Aronson *et al.* [2] revealed DNase only at sites containing also phosphatase activity (cf., however, ref. [16]). In addition, possibly for the same reasons, we were unable to confirm the presence of DNase activity in the ring of cells directly surrounding the splenic lymphatic nodules [2] but did find such activity in the red pulp surrounding the layer of white pulp enveloping the nodules.

In conclusion, attention should once more be directed to the difficulty of pH control during incubation with the film-substrate technique and the accompanying uncertainties resulting therefrom as concerns: (a) localization of enzymes with acid or alkaline pH optima (e.g. DNase I and DNase II); (b) estimations of the relative activities of different enzymes, since the technique does not readily permit of their estimation at the optimal pH.

SUMMARY

The specificity of the film-substrate technique of Daoust, for the gross histochemical localization of ribonuclease and deoxyribonuclease, has been extended by the use of natural and synthetic polynucleotides

as substrates in addition to RNA and DNA. The enzymatic activity of rat spleen and kidney sections has been examined in this way, using as substrates RNA, DNA, apurinic acid, oligo-G and poly-A. The spleen showed no difference in localization of the different enzymes acting on these substrates. The differences in localization obtained in the kidney have been interpreted in terms of ribonuclease, deoxyribonuclease and two non-specific phosphodiesterases. The techniques involved in the substrate-film method, as well as some of its limitations, are described and discussed.

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ORIENTACYJNE HISTOCHEMICZNE BADANIA NAD LOKALIZACJĄ NUKLEAZ

Streszczenie

Rozszerzono specyficzność metody Daousta polegającej na zastosowaniu błony zawierającej substrat dla histochemicznej lokalizacji rybonukleazy i dezoksyrybonukleazy przez wprowadzenie jako substratów poza RNA i DNA również naturalnych i syntetycznych polinukleotydów. Stosując jako substraty RNA, DNA, kwas apurynowy, oligo-G oraz poly-A

zbadano orientacyjnie aktywność enzymatyczną skrawków nerki oraz śledziony szczura. W przypadku śledziony nie stwierdzono różnic w lokalizacji różnych enzymów działających na te substraty.

Różnice w lokalizacji występujące w nerce przypisano działaniu rybo- i dezoksyrybonukleazy oraz dwu niespecyficznych fosfodwuesteraz.

Omówiono stronę techniczną metody błony substratowej wskazując na niektóre jej ograniczenia.

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LUDMIŁA SZARKOWSKA and J. HELLER

STUDIES ON COENZYME Q REDUCTION

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, and Department of Physiological Chemistry, Medical School, Warszawa

Coenzyme Q (ubiquinone) is *in vivo* reduced by both succinic acid and DPNH dehydrogenase systems. The reduced form is readily reoxidized under aerobic conditions of the cell. The reduction of coenzyme Q was examined chiefly in mitochondria, but also with submitochondrial particles. Electron transporting particles had been used for this purpose by Crane *et al.* [3], succinic dehydrogenase - cytochrome *b* complex by Ziegler & Doeg [23], and sarcosomal fragment by Pumphrey, Redfearn & Morton [20].

The study of isolated, soluble components of these complex structural units is an indispensable first approach, as has been pointed out by Green [7]. Our purpose has been to examine the reduction of coenzyme Q in a soluble system of succinic dehydrogenase free of particulate fragments. The influence of some inhibitors and artificial electron acceptors was also studied. A preliminary note was published elsewhere [12].

MATERIALS AND METHODS

Coenzyme Q (CoQ) has been obtained from fresh or frozen beef heart after Lowe *et al.* [15]. The whole tissue has been saponified with the addition of 10 g. of pyrogallol per 150 g. of tissue to prevent oxidation [4]. The saponification was followed by extracting three times with ethyl ether which before use had been shaken with ferrous sulphate and redistilled. The ethyl ether extract was washed several times with water to remove alkali and thereafter rapidly concentrated. Under this treatment water froze out, and was discarded. The extract was then further concentrated under nitrogen until dry. The residue was dissolved in anhydrous ethanol and left overnight at 4°. The sterol substances which appeared in crystalline form were spun off and the supernatant allowed to crystallize in -20° for two days. The sediment formed was separated from the oleic supernatant by centrifugation at -20°, and after dissolution

in anhydrous ethanol allowed to crystallize in the same temperature. After centrifugation, the sediment was dissolved again in ethanol and this solution was used. The coenzyme Q was estimated after reduction with borohydride as a decrease of absorption at 275 mμ. Applying the coefficient $E^{1\%}_{1cm} = 142$ we used to find the purity of our preparations to be about 50%.

Succinic acid or sodium succinate: commercial products were used after three or four recrystallizations. The necessity of such purification was indicated in experiments where the relation between coenzyme Q reduction and succinate concentration was examined. The reduction increased with succinate concentration up to 6 mM: the higher concentrations inhibited the reaction. With purified preparations the inhibition was not observed.

Antimycin A was a Sigma & Co. preparation, N-methylphenazonium methosulphate (phenazine methosulphate) from Light. Triton X-100 was kindly supplied by Rohm & Haas Co., Philadelphia, Pa. Potassium borohydride was a product of Bender & Hobbein, Zürich. 2-methyl-1,4-naphtoquinone (menadione) was from Klawe, Poland.

The preparation of succinic dehydrogenase was obtained from pig heart after Neufeld *et al.* [18]. We used the preparation B in VI stage, or the V stage, fraction 0.3 - 0.4 called subsequently V_0. In our experiments this last fraction was many times more active than the stage VI. All preparations have been kept below 0°.

The enzymic reduction of coenzyme Q was determined after Hatefi *et al.* [9, 10]. Although in our case sucrose was not needed in the medium we retained it to keep the conditions comparable to those of other authors. A medium was prepared consisting of 9 ml. 0.365 M-potassium phosphate, pH 7.2; 4.5 ml. 0.075 M-sodium succinate; 5.35 ml. 0.25 M-sucrose; 2.25 ml. water, and 3.5 mg. of coenzyme Q in 0.9 ml. of ethanol. All solutions before mixing have been brought to pH 7.2. To 0.5 ml. of this medium the compounds investigated were added, and the volume filled up with water to 0.9 ml. After 5 min. at 37° the reaction was initiated by adding 0.1 ml. of enzyme solution of a suitable concentration, and at the end of incubation it was stopped by adding perchloric acid. The controls were composed and incubated in the same way except that in one of them the tested substances, and in the other the enzyme preparation was omitted. After deproteinization, the omitted compounds were supplemented. From all solutions, tests and controls, coenzyme Q was extracted with cyclohexane during 15 min., at variance with the original method which advised to do so for 45 min. The reduction of coenzyme Q was estimated from the decrease of absorption at 275 mμ, using for calculation the coefficient 142.

The activity of succinate dehydrogenase with phenazine methosulphate was estimated manometrically at 37°, following the method of Keilin & King [13]. The medium was of the same composition as had been used for coenzyme Q reduction, except, of course, that coenzyme was omitted. After 10 min. for temperature equilibration the reaction was initiated by pouring out from the sidearm 0.5 ml. of phenazine methosulphate solution (2 mg.).

Protein was estimated after Lowry *et al.* [16] applying reagents for soluble proteins.

RESULTS

Fig. 1 illustrates the reduction of coenzyme Q by succinic dehydrogenase in relation to enzyme concentration. With lower concentrations the reaction velocity is proportional to the amount of enzyme protein applied. With higher concentrations the increase in reaction velocity

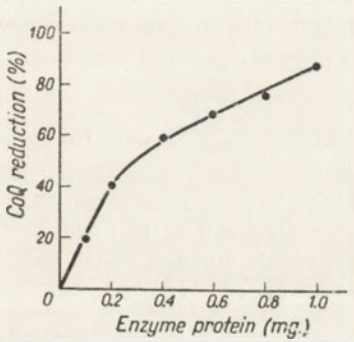

Fig. 1. Coenzyme Q reduction at various enzyme concentrations. In 1 ml. of incubation mixture: 75 µmoles potassium phosphate, pH 7.2; 30 µmoles sucrose; 80 µmoles CoQ; 7.5 µmoles sodium succinate; succinic dehydrogenase preparation stage VI. Incubation 10 min., at 37°

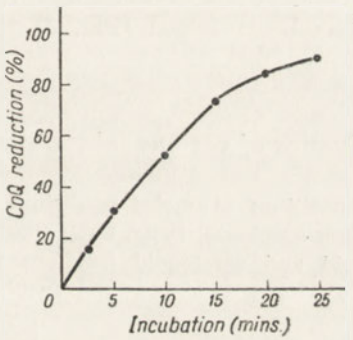

Fig. 2. Time course of coenzyme Q reduction. Composition as in Fig. 1; 0.4 mg. of enzyme protein

diminishes, probably because of a proceeding exhaustion of coenzyme Q. Analogous picture was obtained when the time-course of the reduction was followed with one concentration of the enzyme (Fig. 2).

As our preparation of coenzyme Q was only of 50% purity it seemed interesting to see whether the main impurities have any influence on the reduction of coenzyme Q. Two main substances were taken into account: (1), steroids which were removed to a great extent when the alcoholic solution of coenzyme Q was cooled at 4° for the first crystalliza-

tion; (2), a yellow oleic substance, remaining in solution after the crystallization of coenzyme Q at -20°, which seems to be a carotenoid. The steroid crystals had been washed with anhydrous ethanol until all traces of coenzyme Q were removed. To obtain the carotenoid fraction the liquid remaining after coenzyme Q crystallization was passed through Decalso column, and the column was eluted with *iso*octane. The first fraction of the eluate contained all the pigment.

As can be seen from Table 1 neither the steroids nor carotenoid substances influence the reduction of coenzyme Q. The applied amounts of these substances did certainly surpass the contaminations in our preparations.

As has been shown by Ziegler & Doeg [24] the addition of Triton X-100 detergent increases the rate of coenzyme Q reduction. We could substantiate this finding with our enzymic preparation obtaining almost five-fold increase of the reduction (Table 1). The mechanism of this effect being obscure it seemed of interest to assay the influence of Triton on the activity of succinic dehydrogenase towards phenazine methosulphate. It has been found (Fig. 3) that Triton X-100 stimulates this reaction.

Table 1

Influence of various substances on the reduction of coenzyme Q by succinic dehydrogenase

Composition: 75 μmoles potassium phosphate, pH 7.2; 30 μmoles sucrose; 7.5 μmoles sodium succinate; 80 μg. CoQ; succinic dehydrogenase preparation [18]; Antimycin A, steroid and carotenoid fractions were added in 0.02 ml. ethanol. Final vol. 1 ml. Incubation 10 min. at 37°

Enzyme Preparation	Enzyme (mg. protein)	Addition		CoQ reduced (%)
Fraction V_0	0.04	None		61
	0.04	Steroid fraction,	150 μg.	62
	0.04	Steroid fraction,	300 μg.	60
	0.04	Steroid fraction,	600 μg.	61
	0.04	None		60
	0.04	Carotenoid fraction,	1100 μg.	62
	0.04	Carotenoid fraction,	2200 μg.	59
	0.005	None		11
	0.005	Triton X-100	0.44 μl.	45
	0.005	Triton X-100	0.88 μl.	54
Stage VI	0.45	None		54
	0.45	Antimycin A	30 μg.	55
	0.45	Antimycin A	60 μg.	54
	0.45*	None		85
	0.45*	Antimycin A	30 μg.	84

* Incubated 16 min.

However, after a period of stimulation a decrease in the rate is observed, and with higher doses even a total inhibition.

Having established the optimal conditions for the activity of the enzymic preparation we tried to get some information about the localization of coenzyme Q in respiratory chain by applying inhibitors or artificial acceptors. By this time there existed a controversy between Morton's and

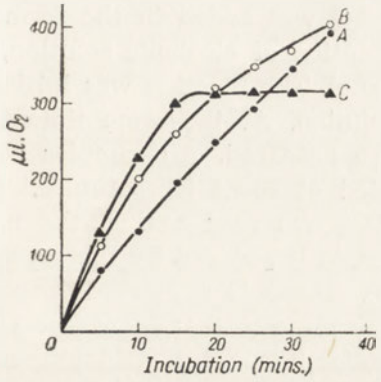

Fig. 3. Influence of Triton X-100 on the reduction of phenazine methosulphate by succinic dehydrogenase. Composition: 225 μmoles potassium phosphate, pH 7.2; 90 μmoles sucrose; 22.5 μmoles sodium succinate; 2 mg. phenazine methosulphate; 0.3 mg. enzyme protein, fraction V_0. Final vol. 3 ml. *A* (●), control; *B* (○), 1.32 μl. Triton X-100 added; *C* (▲), 2.64 μl. Triton X-100 added

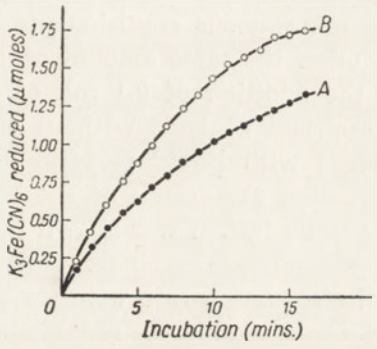

Fig. 4. Influence of coenzyme Q on the reduction of ferricyanide by succinic dehydrogenase. Composition: 219 μmoles potassium phosphate, pH 7.2; 90 μmoles sucrose; 22.5 μmoles sodium succinate; 2.7 μmoles potassium ferricyanide; 1.32 μl. Triton X-100, 0.2 mg. enzyme protein, fraction V_0. Final vol. 3.22 ml. *A* (●), control; *B* (○), with 32.4 μg. CoQ added

Green's group. In 1959 the Green's group [10] claimed to find an inhibition of coenzyme Q reduction in mitochondria by Antimycin A. At variance with this statement Pumphrey & Redfearn, from Morton's group [19] have shown with sarcosome fragments that this reaction is Antimycin A insensitive. In this situation we assayed the soluble succinic dehydrogenase and we found the reduction of coenzyme Q by this preparation to be Antimycin A insensitive (Table 1). Eventually Green's group [8] arrived to the same conclusion.

Doeg, Krueger & Ziegler [5] found that dichlorophenol-indophenol is able to accept electrons from coenzyme Q, and Lester & Smith [14] have shown that the reduction of some tetrazolium compounds like 2,2′,5-tetraphenyl-3,3′-(4,4′-biphenylene) ditetrazolium chloride, 2-*p*-indophenyl-3-*p*--nitrophenyl-5-phenyltetrazolium chloride, and 2,5-diphenyl-3-naphtyltetrazolium chloride is dependent on coenzyme Q. Adding to this list, we

have assayed the influence of coenzyme Q on the reduction of ferricyanide by succinic dehydrogenase. The reduction has been followed spectrophotometrically as decrease of absorption at 400 mμ corresponding to transition of $Fe(CN)_6^{3-}$ into $Fe(CN)_6^{4-}$. The test was performed by mixing 4 ml. 0.365 M-potassium phosphate, pH 7.2, 2 ml. 0.075 M-sodium succinate, 8.8 μl. Triton X-100, 2.4 ml. 0.25 M-sucrose, 9 ml. 0.002 M-$K_3Fe(Cn)_6$ and 2.6 ml. water. Into two photometric cells 3 ml. of this mixture were placed. To one of them 32.4 μg. coenzyme Q was added in the form of 0.12 ml. aqueous emulsion prepared by dilution of alcoholic solution, to the other the same amount of diluted ethanol. The reaction was initiated by the addition of 0.1 ml. of enzyme solution. At the same time two appropriate controls without enzyme were prepared. All samples were covered with liquid paraffin and incubated at room temperature. The decreasing absorption of the enzymatic test was read against the non-enzymatic test. It was shown that ferricyanide is reduced by the succinic dehydrogenase - succinate system (Fig. 4). There is a marked increase in the reduction rate with CoQ, which may indicate that coenzyme Q is an intermediate in the reduction of ferricyanide by succinic dehydrogenase and that its turnover could be a bottleneck of this reaction. The stimulation of the reaction by coenzyme Q was obtained only with very active enzyme preparations, such as can be obtained with Triton X-100. It is to be assumed that with poorly active preparations the content of the endogenous coenzyme Q is sufficient to cope with the maximum of enzyme's efficiency. Therefore the supplementation with exogenous coenzyme Q is in these cases without any effect.

Table 2

Non-enzymic oxidation of coenzyme Q by ferricyanide

28 μmoles $K_3Fe(CN)_6$, 0.14 mg. reduced CoQ, 84 μmoles phosphate, pH 7.2. Final vol. 2.85 ml. Incubation 10 min., at 37°

Composition	E_{275}
Ferricyanide	0
CoQ	0.252
Ferricyanide, CoQ	0.662

In turn the question arose whether the electron transfer from coenzyme Q to ferricyanide is an enzymatic process or not. We reduced an amount of coenzyme Q with borohydride after Hatefi [9] and we tried herewith the non-enzymatic reduction of ferricyanide. Into two tubes with grounded stopcocks were transferred: 1.4 ml. 0.02 M-$K_3Fe(CN)_6$, 0.84 ml. 0.1 M-phosphate buffer, pH 7.2, and 0.56 ml. water. To one sample 0.14 mg. of reduced coenzyme Q in 0.05 ml. of ethanol was added, and to

the control 0.05 ml. of ethanol. Another control contained the reduced coenzyme Q but no ferricyanide. After incubation at 37° during 10 min. 2.8 ml. of 0.1 N-perchloric acid and 2.8 ml. of 95% ethanol were added. Coenzyme Q was then extracted and the absorption at 275 mμ was estimated (Table 2). There was an increase in absorbancy at 275 mμ indicating the oxidation of the reduced form of coenzyme. The oxidation is therefore not mediated by an enzyme, and reminds in this respect the oxidation of dihydroxyphenylalanine observed by Heller & Świechowska [11]. Also methylene blue oxidized non-enzymatically the reduced coenzyme Q, but we were unable to demonstrate any influence of coenzyme Q on the reduction of methylene blue by the succinic dehydrogenase, not even in presence of Triton X-100. Taking into account that the redox-potential of methylene blue is 0.011 v. [17] we can suppose that it can be reduced by coenzyme Q only when the reduced form of the latter is decisively prevailing.

Table 3

Influence of quinones on the reduction of coenzyme Q by succinic dehydrogenase

Composition: 75 μmoles potassium phosphate, pH 7.2; 30 μmoles sucrose; 7.5 μmoles sodium succinate; 80 μg. CoQ; 0.03 ml. ethanolic solution of the respective quinone; 0.02 mg. enzyme protein (fraction V_0). Final vol. 1 ml. Incubation 10 min. at 37°

Quinone	Conc. (mM)	Inhibition (%)
Menadione	0.03	62
Menadione	0.075	91
Menadione	0.15	100
Menadione	0.075	80*
1,4-Naphtoquinone	0.03	40
p-Benzoquinone	0.03	100
2-Methylbenzoquinone	0.03	100

* 0.1 mg. protein.

Many authors have tried to combine *in vitro* the reduction of quinones with the oxidation of succinate in isolated mitochondria [e.g. 21,14]. Some of these compounds like *p*-benzoquinone were quite inert, other like 1,4-naphtoquinone and menadione were reduced only very poorly in comparison with coenzyme Q. We have studied the influence of those compounds on the reduction of coenzyme Q by succinic dehydrogenase and found that *p*-benzoquinone, 2-methylbenzoquinone, 1,4-naphtoquinone and menadione inhibit this reaction. The results presented in Table 3 show that benzoquinones are more efficient inhibitors than the analogous naphtoquinones.

The interference of quinones with the reduction of coenzyme Q can be attributed to the inactivation of the succinic dehydrogenase through an interaction of quinones with active thiol groups of the enzyme. Of course all quinones quoted above exhibit unsubstituted carbon atoms in

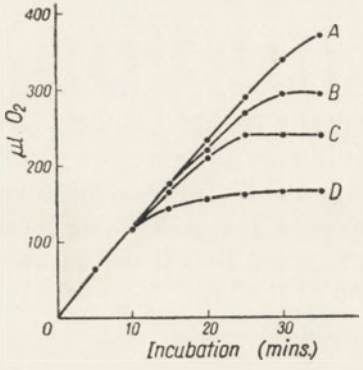

Fig. 5. Influence of menadione on the reduction of phenazine methosulphate by succinic dehydrogenase. Composition: 225 µmoles potassium phosphate, pH 7.2; 90 µmoles sucrose; 22.5 µmoles sodium succinate; 2 mg. phenazine methosulphate; 0.3 mg. enzyme protein, fraction V_0. Final vol. 3 ml. *A*, control; *B*, with 0.0375 mM-menadione; *C*, 0.075 m M-menadione; *D*, 0.15 m M-menadione

the ring. We have therefore examined the influence of menadione on the activity of succinic dehydrogenase towards phenazine methosulphate, which is accepting, according to Singer & Kearney [22] the hydrogen atoms directly from the dehydrogenase. As can be seen in Fig. 5 mena-

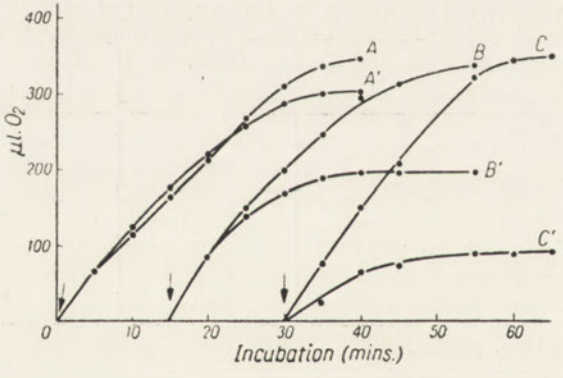

Fig. 6. Influence of preincubation of the enzyme with menadione on the reduction of phenazine methosulphate by succinic dehydrogenase. Composition as in Fig. 5. *A*, *B*, *C*, controls, without menadione. *A'*, *B'*, *C'*, with 0.075 m M-menadione. The preincubation time with menadione was in *A'*, 10 min.; *B'*, 25 min.; *C'*, 40 min., including 10 min. for temperature equilibration. Arrows indicate the addition of phenazine

dione inhibits the phenazine reduction by succinic dehydrogenase after a lag of 25 min. (including the 10 min. for temperature equilibration), whereas the reduction of coenzyme Q (Table 3) is inhibited in 80 - 100% during the first 10 min.

The lag period with phenazine can be understood as the time needed for the inactivation of enzyme by menadione. Alternatively it could be supposed that during this time some products are generated in the reaction with phenazine which inhibit succinic dehydrogenase. To get more information in this respect we examined the influence of preincubation of the enzyme with menadione on the reaction with phenazine. The results are illustrated in Fig. 6. The curves *A'*, *B'* and *C'* show the inhibition after preincubation with menadione during 10, 25 and 40 min., respectively, including 10 min. for temperature equilibration. The curves *A*, *B* and *C* represent controls without the addition of menadione. It can be seen that the longer had been the preincubation with menadione the sooner inhibition was begun. This would indicate that the inhibiting agent is menadione itself and not some reaction product.

DISCUSSION

We have shown that coenzyme Q is reduced by a soluble preparation of succinic dehydrogenase. In the meantime Ziegler & Doeg [24] have found that coenzyme Q is reduced by a soluble preparation of succinic dehydrogenase somewhat different from the one we were using. The reduction in our preparation is Antimycin A insensitive, in accordance with the recent findings of all authors studying this problem in mitochondria and in enzymic preparations. These results seem to substantiate the view that the electrons can pass in mitochondria from succinate to coenzyme Q without cytochrom *b* as an indispensable intermediate carrier.

We have shown that the reduction of ferricyanide by the succinic dehydrogenase is stimulated by the addition of coenzyme Q. Estabrook [6] demonstrated that the reduction of ferricyanide is stimulated three to four-folds by cytochrome *c* in enzymic preparation deprived of this cytochrome. These findings show that ferricyanide is able to accept electrons from all components of the succinic oxydase chain beginning with the dehydrogenase up to the cytochrome *c*. This can be understood taking into account the high positive redox-potential of ferricyanide which is 0.360 v. [17]. The reduction of dichlorophenol-indophenol [5] and of tetrazolium salts [14] by succinic dehydrogenase which is dependent on coenzyme Q and is Antimycin A insensitive seems to suggest that these compounds are acting below the Antimycin A sensitive point of the chain, the more so below the ferricyanide, somewhere between coenzyme Q and cytochrome *c*.

The inhibition of succinic dehydrogenase by quinones such as *p*-benzoquinone, probably by binding of thiol groups, was known for some time [2]. However the influence of menadione is more complex. In the reac-

tion with phenazine there is an inhibition only after some time of contact between the enzyme and menadione. The mechanism of this inhibition is probably of the same kind as that of *p*-benzoquinone. Besides, there is another kind of inhibition to be observed in the reaction of succinic dehydrogenase with coenzyme Q which is visible practically from the very beginning. May be, this other inhibition is a competitive one. Were this the case, the point of action of menadione would be the same as of the antimalaric 2-hydroxy-3-alkylonaphtoquinones, the inhibiting effect of which on succinic oxydase was observed by Ball & Anfinsen [1].

SUMMARY

Coenzyme Q is reduced by a soluble preparation of succinic dehydrogenase. This reduction is Antimycin A insensitive. The reaction is inhibited by quinones like *p*-benzoquinone, *p*-methylbenzoquinone, 1,4-naphtoquinone, and menadione.

Menadione inhibits also the reduction of phenazine methosulphate by succinic dehydrogenase. The inhibition can be observed only after a lag period or after preincubation of the enzyme preparation with menadione.

The reduction of ferricyanide by succinic dehydrogenase is stimulated by coenzyme Q.

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BADANIA NAD REDUKCJĄ KOENZYMU Q

Streszczenie

Rozpuszczalny preparat dehydrogenazy bursztynowej redukuje koenzym Q. Reakcja ta jest niewrażliwa na Antymycynę A. Chinony takie jak *p*-benzochinon, *p*-metylobenzochinon, 1,4-naftochinon i menadion hamują redukcję koenzymu Q. Menadion poza tym hamuje reakcję dehydrogenazy bursztynowej z fenazyną, przy czym hamowanie to występuje dopiero po pewnym czasie lub po preinkubacji menadionu z enzymem. Koenzym Q stymuluje redukcję żelazicyjanku potasu przez dehydrogenazę bursztynową.

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R. NIEMIRO and J. PRZYJEMSKI

THE QUANTITATIVE STUDIES ON RAT TISSUES LYSOLECITHIN

Department of Biochemistry, Medical School, Gdańsk
Head of the Department: Prof. Dr. Wł. Mozołowski

The presence of lysolecithin in lipid extracts of a number of mammalian tissues has recently been reported. Marinetti *et al.* [13] administered ^{32}P-labelled orthophosphate to rats and, using paper chromatography, described a lipid behaving like lysolecithin in intestine, heart, liver, kidney and spleen. Hajdu *et al.* [10] isolated and identified the lysolecithin from plasma, liver, heart, muscle and adrenal medulla. Lysolecithin has also been identified in lipid extracts of normal human serum [9, 11], intestine [1], skeletal muscle [17], and human and rat brain [19]. So far as we are aware the quantitative studies on the lysolecithin content of human serum [9, 15] and purified pig heart cytochrome b-c_1 preparation [12] have only been reported.

It was decided to describe the chromatographic method for quantitative estimation of the mammalian tissues lysolecithin and to determine the content of lysolecithin in different rat tissues.

EXPERIMENTAL

The albino rats aged 2 to 6 months, males and females, were used for this experiment. They were killed by decapitation, and about 1 g. of each tissue tested, was removed and weighed as soon as possible. Usually this operation took 3 - 5 min. Then the tissues were placed in large tubes filled with 10 ml. of chloroform - methanol (2:1, v/v) and cooled by dipping them into freezing mixture (CO_2 and acetone). The tissues were homogenized in an all-glass homogenizer with chloroform - methanol (2:1, v/v) in proportion of 19 ml. per 1 g. of fresh tissue. The extracts were filtered and the filtrate was washed with 1/5th of its volume of distilled water as described by Folch *et al.* [7], and centrifuged. The upper phase was carefully and possibly completely removed and discarded. The lower phase was evaporated to dryness *in vacuo* at no more than 50°

and left overnight in a dessicator over $CaCl_2$ and paraffin under reduced pressure. The dry residue was extracted four times with small volumes (0.5 to 1 ml.) of chloroform. The chloroform extracts were applied to a column containing 3 g. of „alumina". The column was eluted first with 30 ml. chloroform and then with 40 ml. chloroform - methanol (1 : 1, v/v). The first fraction was discarded and the second one containing the choline phosphatides was evaporated to dryness as above, and the residue taken up in 1 or 2 ml. of chloroform - methanol (2:1, v/v).

Samples of choline phosphatides solution equivalent to 100 - 200 mg. of fresh tissue were spotted in duplicates onto the silicic acid impregnated paper and dried in a current of air. Silicic acid impregnated papers 15 × 30 cm. (Whatman No. 3MM) were prepared as described by Lea, Rhodes & Stoll [11]. Chromatograms were developed with chloroform - -methanol (4 : 1, v/v) at room temperature in a sealed tank for 6 - 7 hr. It was found that solvent which had been „aged" for at least a week gave better resolution of phosphatides than the freshly prepared one. Owing to the large amount of phosphatide applied to each spot on the paper, the adequate resolution of choline phosphatides could be achieved by developing the chromatograms three times in the same direction using the same solvent. The chromatograms were stained with 3 mg% solution of Rhodamine G in acetone. The areas corresponding to the lysolecithin were cut off in ultra-violet light, and then eluted by running solvent of methanol — 9 N-HCL in methonol-chloroform-water (80:10:22:5.6, by vol.) using special set in a sealed tank; 2 - 3 ml. of eluate collected from each spot was practically sufficient to wash out all lysolecithin. The speed of elution was about 1 - 2 ml. of eluate per 12 - 15 hr. After evaporation the residue was examined for phosphorus. The recovery of six parallel samples of lysolecithin standards first chromatographed and then eluted was 95 - 98%. The recovery of lysolecithin standard added to the homogenate of different tissues which were taken through the entire procedure of extraction and chromatographic technique was 80 - 85%.

For haemolytic activity test of tissue lysolecithin, the corresponding areas from paper chromatograms were eluted with running solvent of chloroform - methanol (1 : 4, v/v).

Analytical method. Total phosphorus of choline phosphatides and lysolecithin phosphorus was determined by the method of Ernster, Zetterström & Lindberg [5] as modified by Stricland, Thompson & Webster [18]. The alkali-labile P was determined by the method of Schmidt *et al.* [16]. Haemolytic activity of lysolecithin was tested using the procedure of Collier [2].

Reagents. The organic solvents were of analytical grade. "Alumina", aluminium oxide, Hopkin & Williams Ltd., 100 - 200 mesh, Brockman activity 1 - 2. Egg lysolecithin was prepared as described by Marples, Thompson & Webster [14].

RESULTS AND DISCUSSION

Table 1 shows the composition of choline phosphatides of different rat tissue lipid extracts. The total, alkali-labile and lysolecithin phosphorus were directly measured. Lecithin and sphingomyelin P were calculated as follows: lecithin = alkali-labile P *minus* lysolecithin P; sphingomyelin = total P *minus* alkali-labile P. The estimations were performed in duplicate showing close agreement. The amounts of lecithin and sphingomyelin found, are generally similar to the figures given by Dawson [3, 4] for rat and sheep tissues. The concentration of lysolecithin is comparatively low in brain, whole blood and skeletal muscle extracts. In remaining tissues the level of lysolecithin is two or three times higher. The lysolecithin of each tissue described in Table 1 proved to be haemolytic.

Table 1

The content of lysolecithin in different rat tissues

The values are expressed in μmoles P per 1 g. of fresh tissue. The average values ± S. D. are given; in brackets the number of tissue samples tested

Tissue	Choline phosphatides P	Alkali-labile P	Lysolecithin P	Lecithin P	Sphingomyelin P
	a	b	c	b — c	a — b
Brain	27.7 ±1.90 (11)	22.4 ±1.92 (10)	0.39 ±0.088 (9)	22.0	5.3
Lung	17.8 ±2.94 (9)	13.1 ±2.18 (8)	0.55 ±0.128 (9)	12.6	4.7
Kidney	20.0 ±2.03 (10)	14.2 ±1.42 (10)	0.62 ±0.136 (9)	13.6	5.8
Heart	16.1 ±2.89 (10)	13.2 ±1.53 (9)	0.59 ±0.217 (7)	12.6	2.9
Spleen	15.7 ±0.84 (7)	11.3 ±0.57 (7)	0.58 ±0.107 (6)	10.7	4.4
Whole blood	3.3 ±0.23 (5)	2.4 ±0.12 (4)	0.26 ±0.045 (5)	2.1	0.9
Liver	29.7 ±1.2 (4)	25.0 ±0.66 (3)	1.12 ±0.29 (4)	23.9	4.7
Skeletal muscle	9.4 ±0.14 (5)	8.5 ±0.18 (5)	0.33 ±0.076 (3)	8.2	0.9

The composition of lipids as percentage of total choline phosphatides is given in Table 2. The content of blood lysolecithin found, shows close agreement to the figures given by Gjone *et al.* [9], and Phillips [15] for normal human serum. So far as we are aware there are no data on the concentration of lysolecithin in other tissues. No close relation was found between lysolecithin and lecithin content of different tissues. This disproportion speaks in favour of the supposition of naturally occurring lysolecithin.

Table 2

Rat tissue phospholipids as percentage of choline phosphatides

Calculated from data of Table 1

Tissue	Lysolecithin	Lecithin	Sphingomyelin
Brain	1.4	79.4	19.2
Lung	3.1	71.0	26.4
Kidney	3.1	68.0	29.0
Heart	3.7	78.3	17.9
Spleen	3.7	68.2	28.0
Whole blood	7.9	63.6	27.3
Liver	3.8	80.5	15.8
Skeletal muscale	3.5	87.3	9.5

It is possible that the lysolecithin in tissue extracts may have been formed *post-mortem* before removal of the tissue from the body, or that it may have arisen during the procedure involved in its isolation. According to Fishler *et al.* [6] and Fries *et al.* [8] the breakdown of tissue phospholipids autolysed for 1 hr. at 37°, corresponded to 2 - 5% of the total phosphatides. In our experiments the operation since decapitation of the rats till cooling the tissues in freezing mixture took 3 - 5 min. Thompson *et al.* [19] have shown that storage of the total lipid extract at -10° for 48 hr. causes no apparent increase of lysolecithin. Furthermore, when lecithin was taken through the entire procedure of extraction and chromatographic separation, no evidence of lysolecithin formation was observed.

SUMMARY

Using the column and paper chromatographic methods, the lysolecithin content of rat brain, lung, kidney, heart, spleen, whole blood, liver and skeletal muscle was determined.

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ZAWARTOŚĆ LIZOLECYTYNY W TKANKACH SZCZURA

Streszczenie

Posługując się chromatografią na kolumnie z Al_2O_3 oraz na bibule impregnowanej kwasem krzemowym, oznaczono zawartość lizolecytyny w następujących tkankach: mózg, płuca, nerki, serce, śledziona, krew, wątroba, mięsień szkieletowy szczura białego.

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DANIELA BARSZCZ and D. SHUGAR

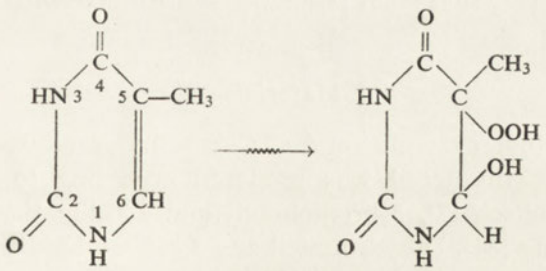
RADIATION CHEMISTRY OF NUCLEIC ACIDS AND THEIR DERIVATIVES

I. SOME PYRIMIDINES, DIHYDROPYRIMIDINES AND HYDRATED PYRIMIDINES

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa

Earlier investigations of the influence of ionizing radiations on nucleic acids were rather general in character and often limited to observations of the gross overall effects, which include decrease in viscosity [30, 31, 3, 4, 5, 6]; destruction of the ultraviolet absorption spectrum [1], indicating ring rupture of some of the base residues; deamination, as well as liberation of some of the purine bases [20, 22, 14]; chain fragmentation, as revealed by liberation of inorganic P and an increase in the number of titratable acid groups [21]. In many instances, however, the radiation doses used were considerably greater than those required to induce biological effects, so that much of this earlier data is not always readily interpretable.

With the increase in our knowledge of the structure of nucleic acids, it has become possible to examine radiation induced changes in greater detail as well as to begin to specify some of the radiation products and their quantitative yields of formation under defined conditions. A typical example was the demonstration by Scholes *et al.* [23] that irradiation of some pyrimidines and nucleic acids in the presence of oxygen led to the formation of relatively stable organic peroxides which, in the case of thymine, were postulated as being the 5-hydroperoxy-6-hydroxy derivatives, as follows:



This deduction was subsequently confirmed by direct chemical synthesis [10] of two of the thymine peroxide isomers. Several further investigations have now been reported [14, 11] in which additional radiation products of purines and pyrimidines have been identified.

Such results represent, indeed, a logical trend in any organized effort to obtain a balanced picture of the biological effects of ionizing radiations, which normally are the consequence of relatively low applied doses. For example the $D_{37\%}$ dose for inactivation of transforming principle in dilute aqueous medium is of the order of 500 - 1000 r., whereas such intracellular effects as mutagenesis may be produced by as little as several r. It follows that such effects as decrease in viscosity of DNA, which requires doses of the order of 10^4 - 10^5 r., represent only the later stages of degradation of these macromolecules.

It seems to us, therefore, that efforts should be concentrated on quantitative measurements of the radiation induced modifications in the fundamental building blocks of polynucleotide chains with a view to pinning down those constituents, or specific structures, which are most susceptible under given conditions. Such results might then be extrapolated to the effects of lower doses on intact acids. An example of such a study is that of Scholes *et al.* [25], which appeared following completion of the present investigation.

Another line of approach, which we have been applying with some success to the photochemistry of nucleic acids [29], involves the employment of synthetic oligo- and polynucleotides which can be prepared by chemical and enzymatic techniques. The use of such models should make it possible to follow quantitatively the behaviour of a given base residue as a function of its position in a polymer chain, the influence of neighbouring bases, as well as of the internucleotide linkages. This is the procedure which we have decided tentatively to adopt and, as starting point, we have examined in somewhat more detail the radiation products of some pyrimidines, with special emphasis on organic peroxide formation.

Because of its importance in the photochemistry of nucleic acids, we have likewise examined the possible formation of pyrimidine hydroperoxides under the influence of ultraviolet radiation at wavelengths above 2400 A. This was considered necessary in view of the general statement of Scholes *et al.* [23] that such peroxides are formed with ordinary ultraviolet lamps [29].

Materials

Uracil, thymine, cytosine and 5,6-dimethyluracil were commercial products; 1,3-dimethyluracil was prepared according to the method of Davidson & Baudisch [7], 5-bromouracil and 1,3-dimethyl-5-bromouracil according to Wang [34]. Dihydrouracil and 1,3-dimethyldihydrouracil were

obtained by hydrogenation of the parent compounds on a rhodium catalyst [13]. The *trans* isomer of thymine hydroperoxide was a gift of Dr. B. Ekert [10]. The photoproduct of 1,3-dimethyluracil (5-hydro-6-hydroxy-1,3-dimethyluracil) was prepared by irradiation of 1,3-dimethyluracil in aqueous medium with a mercury resonance lamp (2537 A) from which wavelengths to the violet of 2400 A were filtered out with an acetic acid filter [27]. Thymine glycol was prepared according to the method of Baudisch & Davidson [2]. Catalase was prepared from beef liver according to the method of Tauber & Petit [32]. Cytochrome c (Sigma) was a commercial product.

Methods

The source of radiation was a 48 Curie disc of cobalt fixed, with its axis vertical, to a steel rod. Samples were contained in 5 ml. bottles. Where desired, the solutions were saturated with oxygen by passing the gas through for 30 min., following which the bottles were closed with cork stoppers and sealed with paraffin. The samples were then placed at required distances from the source in the receptacles of a plastic stand fixed and centred with respect to the source. The doses used varied from 80 - 300 kr. and dosimetry was by means of the ferrous sulphate dosimeter [35].

Solutions were suitably diluted for absorption spectra measurements by means of a Unicam SP-500 spectrophotometer. A Radiometer pH meter with glass electrodes was used for pH measurements.

H_2O_2 was determined with the titanium sulphate reagent according to Eisenberg [8], total peroxides with potassium iodide according to the method of Hochenadel [15], and organic peroxides from the difference between these two.

The solvent systems used for chromatography of irradiation products were as follows (in all instances ascending chromatography with Whatman No. 1 paper): (*A*) *n*-butanol - acetic acid - water (2 : 1 : 1, by vol.), (*B*) *iso*-propanol - ammonia (conc.) - water (7 : 1 : 2, by vol.), (*C*) *n*-butanol - ethanol - water (4 : 5 : 1, by vol.), (*D*) ethanol - ammonia (conc.) (100 : 1, v/v), (*E*) *n*-butanol - acetic acid - water (4 : 1 : 5, by vol.), (*F*) tert. amyl alcohol - *n*-propanol - ammonia (conc.) (65 : 5 : 30, by vol.), (*G*) *n*-butanol - - ethanol - formic acid - water (25 : 7.5 : 5 : 12.5, by vol.).

RESULTS AND DISCUSSION

Destruction of the pyrimidine ring, and/or a transformation of its aromatic character, is most conveniently followed by the disappearance of the characteristic absorption maximum in the quartz ultraviolet, as already made use of by several observers [1, 25]. However, a closer

Table 1

Radiochemical yields for transformation of pyrimidine derivatives (G_t) and for pyrimidine hydroperoxide formation (G_p) in buffered and unbuffered medium

For each substance the yields are given for the lowest (80 kr.) and highest (300 kr.) doses applied, as well as the mean for all doses over this range

Compound	Unbuffered medium					0.01 M-phosphate, pH 7.4				
	G_t		G_p		$\frac{G_p}{G_t}$ (%)	G_t		G_p		$\frac{G_p}{G_t}$ (%)
	Variation with increasing dose	Mean	Variation with increasing dose	Mean		Variation with increasing dose	Mean	Variation with increasing dose	Mean	
Uracil	2.62—3.20	3.03	0.39—0.96	0.72	23.7	2.56—2.42	2.72	0.24—0.31	0.31	11.4
Thymine	2.36—2.80	2.55	0.35—0.59	0.48	18.0	1.50—2.20	1.88	0.15—0.23	0.20	10.6
Cytosine	3.54—3.26	3.46	0.06—0.07*	0.12	3.4	3.84—3.06	3.42	0.05—0.29	0.18	5.3
1,3-Dimethyluracil	3.14—3.00	3.18	0.53—0.45	0.52	16.0	2.03—2.51	2.52	0.14—0.21	0.16	7.9
5,6-Dimethyluracil	4.06—3.88	4.13	0.43—0.60	0.53	12.8	2.99—3.25	3.23	0.02—0.24	0.28	7.9
5-Bromouracil	4.30—2.68	3.54	0.00—0.17	0.08	2.2	4.36—2.65	3.72	0.00—0.09	0.05	1.3
5-Bromo-1,3-dimethyluracil	3.88—2.49	3.28	0.17—0.26	0.25	7.6	3.82—2.37	3.19	0.00—0.00	0.00	—

* Value at 231 kr.; at 300 kr. $G_p = 0$

examination of the entire spectrum during the course of irradiation reveals modifications at shorter wavelengths indicating, by analogy with the absorption spectra of dihydropyrimidines [18], the partial formation of products with saturated 5,6 linkages, in agreement with the known formation of hydroperoxides. This is illustrated in Fig. 1 for irradiated

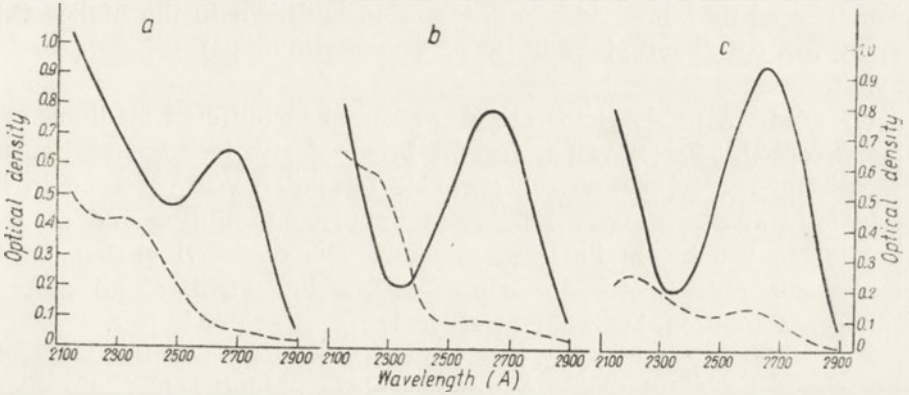


Fig. 1. Absorption spectra at neutral pH prior to (—) and after (- -) submission to a dose of 300 kr. of 10^{-3} M solutions of (*a*) cytosine, (*b*) thymine, (*c*) 1,3-dimethyluracil.

cytosine, thymine and 1,3-dimethyluracil (cf. also Fig. 2, below). Irradiated cytosine, for example, shows the characteristic absorption maximum at about 2350 A of dihydrocytosine; while irradiated dimethyluracil possesses a maximum at about 2200 A similar to that of dihydrodimethyluracil. This latter maximum is partially obscured by H_2O_2 formed during irradiation and is clearly resolved, as in Fig. 1, only following removal of H_2O_2 with catalase.

The G values (number of molecules transformed per 100 *e*V dissipated in the medium) for formation of total radiation products, G_t, were calculated from the decrease in absorption at the principal maximum and are given in Table 1 for 10^{-3} M solutions at neutral pH (buffered and unbuffered) following exposure to doses of from 80 to 300 kr. The G_t values are not constant but show some variation with the applied dose. The table therefore gives the variation in G_t as the dose is varied from the lowest to the highest applied. In Table 1 are also presented the G values for pyrimidine hydroperoxide formation, (G_p), over the same range of applied doses. The final column gives the percentage of transformed molecules which make up peroxides, using average values of G_p and G_t over the range of doses applied. Attention is drawn to the following points:

(a) The G_t values for most compounds vary only about 10% from the mean over the range of doses applied. For the bromo derivatives.

however, these variations are quite marked. In most instances the yield decreases with increasing dose, but there are several examples where the yield is higher at higher doses.

(b) The G_t values are usually somewhat lower in buffered, as compared to unbuffered, solution with the exception of bromouracil for which it is higher, and of 5-bromo-1,3-dimethyluracil, for which the G_t value is unaffected by buffer addition. Attention is drawn to the higher value of G_t for 5,6-dimethyluracil (i. e. 6-methylthymine) as compared to thymine.

(c) Hydroperoxide yields are also reduced in buffered medium, cytosine excepted. The G_p values for the bromo derivatives are particularly interesting. For 5-bromouracil, peroxide formation was observed in low yield only at doses above 100 kr. in unbuffered solution and at doses superior to 200 kr. in buffered medium. The dimethyl analog, on the other hand, forms peroxides, albeit with a low yield, at all doses in unbuffered solution, but none at all in the presence of buffer.

Even more striking is fact that the bromo derivatives, although giving appreciably lower or zero yields of peroxides, exhibit a higher radiation sensitivity (i. e. higher G_t values) than thymine. This is in agreement with the reported higher radiation sensitivity of biological material containing bromouracil in place of thymine in its DNA [37, 19].

(d) For cytosine in unbuffered medium the G_p values in the table are not fully representative. G_p initially increased with increasing dose to a value of 0.18 at 160 kr. and then diminished at higher doses to a value of zero at 300 kr. Note in particular that in buffered medium there is a considerably higher yield of hydroperoxides.

Our values of G_t are somewhat higher, and of G_p lower, than those reported by Scholes *et al.* [25] for several of the above compounds. The reasons for these discrepancies are not clear. From the data of Ekert & Monier [9] for 10^{-3} M-thymine in water under O_2 we find their value of G_p to be 0.7, about the same as our own.

pH changes in unbuffered medium: In unbuffered medium, irradiation is accompanied by a decrease in pH, as shown in Table 2, with the exception of cytosine. For the latter compound this is explicable in the light of the finding of Ekert & Monier [11] that cytosine undergoes deamination following peroxide formation, a result also to be expected in view of the known instability of cytosine derivatives containing a 5,6 saturated bond [13, 18]. The pH decrease is also most marked for the bromo derivatives, notwithstanding their low peroxide yields, and may be associated with the higher overall radiochemical yields exhibited by them.

Irradiation in air: Irradiation is normally conducted under an oxygen atmosphere in order to increase the yield of peroxides. It is therefore

of interest to examine peroxide formation in an atmosphere of air. This was done for thymine and dimethyluracil at a concentration of 10^{-3} M and an applied dose of 120 - 330 kr. The G_t values under these conditions were practically unchanged but the G_p yields were decreased to one-half. These are the values which would probably have to be taken into account in irradiation of biological material.

Table 2

Changes in pH resulting from irradiation of 10^{-3} M unbuffered solutions of pyrimidine derivatives submitted to a dose of ~ 300 kr.

Compound	pH	
	before irradiation	after irradiation
Uracil	6.7	4.0
Thymine	6.7	5.2
Cytosine	6.5	6.6
1,3-Dimethyluracil	7.0	4.2
5,6-Dimethyluracil	7.0	4.2
5-Bromouracil	6.8	3.2
5-Bromo-1,3-dimethyluracil	7.1	3.1

Influence of concentration: For thymine and dimethyluracil (unbuffered at pH ~ 6.5) under oxygen at a concentration of 10^{-4} M and an applied dose of 80 kr., the values of G_t were reduced, respectively, to 0.92 and 1.08. Peroxide formation, on the other hand, increased considerably; the G_p values for thymine and dimethyluracil were, respectively, 0.75 and 0.75, so that the percentage peroxide formation was 82 and 70.

It is clear from the foregoing that it will be no simple matter to extrapolate the behaviour of the free bases to that likely prevailing in nucleic acids and that it may be necessary to employ model polynucleotide chains to determine the extent of formation of pyrimidine peroxides to be expected in natural nucleic acids.

Radiation sensitivity of thymine hydroperoxides: It was tacitly assumed in the foregoing results that organic peroxides are radiation resistant. This is, however, a most unlikely possibility.

Synthetic *trans* thymine hydroperoxide was dissolved in distilled water at a concentration of 100 μg./ml. and the solutions saturated with oxygen prior to irradiation. Under analogous conditions a mixture of thymine and thymine hydroperoxide was irradiated at concentrations prevailing in the experiments (above) on radiation induced formation of peroxides. The results are illustrated in Table 3.

An examination of the alkaline instability of the product(s) of irradiation of thymine peroxide (by following the rate of disappearance of

the absorption at 2300 A in 0.1 N-NaOH, see Fig. 2 below) demonstrated the formation of a new compound for which the half-time of alkaline decomposition, $t_{1/2}$ was 1.2 min. as compared to 12 min. for thymine peroxide.

It follows from the above results that thymine peroxide itself is radiation-sensitive and that the peroxide group is removed as a result of irradiation, possibly accompanied by rearrangement. The disappearance of organic peroxide groups is dependant on the thymine hydroperoxide concentration and on the radiation dose.

Table 3

Effect of irradiation on thymine hydroperoxide in solution

Compound	Irradiation dose (kr.)	Reaction with μM/l.		Difference (μM/l.)	% of initial value	G
		KI	$Ti_2(SO_4)_3$			
Thymine peroxide	0	697	0	697	100	—
Thymine peroxide	255	627	321	306	44	1.6
Thymine peroxide	0	546	0	546	100	—
Thymine peroxide	165	569	203	366	67	1.1
Thymine peroxide	0	261	0	261	100	—
Thymine peroxide	147	396	224	172	66	0.6
Thymine	0	0	0	0	—	—
Thymine	147	275	112	163	—	—
Thymine + thymine peroxide*	0	254	0	254	100	—
Thymine + thymine peroxide	147	381	85	183**	51	0.9

* Mixture containing 126 μg./ml. thymine and 46 μg./ml. thymine hydroperoxide.

** This value was obtained by subtraction from 381 of 85 (amount of H_2O_2 formed) and 163 (amount of thymine peroxide formed from thymine in the mixture).

It consequently follows that the yields (G_p) for organic peroxide formation in irradiated solutions are only apparent yields representing the difference between peroxide formation and destruction. This may be the source of the low observed peroxide yields for 5-bromouracil.

Reaction of thymine hydroperoxides with p-*dimethylaminobenzaldehyde:* Alkalization of dihydropyrimidines results in the opening of the 3,4 bond with the formation of ureido acids which may then react with p-dimethylaminobenzaldehyde; this principle is made use of to locate dihydropyrimidines on paper chromatograms [12, 18]. A similar procedure was found to be applicable to the detection of pyrimidine peroxides which also possess a saturated 5,6 bond, and which react with p-dimethylaminobenzaldehyde only after treatment with alkali. It was shown spec-

trally that treatment of such peroxides with alkali results in opening of the 3,4 bond. Like dihydrothymine [18], thymine peroxide exhibits an absorption maximum in alkaline medium at 2300 A, the extinction of which decreases with opening of the 3,4 bond. The half-time for ring opening in 0.1 N-NaOH was found to be about 12 min. at room temperature, as compared to 6.2 min. for dihydrothymine [18]. The *trans* form of thymine peroxide reacts with *p*-dimethylaminobenzaldehyde to give a blue spot, while the *cis* form gives a yellow spot, the colour intensity being proportional to the amount of substance on the chromatogram.

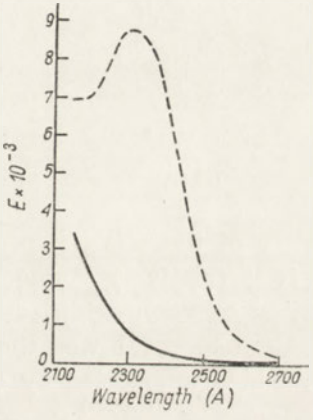


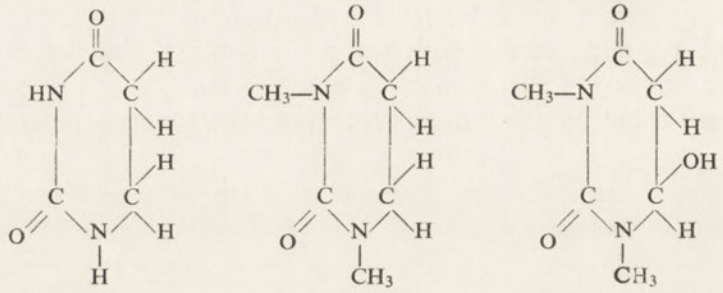
Fig. 2. Absorption spectrum of thymine hydroperoxide at neutral pH (—) and at pH 12 (— —). The latter spectrum is only transitory, due to alkaline lability of the compound (see text for details)

About 4 μg. substance is required to give a well-defined colour as compared to about 8-10 μg. with methanolic KI. It is therefore not only 2-3 times more sensitive but also distinguishes between the two isomers.

The above reagent demonstrated also the presence of several additional blue and yellow spots in irradiated solutions of thymine other than peroxides. Five such spots were noted following chromatography in solvent system *A*. Three of these reacted with the reagent without prior treatment with NaOH and hence must be products resulting from ring opening as a result of irradiation. One of these was identified as urea (R_F 0.62), the second as β-ureidobutyric acid (R_F 0.78), while the third (R_F 0.35) was unidentified. Prior treatment with NaOH, followed by spraying with the reagent, revealed one yellow spot corresponding to dihydrothymine in fairly high yield. In addition there were two spots with R_F values of 0.52 (corresponding to thymine peroxides, the isomers of which are not separated in this solvent) and 0.45 (unidentified).

It is difficult to decide unequivocally whether the β-ureidobutyric acid is a direct product of irradiation of thymine or whether it is one of the subsequent products of decomposition. In view of the presence of appreciable quantities of dihydrothymine, it is most likely the direct product of decomposition of this latter.

Irradiation of pyrimidine derivatives containing a saturated 5,6 bond: In addition to thymine hydroperoxide, three such compounds were examined: 5,6-dihydrouracil, 1,3-dimethyldihydrouracil and 1,3-dimethyl--5-hydro-6-hydroxyuracil:



5,6-dihydrouracil (U.H_2)

1,3-dimethyldihydrouracil (DMU.H_2)

1,3-dimethyl-5-hydro-6-hydroxyuracil (DMU.H_2O)

The latter of these is the product of ultraviolet (2537 A) irradiation of DMU; it is relatively stable at room temperature and neutral pH but, on heating or upon acidification or alkalization, it reverts to DMU and acidification to pH 1 was the criterion used for estimating the amount of DMU · H_2O unaffected by γ-irradiation.

Irradation of a 10^{-4} M solution of U.H_2 results in the formation of some organic peroxides, dependant on the dose and the presence of oxygen, but in relatively low yield, the G_p not exceeding 0.2. Such peroxide formation must have been preceded by oxidation of the pyrimidine ring (see next paragraph).

For DMU · H_2 the yield of peroxides was considerably higher, the value of G_p being about 0.7. No convenient method exists for the quantitative determination of DMU · H_2 in the presence of other derivatives with saturated 5,6 bonds, hence no values for G_t were recorded. Paper chromatography of the irradiation products in solvent *B* demonstrated the presence of small quantities of DMU and 3-methyluracil, up to about 5% of the starting product. In addition there were two products exhibiting fluorescence under the ultraviolet lamp with R_F values of 0.1 and 0.53. The latter is most likely identical to that resulting from irradiation of DMU under the same conditions. The former has not been identified. These products are not the same as the fluorescent spots obtained by chromatography of the irradiation products of DMU · H_2O (see below).

A 10^{-4} M solution of 1,3-DMU.H_2O was irradiated with a total dose of 28 kr., lyophilized and then submitted to chromatography in solvent *B*. Two spots were found under the UV lamp with R_F values of 0.89 and 0.77; these were identified as 1,3-DMU and 3-methyluracil, respectively, on the

basis of their R_F values, as well as elution followed by an examination of their absorption spectra as a function of pH [26]. Following a dose of 100 kr. the solution contained smaller quantities of two additional products with R_F values of 0.57 and 0.5, corresponding to 1-methyluracil and uracil; at the same time the amount of 3-methyluracil was increased appreciably and attained 20% of the starting product. These products were not detected in the solution immediately after irradiation (if they had been present, they would have shown up by means of their spectra), but only after exposure to the alkaline solvent used for chromatography. With an increase in dose to 600 kr., chromatography of the radiation products in the same solvent system exhibited the presence of only uracil and additional ultraviolet absorbing component with an R_F of 0.79, as well as a fluorescent component with an R_F of 0.15. The ultraviolet absorbing spot possessed an absorption maximum at 2575 A at pH 7 which was shifted to 2825 A at pH 13 with the concomitant appearance of an additional maximum at 3350 A; this substance made up about 15% of the starting product (assuming its extinction coefficient at neutral pH to be approximately that of uracil), but has not been identified. The amount of uracil in this case was about 15%.

It is clear that in the initial stages of the reaction, extensive demethylation occurs at position 1 and, to a smaller extent, at position 3. The final formation of free uracil is obviously due to demethylation first at position 1, then at position 3. It is of interest to note that the extensive demethylation observed is completely absent in irradiated 1,3-DMU. The greater facility with which demethylation occurs at position 1 is in agreement with the known fact that, in reduced pyrimidine glycosides, the glycosidic linkage is considerably more labile. The presence of the water molecule at the 5,6 bond also inhibits attack by free radicals since irradiation of a similar solution of DMU at such high doses produces complete transformation to reaction products no longer containing the aromatic ring.

The yield of organic peroxides from DMU.H_2O is somewhat lower than in the case of DMU·H_2, G_p being about 0.56.

Three additional products resulting from irradiation of DMU.H_2O and DMU.H_2 were detected, following chromatography in solvent *E*, by spraying with 0.5 N-NaOH and then *p*-dimethylaminobenzaldehyde to give yellow spots. One of these with R_F 0.38, was common to both substances. The other two possessed R_F values of 0.54 and 0.73, but none of these was identified.

Ultraviolet induced formation of organic peroxides: Scholes *et al.* [23] reported that nucleic acids and their derivatives give rise to peroxides under the influence of ultraviolet radiation. This is a matter of considerable importance in the photochemistry of nucleic acids, and attention has been

drawn to the fact that the source used by the foregoing authors was such that emission of short wavelengths (below 2200 A) was probably appreciable and that any peroxide formation observed was most likely due to the effect of such short wavelengths acting in part *via* solvent molecules and dissolved oxygen [27, 28]. We have consequently examined the influence of wavelengths to the violet and red of 2300 A on the formation of organic peroxides from several pyrimidine derivatives.

Solutions of the thymine, uracil, 1,3-DMU, 5-BrU and cytosine (10^{-4} or 10^{-3} M) were irradiated with a British Thermal Syndicate mercury resonance lamp in quartz cuvettes fitted with ground joints containing inlet and outlet tubes for introducing oxygen. Solutions were irradiated at a distance of 3.5 cm. so that the incident intensity at 2537 A was 3.3×10^{-7} einsteins/cm.^{2}/min. For irradation in the presence of oxygen, the gas was passed through the solution in the cuvette for 15 min. prior to, and during, irradiation. A 5 mm. aqueous acetic acid (1:1) filter was used for eliminating wavelengths below 2400 A. Prior to and following irradiation, measurements were made of the following: ultraviolet absorption spectrum, H_2O_2 with titanium sulphate and total peroxide with potassium iodide. The results are set forth in Table 4.

Table 4

Influence of ultraviolet irradiation on formation of organic peroxides from pyrimidine derivatives in aqueous solution in presence of oxygen and air

Radiation with filter included only wavelengths above 2400 A; that without filter included traces of radiation down to 1849 A

Compound	Conc. (M/l.)	In presence of	Yield organic peroxides (μM/l.)			
			Irradiation time	Without filter	Irradiation time	With filter
Thymine	10^{-4}	O_2	25 min.	0	5 hr.	0
Thymine	10^{-3}	O_2	3 hr.	60	5 hr.	0
Thymine	10^{-3}	air	3 hr.	6	—	—
Uracil	10^{-4}	O_2	20 min.	7	3 hr.	0
Uracil	10^{-3}	O_2	4 hr.	13	—	—
Uracil	10^{-3}	air	4 hr.	0	—	—
1,3-DMU	10^{-3}	O_2	5 hr.	2	—	—
5-BrU	10^{-4}	O_2	20 min.	0	—	—
Cytosine	10^{-4}	O_2	20 min.	0	—	—

For low concentrations of H_2O_2 the titanium sulphate reaction gives values somewhat on the high side as compared to KI, the error being of the order of 6 - 20%, which must be taken into account for the 10^{-4} M solutions. If, however, we examine the results for the 10^{-3} M solutions, it

may be concluded that, even in the absence of the filter, there is practically no formation of peroxides, or at any rate less than 1% of the number of molecules which have been photochemically transformed, as estimated from changes in absorption spectra. On the other hand there was a slight "after-effect"; if irradiated solutions were maintained at room temperature, minimal formation of organic peroxides was observed. With filtered radiation there was no formation of measureable quantities of organic peroxides.

As a separate control of the foregoing, distilled water was irradiated under identical conditions, with filter and in presence of oxygen. The water was twice glass-distilled over permanganate. Following 5 hr. irradiation, 5.6 μM/liter H_2O_2 was found in irradiated solvent by the method of Hochenadel [15]. It is conceivable that such a quantity of H_2O_2 may result in the formation of organic peroxides; if so, these latter are formed in too small a concentration to estimate by our methods.

Chromatography of products of irradiation of DMU and thymine: A number of additional products were isolated by paper chromatography of cobalt irradiated 10^{-3} M solutions of DMU and thymine, but positive

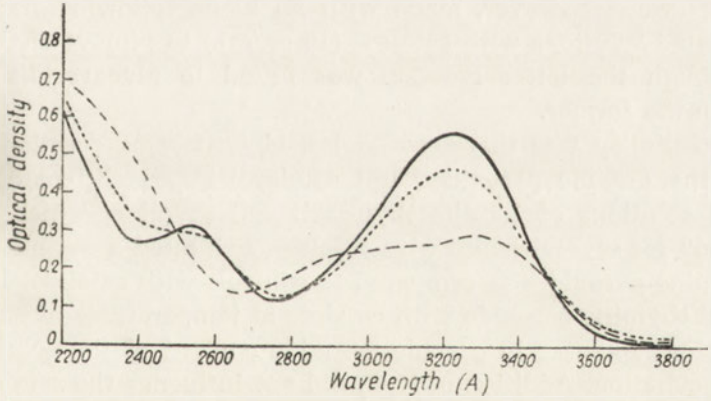


Fig. 3. Absorption spectra at pH values indicated of one of the products of irradiation of 1,3-dimethyluracil. Irradiation product eluted from paper chromatogram following chromatography in solvent B, in which it has an R_F of 0.57. (—), at neutral pH; (— —), at pH 2; (- - -), at pH 12

identification in most instances proved rather difficult of achievement. Irradiated solutions were first lyophilized, then dissolved in a small quantity of water for spotting on the chromatograms.

When viewed under a "mineralight" (2537 A) or a mercury lamp with a Wood's filter (3600 A), irradiated DMU was found to give two strongly fluorescent spots with R_F values in solvent *B* of 0.57 and 0.74 as compared to 0.92 for DMU. Both spots react with KI. An eluate of the upper spot

showed no characteristic absorption at neutral pH, but exhibited a maximum at 2825 A at pH 2 and another at 3175 A at pH 12. The absorption spectrum of the lower spot as a function of pH is shown in Fig. 3.

With solvent system *B*, irradiated thymine gave an absorbing spot with an R_F of 0.68 (thymine 0.78), an eluate of which showed no maximum at pH 7, but a maximum at 2650 A at pH 2 and at 2875 A at pH 12.

Irradiated thymine was also run in solvent systems *C*, *D*, and *E* and, following conversion to the dinitrophenylhydrazine derivatives, in the solvent *F* [17], with application of the following staining reactions: alkaline $AgNO_3$ [33], 1% methanolic bromocresol green, and *p*-dimethylaminobenzaldehyde [16]. This led to the identification of urea, formic acid and pyruvic acid. These are, however, of little interest since they must represent final products of degradation; all three are, in fact, obtained by mild hydrolysis of thymine hydroperoxide [36]. Some additional products were also located which gave reactions for keto, aldehyde and ureido groupings.

Influence of catalase on pyrimidine hydroperoxides: In most of the above work, organic peroxides in irradiated solutions were estimated from the difference between the reaction with KI and titanium sulphate. Some estimations were, however, made with KI alone following treatment of the irradiated solutions with catalase (15 μg/ml.) to remove H_2O_2. Surprisingly enough the latter reaction was found to give results 15 - 80% lower than the former.

The possibility was therefore envisaged that some reaction occurred between the organic peroxides and catalase. Paper chromatography of irradiated solutions of 1,3-dimethyluracil and thymine, which had been "incubated" for several hours with catalase, exhibited a complete absence of pyrimidine peroxides as compared to controls with catalase. Incubation of isolated thymine peroxide with catalase at temperatures of 22° and 38° led to a slow decrease in organic peroxide content of the solutions with time of incubation. Addition of H_2O_2 did not influence the rate of decomposition of thymine peroxide by catalase.

That this is not an enzymatic reaction was shown by the fact that heat-inactivated catalase (5 min. at 100°) was only slightly less "active" against hydroperoxides. A similar conclusion follows from the fact that the concentration of catalase required to obtain an observable decomposition of peroxides is of the order of 3 μg./ml., much higher than that required for enzymatic decomposition of H_2O_2. An equivalent molar concentration of cytochrome *c* was likewise found to cause a time-dependant decrease in organic peroxide content, although at a considerably lower rate than with catalase (Fig. 4). That this reaction is not entirely lacking in specificity was demonstrated by the absence of any effect with a protein such as ribonuclease, which is basic like cytochrome *c*. No effect could be

observed with ferric ions at a concentration equivalent to that contained in catalase. It is therefore possible that the action of catalase and cytochrome c is linked in some way with the presence of the heme group.

The product resulting from the decomposition of thymine peroxide by catalase could not be positively identified. It appears, however, like thymine peroxide, to contain the intact pyrimidine ring with a saturated 5,6 bond. The decomposition of this product in alkaline medium, pH 12,

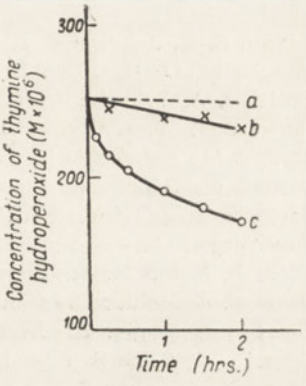


Fig. 4. Transformation of thymine hydroperoxide by catalase and cytochrome c at neutral pH and 38° and a concentration of 4×10^{-7} M. Concentration of thymine hydroperoxide shown on ordinates. (a), Thymine hydroperoxide alone; (b), in presence of cytochrome c; (c) in presence of catalase

as followed by the rate of decrease in optical density at 2300 A, proceeded with a $t_{1/2}$ of about 8 min. at 23°. This is fairly close to the $t_{1/2}$ for decomposition of glycol thymine under the same conditions, 8.8 min., but attempts to confirm this chromatographically were unsuccessful.

SUMMARY

A study has been made of the radiation-induced transformation of a number of pyrimidine derivatives under various conditions, and with particular reference to formation of pyrimidine hydroperoxides. The latter exhibit the properties of dihydropyrimidines, a fact which is useful for their chromatographic identification with increased sensitivity. Other products containing a saturated 5,6 linkage are also formed as a result of irradiation, e.g. dihydrothymine from thymine. The peroxides themselves are radiation sensitive, the initial products containing the intact ring with a saturated 5,6 bond. N-alkylated pyrimidines with a saturated 5,6 bond readily undergo dealkylation, without opening of the pyrimidine ring, in relatively high yield. Quantitative data are presented for formation of some of the radiation products under given conditions.

No formation of organic peroxides results initially from the ultraviolet irradiation of pyrimidine derivatives, even in an oxygen atmosphere, at wavelengths to the red of 2400 A. There is, however, some evidence for a small "after-effect". To the violet of 2200 A there is some formation of

peroxides, a fact of some importance in the photochemistry of nucleic acids.

Both catalase and cytochrome *c*, in relatively high concentration, provoke the decomposition of pyrimidine peroxides; but the reaction is not an enzymatic one. The resulting product, in the case of thymine hydroperoxide, appears to resemble thymine glycol.

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CHEMIA RADIACYJNA KWASÓW NUKLEINOWYCH I ICH POCHODNYCH

I. PIRYMIDYNY, DWUHYDROPIRYMIDYNY I HYDRO-HYDROKSYPIRYMIDYNY

Streszczenie

Badano w różnych warunkach wpływ promieni γ na pochodne pirymidynowe, ze szczególnym uwzględnieniem powstawania wodoronadtlenków pirymidynowych. Te ostatnie związki wykazują właściwości dwuhydropirymidyn; zostało to wykorzystane do zwiększenia czułości ich chromatograficznej identyfikacji. W wyniku napromieniowania powstają również inne produkty zawierające nasycone wiązanie 5,6 np. dwuhydrotymina z tyminy. Same nadtlenki są także wrażliwe na promieniowanie, a powstające produkty zawierają nienaruszony pierścień z nasyconym wiązaniem 5,6. N-alkilowane pirymidyny posiadające nasycone wiązanie 5,6 łatwo odszczepiają grupy alkilowe, bez otwierania pierścienia pirymidynowego, ze stosunkowo wysoką wydajnością. Przedstawione zostały ilościowe dane dotyczące powstawania w danych warunkach niektórych produktów.

Naświetlanie ultrafioletem pochodnych pirymidynowych przy długościach fali powyżej 2400 A nie powoduje początkowo powstawania nadtlenków organicznych nawet w obecności tlenu. Zaobserwowano jednak powstawanie niewielkiej ilości wodoronadtlenków organicznych po przerwaniu naświetlania. Naświetlanie ultrafioletem do 2200 A wywołuje powstawanie niewielkich ilości nadtlenków organicznych; fakty te mają znaczenie w fotochemii kwasów nukleinowych. Zarówno katalaza jak i cytochrom *c*, w stosunkowo wysokich stężeniach wywołują rozkład wodoronadtlenków pirymidynowych, nie jest to jednak reakcja enzymatyczna. Powstający produkt, w wypadku wodoronadtlenku tyminy, jest podobny do glykolu tyminy.

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МАРИЯ САРНЕЦКА-КЕЛЛЕР

ПЕПТИДНАЯ СТРУКТУРА ПРОТЕИНОВЫХ КИСЛОТ

Резюме

Исследованы соединения, входящие в состав смеси, изолированной из мочи С. Бондзинским и названные им протеиновыми кислотами. Изучены общие свойства этих соединений и проведено сравнение их с данными, описанными до сих пор в литературе. Показано, что качественный состав смеси протеиновых кислот, изолированных из мочи, является относительно постоянным. Обоснован пептидный характер этих веществ. Установлено, что в нормальной моче протеиновые кислоты составляют 0,68 - 1,27% общего азота мочи, в то время как на долю пептидов, содержащихся в смеси протеиновых кислот, приходится 16 - 21% всех соединений мочи, дающих положительную биуретовую реакцию. Протеиновые кислоты, полученные из мочи больных, показывают количественное различие в содержании азота и пептидов по сравнению с нормальной мочой.

Исследован пептидный состав протеиновых кислот. Показано, что разделение протеиновых кислот на антокси-, аллокси- и оксипротеиновые кислоты по методу, предложенному Бондзинским и сотрудниками, не дает разделения смеси на определенные пептидные фракции и связано с большими померями сфракционируемого материала.

Применение высоковольтного электрофореза и хроматографии на бумаге позволило изолировать из смеси протеиновых кислот 22 соединения, окрашивающиеся нингидрином и изатином. 14 из них были идентифицированы как пептиды и определен их аминокислотный состав. У 8 пептидов определены N- и C концевые аминокислоты

Р. ПАКУЛА, В. ВАЛЬЧАК и Д. ШУГАР

ИНАКТИВИРОВАНИЕ МАРКЕРОВ СТРЕПТОМИЦИНО-УСТОЙЧИВОСТИ ТРЕХ ВИДОВ БАКТЕРИЙ ИОНИЗИРУЮЩИМ ИЗУЧЕНИЕМ

Резюме

При помощи γ-лучей Co^{60} инактивировали маркеры стрептомицино-устойчивости трансформирующего агента. Были исследованы трансформирующие агенты стрептококков, диплококков воспаления легких и штамма H. influenzae параллельно в 0,15 м растворе NaCl и в сухом состоянии пленки. В растворах установлены незначительные, но определяемые различия в чувствительности агентов исследуемых штаммов. В опытах с пленками эти различия оказались значительно более отчетливыми, особенно в присутствии предохраняющих веществ, таких как вытяжка из дрожжей. Эта вытяжка усложняла характер кривых, иллюстрирующих инактивацию маркеров, при этом чувствительность данной DNA, по сравнению с другими, изменялась в зависимости от использованной дозы облучения.

Используя теорию попаданий, можно вычислить, что минимальный молекулярный вес маркеров устойчивости к стрептомицину составляет около 10^5. Однако, сложный характер кривых инактивации, зависимость их характера от концентрации DNA и влияние вытяжки дрожжей вызывают серьезные сомнения в отношении данных, полученных из расчетов.

Полученные результаты были сопоставлены с результатами других авторов и подвергнуты обсуждению.

ГАЛИНА СЕРАКОВСКА и Д. ШУГАР

НЕКОТОРЫЕ ДАННЫЕ О ГИСТОХИМИЧЕСКОМ ИССЛЕДОВАНИИ ЛОКАЛИЗАЦИИ НУКЛЕАЗ В ТКАНИ

Резюме

Расширена специфичность метода Дауста, основанного на применении пленки, содержащей субстрат, для гистохимического определения локализации рибонуклеазы и дезоксирибонуклеазы. Это достигнуто введением в качестве субстрата, кроме RNA и DNA, также естественных и синтетических полинуклеотидов. Была исследована

приблизительная энзиматическая активность срезов почки и селезенки крысы при использовании в качестве субстратов RNA, DNA, апуриновой кислоты, олигогуаниловой и полиадениловой кислот.

В срезах селезенки не установлены различия в локализации различных ферментов, действующих на эти субстраты.

Различия в локализации ферментов, отмеченные в срезах почки, связаны с действием рибо- и дезоксирибонуклеаз и двух неспецифических фосфодиэстераз.

Обсуждается техническая сторона метода субстратной пленки и указаны некоторые ограничения этого метода.

ЛЮДМИЛА ШАРКОВСКА и И. ГЕЛЛЕР

ИССЛЕДОВАНИЕ ВОССТАНОВЛЕНИЯ КОЭНЗИМА Q

Резюме

Растворимый препарат дегидрогеназы янтарной кислоты восстанавливает коэнзим Q. Эта реакция не чувствительна к антимицину А. Хиноны, такие как, например, *p*-бензохинон, *p*-метилбензохинон, 1,4 нафтохинон и менадион тормозят восстановление коэнзима Q. Менадион, кроме того, тормозит реакцию дегидрогеназы янтарной кислоты с феназином. Это торможение наступает только через некоторое время или в результате предварительной инкубации менадиона с ферментом. Коэнзим Q стимулирует восстановление феррицианида калия дегидрогеназой янтарной кислоты.

Р. НЕМИРО и Е. ПШИЕМСКИ

КОЛИЧЕСТВО ЛИЗОЛЕЦИТИНА В ТКАНЯХ КРЫСЫ

Резюме

При помощи хроматографии на колонке с Al_2O_3 и хроматографии на бумаге, пропитанной кремниевой кислотой, определено содержание лизолецитина в мозгу, легких, почках ,сердце, селезенке, крови, печени и скелетных мышцах белой крысы.

ДАНИЕЛЯ БАРЩ и Д. ШУГАР

РАДИАЦИОННАЯ ХИМИЯ НУКЛЕИНОВЫХ КИСЛОТ И ИХ ПРОИЗВОДНЫХ

I. Пиримидины, дигидропиримидины и гидро-гидроксипиримидины

Резюме

В разных условиях исследовано влияние γ-лучей на производные пиримидина. Особое внимание обращено на образование гидроперекисей пиримидинов, обладающих свойствами дигидропиримидинов. Это было использовано для увеличения чувствительности метода хроматографической идентификации этих веществ. В результате облучения образуются также и другие продукты, содержащие насыщенные связи в положении 5,6, например дигидротимин из тимина. Сами перекиси также чувствительны к облучению, образующиеся при этом продукты содержат неразрушенное кольцо с насыщенным связями в положении 5,6. N-алкилпиримидины, содержащие насыщенные связи в положении 5,6, легко отщепляют алкильные группы без развертывания пиримидинового кольца при относительно высоком выходе. В работе представлены количественные данные, касающиеся образования в данных условиях некоторых продуктов.

Облучение производных пиримидина ультрафиолетом при длине волны свыше 2400 Å не вызывает сначала образования органических перекисей даже в присутствии кислорода. Отмечено, однако, образование небольшого количества органических гидроперекисей после прекращения облучения. Облучение ультрафиолетом до 2200 Å вызывает образование небольших количеств органических перекисей. Эти факты имеют значение в фотохимии нуклеиновых кислот. Как каталаза, так и цитохром в относительно высоких концентрациях вызывают распад гидроперекисей пиримидинов. Эта реакция, однако, не является энзиматической. Образующийся продукт в случае гидроперекиси тимина имеет сходство с гликолем тимина.

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WYTYCZNE DLA AUTORÓW

1. Dokładne zapoznanie się przez autora z treścią „wytycznych" jeszcze przed przystąpieniem do pisania pracy i skrupulatne stosowanie się do nich bardzo ułatwia pracę redakcji; niestosowanie się do „wytycznych" może bardzo znacznie opóźnić druk pracy.

2. Acta Biochimica Polonica publikują prace biochemiczne i biofizyczne oraz z dziedzin pokrewnych, zawierające nie ogłoszone dotychczas wyniki badań doświadczalnych. Prace powinny zawierać element nowości naukowej. Przysłanie pracy do redakcji uważa się za równoznaczne ze stwierdzeniem, że praca nie została opublikowana ani złożona do druku w innym czasopiśmie.

3. Acta Biochimica Polonica drukują prace w języku angielskim. Prace pisane po polsku będą, ewentualnie z ramienia redakcji, przełożone na język angielski; tłumaczenie będzie przesłane autorowi do zatwierdzenia. Przy składaniu maszynopisu w języku polskim należy obok polskich nazw technicznych podać nazwy angielskie, co bardzo ułatwi pracę tłumacza i zmniejszy możliwości błędów.

4. Prace powinny być pisane możliwie zwięźle, ale zwięzłość nie powinna powodować niejasności przedstawienia. Artykuły są przeznaczone przede wszystkim dla czytelnika wyspecjalizowanego w danym temacie, mimo to artykuły powinny być zrozumiałe dla ogółu czytelników. Opis doświadczeń powinien być taki, by mógł je powtórzyć każdy, kto dysponuje odpowiednim materiałem, wyposażeniem i techniczną sprawnością.

5. Na ogół najlepiej odpowiada celowi następujący układ pracy: a. Zwięzły wstęp wyjaśniający cel i założenie pracy. Dla nawiązania łączności z poprzednio ogłaszanymi pracami na ten temat należy wskazać piśmiennictwo w monografiach, postępach, przeglądach itp.; obszerne omawianie piśmiennictwa i obciążanie pracy licznymi odsyłaczami nie odpowiada charakterowi pisma jakim są Acta Biochimica Polonica. b. Opis metod doświadczalnych, charakterystyka, pochodzenie lub sposób preparatywnego uzyskania używanych chemikaliów oraz opis szczegółów, które są potrzebne dla powtórzenia opisanych doświadczeń i sprawdzenia przeprowadzonych obliczeń. W odniesieniu do metod ogłoszonych drukiem, jeżeli stosowano je ściśle zgodnie z opisem, wystarczy powołanie się na nie z podaniem odpowiedniego odsyłacza do piśmiennictwa; jeżeli jednak wprowadzono jakiekolwiek, nawet pozornie niewielkie, odchylenia od pierwotnej metody, należy to wyraźnie zaznaczyć; stwierdzenie, że posługiwano się odnośną metodą „z niewielkimi modyfikacjami" nie jest dopuszczalne. c. Opis wyników ujęty możliwie zwięźle, najlepiej w postaci tablic lub wykresów. Tablice powinny być opatrzone wyjaśniającym opisem, zawierającym odpowiednie dane, jak warunki doświadczalne, liczba zwierząt, wielkość dawek, stężenie składników itp. Gdy mamy do czynienia z większą liczbą w zasadzie podobnych wyników, nie należy podawać ich wszystkich, lecz podać liczbę wyników indywidualnych, wartość średnią oraz dyspersję (standard deviation). Wykresy zdające sprawę z wykonanych doświadczeń powinny wyraźnie podawać punkty doświadczalne, ewentualnie z zakresem rozrzutu. Przedstawienie tych samych wyników i w tabli-

cach i w wykresach może mieć miejsce jedynie w wyjątkowych, szczególnie uzasadnionych wypadkach. d. Dyskusja, której celem jest uwydatnienie znaczenia uzyskanych wyników w odniesieniu do obecnego stanu zagadnienia. W dyskusji nie należy powtórnie omawiać wyników. e. Streszczenie obejmujące około 3% objętości pracy, ewentualnie ujęte w punktach opatrzonych kolejną numeracją. Streszczenie powinno podawać wyniki pracy w miarę możności z głównymi danymi liczbowymi. f. Cytowana literatura w alfabetycznej kolejności autorów. W wykazie należy podawać kolejno: numer pozycji, nazwisko autora, pierwsze litery imion, przyjęty skrót tytułu czasopisma, tom, początkową stronę artykułu i rok wydania. Jeżeli cytowany artykuł ma kilku autorów, należy w wykazie literatury podać nazwiska i początkowe litery imion wszystkich autorów. Dla cytowanych książek (nie czasopism) należy podać także tytuł książki, wydawcę, oraz miejsce i rok wydania. Powołanie się w tekście na odnośną pozycję cytowanej literatury następuje przez wymienienie numeru pozycji wykazu w nawiasie kwadratowym, np. [3]. Wzmianki o „nieogłoszonych doświadczeniach", „informacjach osobistych" itp. należy umieszczać w tekście lub w uwagach na dole strony, a nie w cytowanej literaturze.

6. Oprócz streszczenia lub wniosków, zawartych w tekście pracy, autor powinien przysłać streszczenie podające cel pracy, jej treść i wyniki liczbowe w 50 do 100 słowach, tak by redakcja mogła je przesłać do redakcji czasopism referatowych, jak Biological Abstracts, International Abstracts of biological Sciences; umożliwi to ogłoszenie streszczenia bezpośrednio po ukazaniu się pracy w druku.

7. Na pierwszej stronie pracy należy podać: imię i nazwisko autora(ów), tytuł możliwie zwięzły, ale jasno charakteryzujący treść pracy, i zakład naukowy, w którym wykonano pracę.

8. Skróty, jednostki miar, nomenklatura, powinny być zgodne z tymi, którymi posługuje się The Biochemical Journal. Szczególną troską redakcji jest dążenie do wprowadzenia nomenklatury enzymów i koenzymów zgodnie z zaleceniami Enzymatycznej Komisji Międzynarodowej Unii Biochemicznej. Szczegóły tych zaleceń oraz ich uzasadnienie znajdują się w książce: Report of the Commission on Enzymes of the International Union of Biochemistry, Pergamon Press 1961. Używane w pracy skróty należy wyjaśnić; ani w tytule ani w streszczeniu nie można podawać skrótów, lecz pełne nazwy.

9. Forma zewnętrzna maszynopisu i przygotowanie go do druku. Prace należy przesyłać redakcji w postaci gotowej do druku w dwóch egzemplarzach pisanych na maszynie jednostronnie, z marginesem szerokości około 4 cm po lewej stronie i około 1 cm po prawej stronie, z podwójną interlinią oraz z numeracją stron. Pierwszy egzemplarz należy pisać na papierze piśmiennym białym. Jako nawiasu zwykłego należy używać znaków ( ), a nie / /. Na podobnych kartkach poza tekstem pracy należy umieścić: a) streszczenie po angielsku i po polsku, b) cytowaną literaturę, c) tablice, d) rysunki lub fotografie wraz z objaśnieniami również w dwóch egzemplarzach, e) pełne imię i nazwisko autora(ów) oraz dokładny adres(y) dla przesłania korekty oraz skróconą wersję tytułu nie przekraczającą 60 miejsc literowych („żywą paginę"). Należy również podać nazwisko i adres osoby upoważnionej do przeprowadzenia korekty w przypadku nieobecności autora. W tekście należy zaznaczyć ołówkiem na marginesie w przybliżeniu miejsca, w których powinny być umieszczone tablice i rysunki. Rysunki i fotografie należy wykonać w postaci nadającej się do reprodukcji lub przerysowania. Każdy rysunek lub fotografia winny być przygotowane na oddzielnej kartce. Na odwrocie każdego rysunku lub fotografii należy podać ołówkiem: nazwisko autora, pierwsze słowa tytułu pracy, kolejny numer rysunku oraz pierwsze słowa legendy, która ma być pod nim umieszczona. Do rysunków i fotografii należy dołączyć wykaz na maszynie zawierający kolejne numery wraz z tytułami i legendą. Należy unikać podawania na rysunkach objaśnień tekstowych. Na fotografiach nie należy dopisywać żadnych

oznaczeń; jeśli fotografie takich oznaczeń wymagają, należy umieścić je na przypiętej do fotografii kalce technicznej. Nadmierna ilość rysunków może być wykonana wyłącznie na koszt autora. Prace nie odpowiadające powyższym wymaganiom zostaną przepisane na koszt autora.

10. Redakcja nie uważa się za upoważnioną do przeprowadzania jakichkolwiek zmian w pracy bez zgody autora. Dla dokonania zmian uważanych przez redakcję za celowe odsyła się jeden egzemplarz pracy autorowi, drugi pozostaje w aktach redakcji. Po ostatecznym uzgodnieniu tekstu maszynopisu autor nie powinien wprowadzać żadnych zmian w korektach. W wyjątkowych przypadkach konieczności wprowadzenia takich zmian (poza poprawieniem błędów drukarskich) ich koszty ponosi autor.

11. Autora obowiązuje korekta autorska, którą należy zwracać redakcji w ciągu trzech dni. Zaleca się wykonanie korekty ołówkiem kolorowym, barwy odmiennej od ołówka korektora. Autor otrzymuje bezpłatnie 25 egzemplarzy odbitek pracy. Żądanie większej ilości odbitek winno być wyrażone na piśmie jednocześnie ze złożeniem pracy w redakcji, najpóźniej przy pierwszej korekcie szpaltowej. Koszt dodatkowych egzemplarzy ponosi autor.

12. W razie nie przyjęcia pracy do druku jeden jej egzemplarz pozostaje w aktach redakcji.

ERRATA

P. Szafrański, Hanna Wehr and T. Gołaszewski

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P. 280, line 4 *for* Diethylaminoethylcellulose (ECTEOLA) *read* ECTEOLA

P. 285, line 3 under Fig. 1 *for* Diethylaminoethylcellulose (ECTEOLA) *read* ECTEOLA