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K. GOLANKIEWICZ, MARIA DEZOR and M. WIEWIÓROWSKI

FURTHER INFORMATION ON THE BEHAVIOUR OF ORNITHINE AND β-AMINOPIPERIDON IN AQUEOUS SOLUTIONS AND SOME SPECTRAL DATA ON β-AMINOPIPERIDON AND ITS SALTS

Department of Organic Chemistry, University, Poznań; and Institute of Biochemistry and Biophysics, Polish Academy of Sciences

1. Hydrolysis of β-aminopiperidon (β-AP) to ornithine and lactamization of ornithine to β-AP was studied at different temperature and pH values. 2. The mechanism of lactamization of ornithine was discussed and compared with the lactamization of lupanic acid. 3. On the basis of the infrared-absorption spectra it was suggested that in the solid state β-AP, similarly as α-piperidon, forms strong associate dimers (N—H ··· O=C). These dimers are stable in CCl_4 but are destroyed in $CHCl_3$ solution. The infrared spectra of the β-AP salts showed that there are also dimer-type associations of a different type ($^+NH_3$ ··· ··· O=C).

Preliminary informations on the behaviour of ornithine (Orn) and β-aminopiperidon (β-AP) in aqueous solution were reported previously [2]. In this paper, more precise observations concerning the lactamization of Orn to β-AP, and hydrolysis of β-AP to Orn are presented.

METHODS

β-AP perchlorate was used because the free base is highly hygroscopic and difficult to handle. Samples containing 10 mg. of β-AP (18.8 mg. of perchlorate) and 10 mg. of Orn (12.8 mg. of Orn·HCl) were dissolved separately in 1 ml. of the following nine solutions: NaOH, 0.02, 0.05, 0.1 and 0.2 N; water; HCl, 0.02, 0.05, 0.1 and 0.2 N. The samples were sealed in glass tubes and kept in a thermostat at the following temperatures: 4°, 20°, 60° and 100°. At different time intervals, samples of 10 μl. were taken from each solution and separated by high-voltage electrophoresis in pyridine buffer, pH 5.9 (1 hr., 80 v/cm.). The electrophoretograms were stained with ninhydrin and the amounts of Orn and β-AP were determined in a Locarte-Laurence Recording Densitometer, using standard curves prepared with mixtures of varying amounts of Orn and β-AP (Fig. 1). The adopted method does not allow to determine quantitatively in a mixture of these two com-

pounds an amount of Orn greater than 60% and an amount of β-AP greater than 80%. In samples in which the content of β-AP or Orn did not exceed, respectively, 80 and 60%, the experimental error was about 6% and either substance was determined separately in each solution. In other samples, only the content of the minor component of the mixture was determined and the content of the other one was calculated. Each determination was made in triplicate.

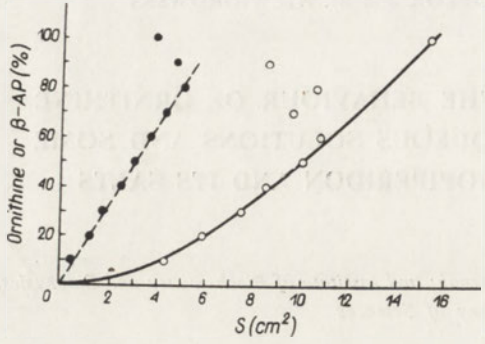


Fig. 1. Standard curves representing (○), the amount of ornithine in the presence of β-AP and (●), the amount of β-AP in the presence of Orn, determined densitometrically after paper electrophoresis and staining with ninhydrin.

The rate of hydrolysis of β-AP and α-piperidon in 4 N-HCl at 80° was determined spectrophotometrically at 224 mμ.

Reagents: Orn·HCl was a Reanal (Budapest, Hungary) product; β-AP was prepared from Orn·HCl according to a recently published method [2].

RESULTS AND DISCUSSION

Figures 2 - 5 represent the rate of hydrolysis of β-AP to Orn (curves 1′ - 9′) and lactamization of Orn to β-AP (curves 1 - 9) in aqueous solutions of sodium hydroxide and hydrochloric acid, and in water, at different temperatures. pH values of the solutions are presented in Table 1. Within about 50 hr. at 60° (Fig. 2c), and about 100 hr. at 4° (Fig. 2a) and 20° (Fig. 2b), only at pH of about 12 (samples 1, 1′) the equilibrium between β-AP and Orn was reached. At 60° at pH about 9 (samples 2, 2′), although the reaction was highly advanced, the equilibrium was not attained after 48 hr. The increase in hydroxyl ion concentration not only accelerated the reaction but also shifted the equilibrium state to the right:

$$\beta\text{-AP} + H_2O \rightleftharpoons \text{Orn}$$

Table 1

pH *values of the reaction solutions*

One ml. of the solution contained 12.8 mg. of Orn·HCl (samples 1 - 9) or 18.8 mg. of β-AP·$HClO_4$ (samples 1′ - 9′).

Solvent	NaOH (N)				Water	HCl (N)			
	0.2	0.1	0.05	0.02		0.02	0.05	0.1	0.2
pH	12	9.0	8.2	7.5	2.9	1.8	1.5	1.0	0.08
Sample no.	1; 1′	2; 2′	3; 3′	4; 4′	5; 5′	6; 6′	7; 7′	8; 8′	9; 9′

At 100° (Fig. 3) the equilibrium between β-AP and Orn was attained in the sodium hydroxide media of 0.2 N (samples 1, 1′), 0.1 N (samples 2, 2′) and 0.05 N (samples 3, 3′) after 2, 8 and 10 hr., respectively. In solutions of 0.02 N-NaOH (samples 4, 4′), 0.1 N- and 0.2 N-HCl (samples 8, 8′ and 9, 9′) after 16 hr. the reaction was so advanced that it was possible to determine by extrapolation the time

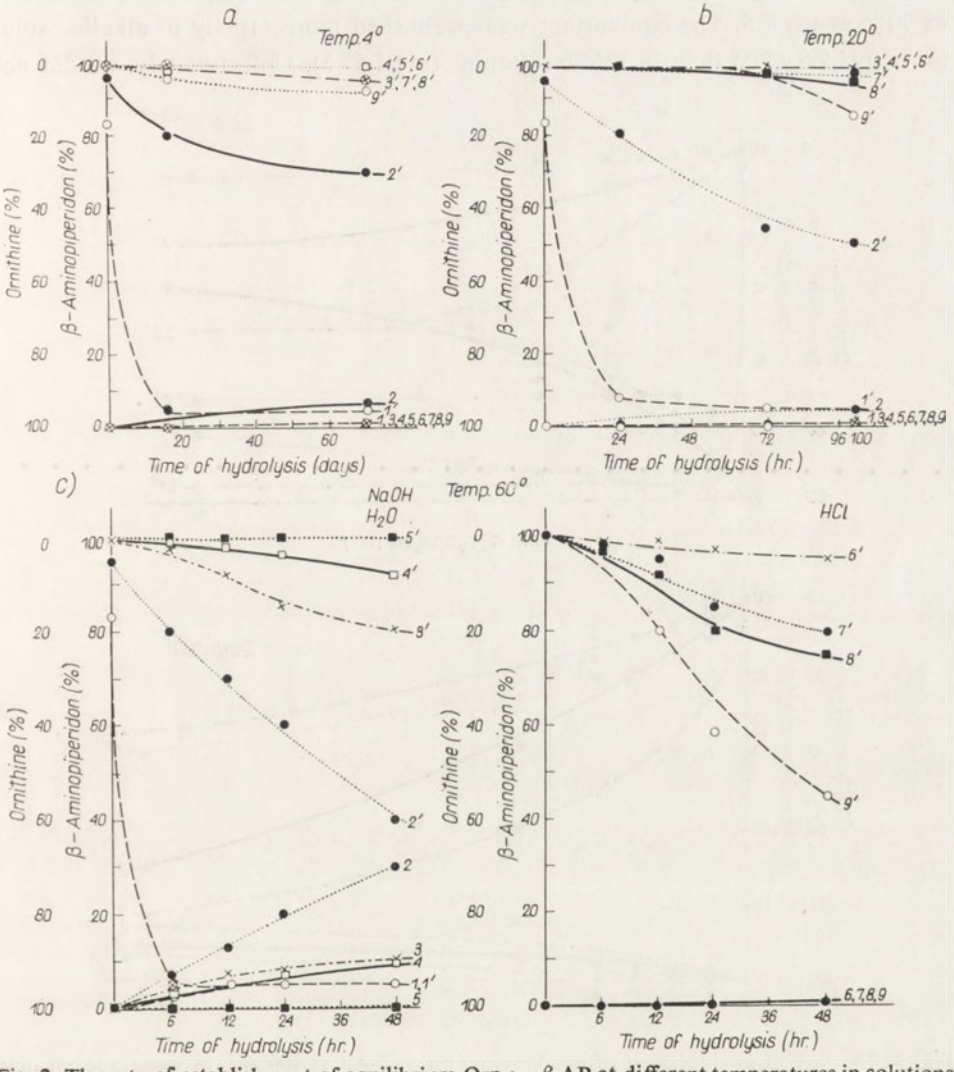


Fig. 2. The rate of establishment of equilibrium Orn ⇌ β-AP at different temperatures in solutions of different pH values. (*a*), At 4°; (*b*), at 20°; (*c*), at 60°. For composition of the samples see Table 1.

needed for attainment of the equilibrium state. It was 18, 24, and 17 hr., respectively. In the remaining three solutions (water, samples 5, 5′; 0.05 N- and 0.02 N-HCl, samples 6, 6′ and 7, 7′) during 16 hr. the rate of the reaction was very slow. Nevertheless, also for these solutions the equilibrium state and the time needed for its attainment could be calculated by extrapolation.

In Fig. 4 the effect of pH at 100°, the time needed for attainment of the equilibrium state, and the percentage of β-AP in the equilibrium state, are presented. It may be seen that in more acidic and more basic solutions the equilibrium is shifted distinctly towards Orn. In solutions of pH about 1.8 and about 8.5 the content of β-AP in the equilibrium state was about 40 - 50%, with a maximum of 70% at pH 7.5. The equilibrium was established more rapidly in alkaline solutions (pH 9.8 - 7.5) than in acidic solutions (pH 1.5 - 3). Unfortunately, we did not

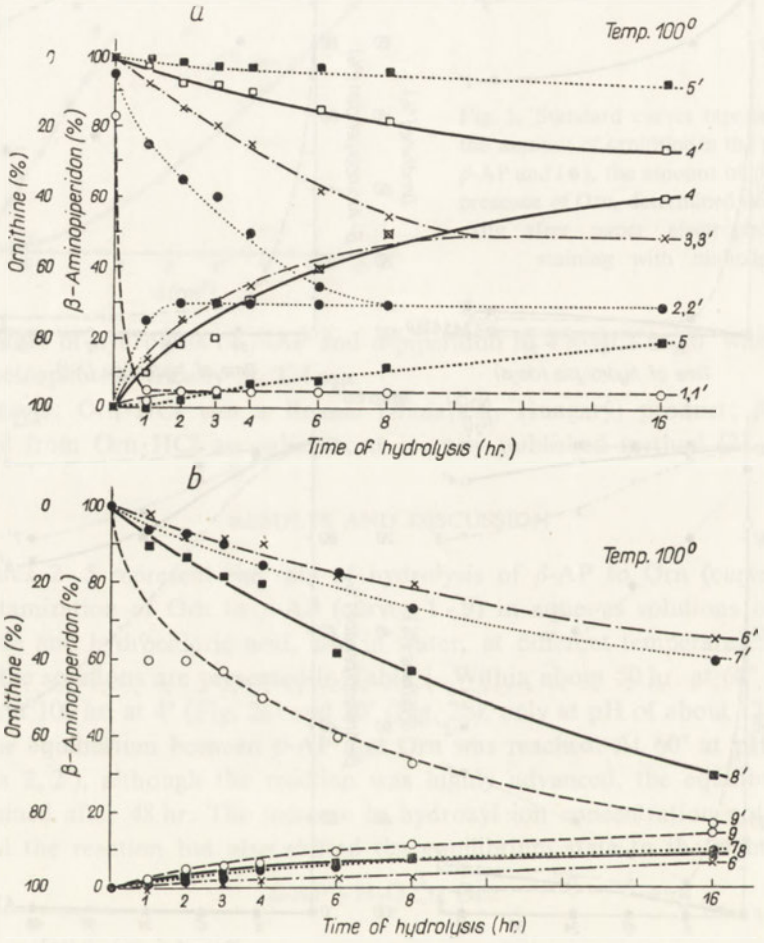


Fig. 3. The rate of establishment of equilibrium Orn ⇌ β-AP at 100° (*a*), in water and alkaline solutions, and (*b*), in acidic solutions. For composition of the samples see Table 1.

investigate the reaction at pH 3.0 - 7.4. Nevertheless, it can be anticipated from the shape of the curves that in this range of pH values the rates both of hydrolysis of β-AP and of lactamization of Orn are very low, and it seems possible that there is such a value of pH (probably about pH 7) at which both these reactions are so slow as to be practically absent.

The rather low rate of lactamization and hydrolysis in neutral or nearly neutral media is very unexpected because, as reported previously [2], the methyl ester of ornithine is immediately condensed to β-AP. Therefore it could have been expected that in aqueous solution of pH about 7 the lactamization of Orn also would be very fast. This supposition was also based on the well-known behaviour of the free methyl ester of lupanic acid as well as of the free lupanic acid itself which rapidly condensed to lupanine [5, 6, 4]. Therefore it is possible to characterize these two compounds only as their salts, e.g. dihydrochlorides.

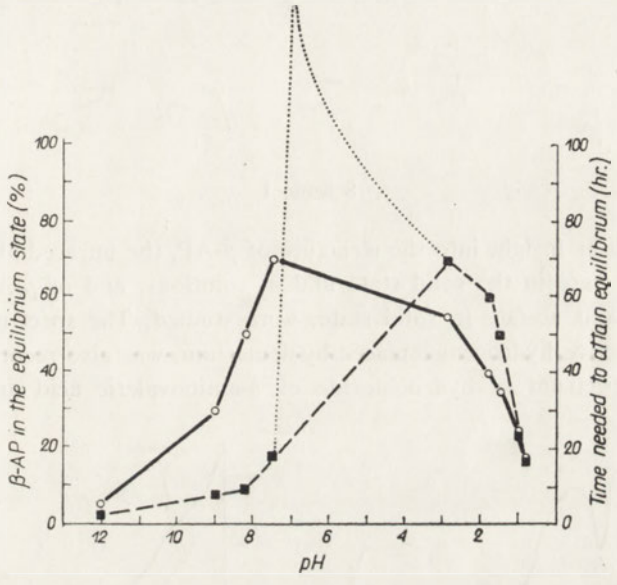


Fig. 4. The effect of pH (■) on the time needed for attaining the equilibrium Orn ⇄ β-AP at 100° and (○), the percentage of β-AP in the equilibrium state.

Although there are some similarities between the two reactions:

$$\beta\text{-AP} \rightleftarrows \text{Orn}$$

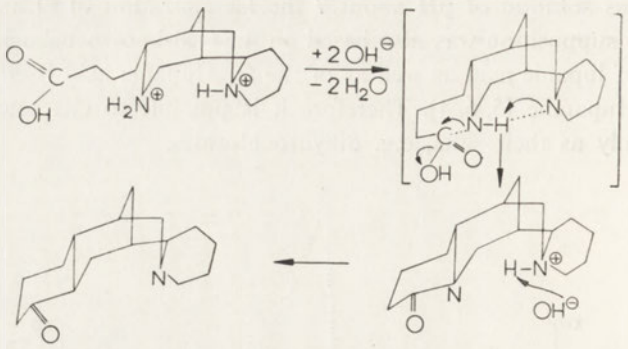
and

$$\text{lupanine} \rightleftarrows \text{lupanic acid},$$

they differ in some respects. In contrast to lupanic acid, free ornithine does not undergo spontaneous cyclization. In the first system, the equilibrium is established faster in alkaline solution, and in the second one in acidic solution; this seems to be due to different position of amino groups in these two amino lactams and two diamino acids.

The behaviour of lupanine and lupanic acid in the reactions of hydrolysis and lactamization was explained only after elucidation of some subtle details of their structure. The infrared-absorption spectra of the alkaloids of the sparteine group demonstrated the presence in the free lupanic acid of a strong hydrogen bond between secondary and tertiary amino groups. This bond would favour the nuc-

leophilic attack of the secondary amino group nitrogen on the carbon of the carbonyl group which would lead to the lactamization. The mechanism of lactamization of lupanic acid is shown in Scheme 1.



Scheme 1

To gain better insight into the structure of β-AP, the infrared-absorption spectra of the free base in the solid state and in solutions, and of β-AP perchlorate, hydrochloride and acetate in solid state, were studied. The spectrum of perchlorate with the active hydrogens replaced by deuterium, was also recorded. For comparison, the spectrum of hydrochloride of δ-aminovaleric acid and the spectra

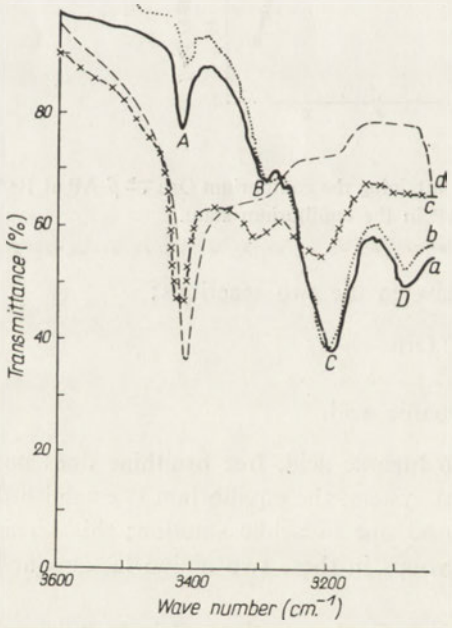


Fig. 5

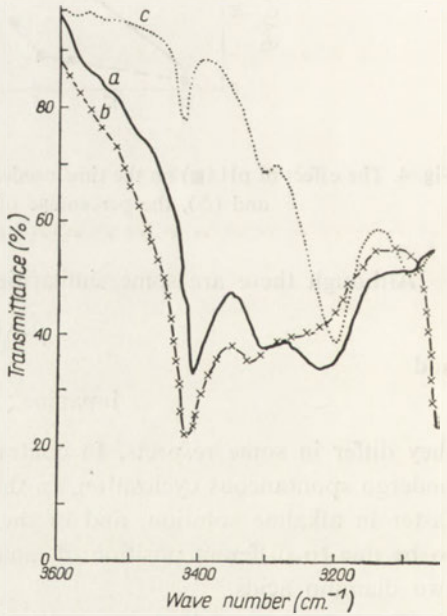


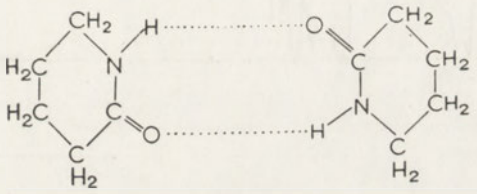
Fig. 6

Fig. 5. The region 3600 - 3050 cm.$^{-1}$ of the spectra of α-piperidon (*a* and *b* in CCl_4, 0.1 and 0.6 mm. cells; and *c* and *d* in $CHCl_3$, 0.1 and 0.6 mm. cells).

Fig. 6. The region 3600 - 3050 cm.$^{-1}$ of the spectra of β-AP (*a* and *b* in $CHCl_3$, 0.1 and 0.6 mm. cells; and *c* in CCl_4, 0.6 mm. cells).

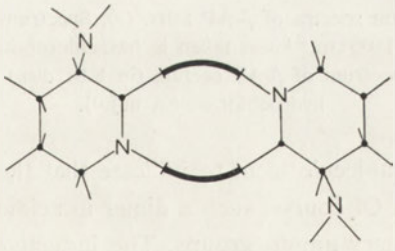
of α-piperidon in CCl_4 and $CHCl_3$ were made. As mentioned before, the rates of hydrolysis of β-AP and α-piperidon were also determined spectrophotometrically. The obtained data showed that α-piperidon was hydrolysed in 4 N-HCl almost immediately whereas β-AP under these conditions was hydrolysed more slowly ($t_{1/2}$ at 80° was about 7 min.).

The infrared-absorption spectra of α-piperidon in CCl_4 (*a* and *b*) and in $CHCl_3$ (*c* and *d*) are shown in Fig. 5. The spectra *a* and *c* were taken using concentrated solutions in 0.1 mm. cells. The spectra *b* and *d* were taken using properly diluted solutions in 0.6 mm. cells. The region 3600 - 3050 cm^{-1} was chosen, and from the positions of the ν NH conclusions were drawn concerning the grade and character of the association of α-piperidon. In the spectra, four maxima (*A* - *D*) are visible. In both CCl_4 solutions the absorption maxima *C* and *D* were the greatest. The dilution caused only a small increase of the weak maximum *A* and a decrease of the maximum *D*. On the other hand, in both $CHCl_3$ solutions the maximum *A* was the most intensive one, and in the dilute solution it was the only maximum, with small shoulders in the places of the maxima *B* and *C*. Since only the maximum *A* is connected with ν N—H of free non-bonded NH group [3], there is a predominance of monomeric α-piperidon only in the dilute $CHCl_3$ solution whereas in CCl_4 the strong associate dimer predominates even in dilute solution (Scheme 2).



Scheme 2

Such structure would favour the protonization of nitrogen and oxygen atoms in acid solution facilitating the nucleophilic attack of water molecule on carbonyl carbon atom, and after breaking of the C—N bond, δ-aminovaleric acid would be obtained. Of course, in water solutions the concentration of the dimer of α-piperidon is low but it is probable that this form is particularly susceptible to hydrolysis.



Scheme 3

The infrared-absorption spectra of β-AP in $CHCl_3$ (Fig. 6) in the region 3600 - - 3050 $cm.^{-1}$ are very similar to those of α-piperidon. This seems to indicate that

introduction of amino group to the molecule of α-piperidon did not decrease the tendency to the dimer-type association, and the absence of a maximum at 3500 cm.$^{-1}$ in the CCl_4 solution suggests that also amino groups are associated.

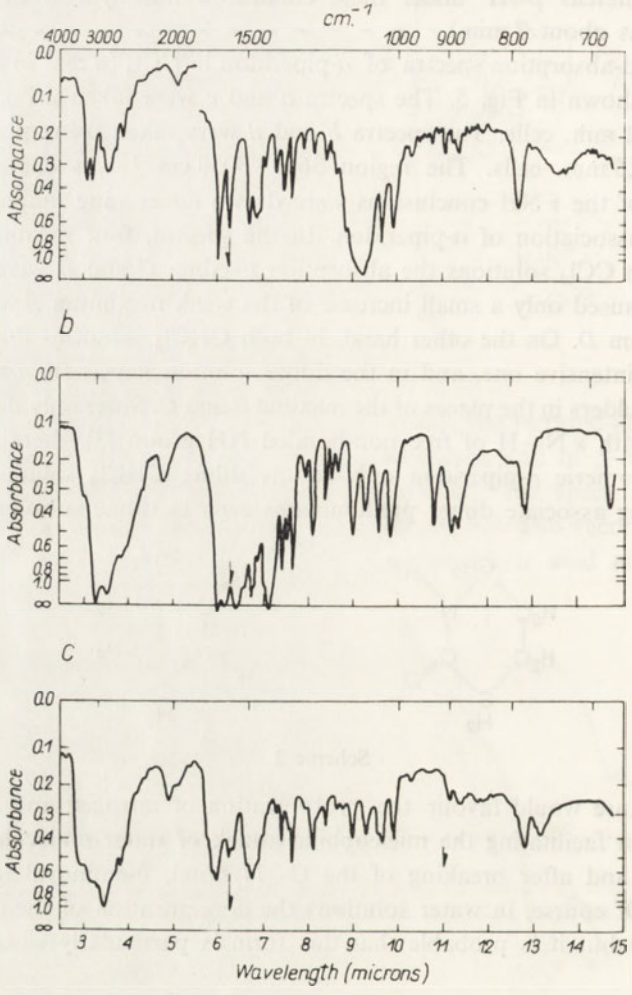
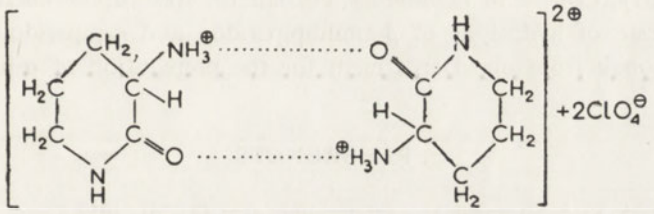


Fig. 7. The infrared-absorption spectra of β-AP salts. (*a*), Spectrum of β-AP perchlorate in the solid state (the region 4000 - 1800 cm.$^{-1}$ was taken in hexachlorobutadiene and the region 1800 - - 650 cm.$^{-1}$ in nujol); (*b*), spectrum of β-AP acetate (in KBr disk); and (*c*), spectrum of β-AP hydrochloride (in nujol).

Studies of the β-AP molecule seem to indicate that the structure of this dimer is as shown in Scheme 3. Of course, such a dimer in acidic solution would be protonated first on the primary amino groups. The inductive effect of $^{+}NH_3$ group would reduce the electropositivity of carbonyl carbon atom and the steric effect would hinder the protonation of oxygen atom. This would explain why the hydrolysis of β-AP is slower than that of α-piperidon. Unfortunately, we are unable

to explain the absence of $\nu_{as}\,NH_2$ at about 3500 cm.$^{-1}$ in dilute $CHCl_3$ solution in which this type of association should be destroyed.

The infrared-absorption spectra of the salts of β-AP are dissimilar and depend on the presence or absence of water of crystallization. The spectrum of β-AP perchlorate (Fig. 7a) seems to be very interesting. It was taken in hexachlorobutadiene (region of 4000 - 1800 cm.$^{-1}$) and in nujol (region of 1800 - 650 cm.$^{-1}$). The perchlorate anion has practically no proton-acceptor properties, therefore the position of the free $^+NH_3$ *a* should be at 3100 cm.$^{-1}$ [1], whereas in the spectrum of β-AP perchlorate this band is missing and instead there are several bands of decreasing intensity in the region of 3000 - 2540 cm.$^{-1}$ which are shifted after deuteration toward 2200 cm^{-1}. Thus they can be ascribed to hydrogen bonded $\nu\ ^+NH_3\cdots B$. This suggests the presence of another kind of association between monoprotonated cations of β-AP (dimerization ?) than in the free base. This possibility is supported by the presence of two bands at 3320 and 3220 cm.$^{-1}$ which can be ascribed to two differently associated ν N—H. On the basis of this evidence it seems possible to suggest the mechanism of dimerization of β-AP perchlorate shown in Scheme 4.



Scheme 4

The association between individual dimers is due to hydrogen bonds N—H···N. The position of ν C=O at 1680 cm.$^{-1}$ and $\beta\ ^+NH_3$ at 1610 cm.$^{-1}$ (which is shifted after deuteration to 1250 cm.$^{-1}$) seems to support this suggestion.

The infrared-absorption spectrum of β-AP acetate (Fig. 7b) is much more complicated as in the region of 3300 - 2500 cm.$^{-1}$ the bands are very compact, but there are no maxima at 3320 and 3220 cm.$^{-1}$. On the other hand, the positions of ν C=O and $\beta\ ^+NH_3$, similar to those found for the perchlorate salt, suggest that the dimerization of monocations of β-AP in acetate is essentially the same. The main difference between them is that in acetate there is no further association of dimers because ν NH is associated with acetic anion (cf. bands at 1560 and 1390 cm.$^{-1}$). Such structure of this salt is supported by the fact that it can easily be sublimed whereas β-AP perchlorate is decomposed during sublimation.

The infrared-absorption spectrum of β-AP hydrochloride (Fig. 7c) differs very much from the spectra of β-AP perchlorate and acetate. The position of ν C=O is at about 1720 cm.$^{-1}$ which indicates that in the crystal lattice the carbonyl oxygen does not play the part of proton-acceptor. The spectrum in the region of 3300 - - 2200 cm.$^{-1}$ is very difficult to interpret. The strongly shaped band at about 2600 cm.$^{-1}$ suggests that first of all there is association of $^+NH_3$ with the chloride ion.

The spectral analyses of β-AP were made in anhydrous media or in solid state, and it is obvious that in aqueous medium at different pH values the properties of the molecule may be fundamentally changed; therefore in future studies the infrared spectra of β-AP will be taken in aqueous media, and the equilibrium between Orn and β-AP will be studied in solutions at pH 4 - 8.

From the presented experiments it appears that in investigations concerning biological fluids in which ornithine is present, the possibility of the occurrence of β-aminopiperidon should be taken into account as in the pH range of 2 - 8 the equilibrium of the reaction β-AP $\rightleftharpoons$ Orn is shifted strongly in the direction of β-AP. Although the rate of lactamization of Orn is very slow, it cannot be excluded that the reaction may be accelerated by some enzymic system.

The authors wish to thank Prof. Dr. J. Pawełkiewicz from the Department of Biochemistry, College of Agriculture, Poznań, for making available the Locarte-Laurence Recording Densitometer; Dr. W. Meissner from the Department of General Chemistry, College of Economics, Poznań, for spectrophotometric determination of the rate of hydrolysis of β-aminopiperidon and α-piperidon in 4 N-HCl; and Dr. J. Dobak from our Department for the preparation of α-piperidon.

REFERENCES

[1] Ebsworth E. A. V. & Sheppard N. - *Spectrochim. Acta* **13**, 261, 1959.
[2] Golankiewicz K. & Wiewiórowski M. - *Acta Biochim. Polon.* **10**, 443, 1963.
[3] Huisgen R., Brade H., Walz H. & Glogger J. - *Chem. Ber.* **90**, 1437, 1957.
[4] Meissner W. & Wiewiórowski M. - *Bull. Acad. Polon. Sci., Ser. Sci. Chim.* **10**, 591, 1962.
[5] Suszko J., Wiewiórowski M. & Meissner W. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **7**, 87, 1959.
[6] Suszko J., Wiewiórowski M. & Meissner W. - *Roczniki Chemii* **33**, 1015, 1959.

DALSZE INFORMACJE NA TEMAT ZACHOWANIA SIĘ ORNITYNY I β-AMINOPIPERYDONU W ROZTWORACH WODNYCH ORAZ PEWNE DANE O WŁAŚCIWOŚCIACH SPEKTRALNYCH β-AMINOPIPERYDONU I JEGO SOLI

Streszczenie

1. Badano hydrolizę β-aminopiperydonu (β-AP) do ornityny i laktamizację ornityny do β-AP w różnych temperaturach i w różnych pH.

2. Przedyskutowano mechanizm laktamizacji ornityny i porównano z laktamizacją kwasu lupaninowego.

3. Na podstawie widm absorpcyjnych w podczerwieni wysunięto sugestię, że w stanie stałym zarówno β-AP jak i α-piperydon tworzą silne dimery asocjacyjne (N—H ··· O=C). Dimery te są trwałe w CCl_4, ale ulegają rozbiciu w roztworze chloroformowym. Widma w podczerwieni soli β-AP wykazały, że również w solach istnieją dimery asocjacyjne, ale innego typu niż w wolnych zasadach, a mianowicie $^+NH_3$ ··· O=C.

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W. DRABIKOWSKI, HALINA DOMINAS and MARIA DĄBROWSKA

LIPID PATTERNS IN MICROSOMAL FRACTIONS OF RABBIT SKELETAL MUSCLE

Department of Biochemistry, Nencki Institute of Experimental Biology, Warszawa

1. Heavy microsomes (fraction sedimented between 8 000 and 28 000 *g*) contain about 54% of proteins and about 22% of total lipids, calculated for the dry weight. Cholesterol accounts for about 6% and phospholipids for about 50% of total lipids. Phosphatidylcholine accounts for about 75% of phospholipids, whereas other phospholipids are present only in small amounts. 2. The lipid pattern of light microsomes (fraction sedimented between 28 000 and 100 000 *g*) is similar to that of heavy microsomes .3. The final 100 000 *g* supernatant contains about 66% of protein and only about 0.2% of lipids. Phospholipids were found in this fraction only in traces; cholesterol accounts for about 12% of total lipids.

It has been recently established that the fraction of the muscle microsomes consists of vesicles which are fragments of sarcoplasmic reticulum, an intercellular membrane system. In connection to relaxing factor activity the microsomal fraction shows the ability to accumulate Ca, coupled with splitting of ATP [8, 9, 17, 18, 33].

It was observed earlier by Lorand and coworkers [25, 26] and by Ebashi [7] that treatment of microsomes with organic solvents, deoxycholate or phospholipase C, completely abolished their relaxing activity. These observations were consistent with the fact that the preparations of the so-called sarcoplasmic ATPase, obtained several years ago by Kielley & Meyerhof [22], lost their activity after treatment with phospholipase C. These preparations were shown later by Ebashi [7] to possess the relaxing factor activity.

All these observations might suggest that some lipid-protein combination could be responsible for the biological effect of muscle microsomal fraction, and such idea was first postulated by Lorand & Molnar [25]. The recent findings of the important role of lipids in the function of various biomembranes (for instance in the case of mitochondrial membranes [cf. 13]) might also support this idea.

Contrary, however, to a good deal of information concerning the content and composition of lipids in microsomes of various tissues [cf. 39], there have been up to recently only scarce data on the content of total lipid material in muscle microsomes [25, 26]. As recently as in 1964 Hasselbach [16] stated in his excellent

revue that the chemical composition of the lipid material of muscle vesicles still remained unknown. Before starting investigations on the possible involvement of lipids in the function of muscle vesicles it seemed therefore necessary to get more information about the content and composition of lipids in this fraction of muscle, and this was the aim of the present investigation [1].

Since it was already shown that the relaxing factor activity is localized in the heavy microsomal fraction, i.e. in the fraction of muscle homogenate sedimenting between about 8000 *g* and about 28 000 *g* [34] special attention was paid to that fraction. For comparison, lipid content and composition was also determined in light microsomes (fraction sedimenting between 28 000 *g* and 100 000 *g*) and in the final supernatant.

During the progress of this work two publications appeared [32, 43] in which some data about the lipid composition in muscle vesicles have been reported. In one of the papers Martonosi [32] also demonstrated the direct involvement of phospholipids in the accumulation of Ca and in the ATPase activity of muscle microsomes.

MATERIAL AND METHODS

Preparation of cell fractions

The leg and back rabbit muscles were excised and immediately chilled in ice. They were then carefully freed from visible fat, ground with a meatgrinder and subsequently homogenized in a Waring Blendor for 1.5 min. with 4 vol. of ice-cold 0.1 M-KCl, containing either 5 mM-histidine, pH 7.2, or 5 mM-phosphate buffer, pH 7.0 [34]. In some experiments 5 mM-K-oxalate was also present. Cell debris, myofibrils and mitochondria were removed by two subsequent centrifugations for 20 min. each; the first at 2000 *g*, the second at 8000 *g*. After each centrifugation the fat layer which accumulated on the top was carefully removed. The obtained supernatant was filtered through several layers of gauze and was subjected to two subsequent centrifugations for 1 hr. each in the preparative Spinco Ultracentrifuge, model L; the first at 28 000 *g*, the second at 100 000 *g*, yielding two microsomal fractions and the final supernatant. These fractions will be referred to later as heavy microsomes (fraction sedimenting between 8 000 and 28 000 *g*), light microsomes (fraction sedimenting between 28 000 and 100 000 *g*) and the final 100 000 *g* supernatant. The whole procedure was performed at 2° - 4°.

Extraction of lipids

The microsomal pellets were extracted several times with a mixture of hot chloroform - methanol (2:1, v/v) [11] in a mortar by grinding with silica sand. All extracts were collected by decantation and filtered through cotton wool. The sol-

[1] A preliminary report of some of the results was already presented in 1963 [6].

vents were evaporated either under reduced pressure at room temperature or under nitrogen in a hot water bath. The crude lipids obtained were reextracted with a mixture of petroleum ether - chloroform (2:1, v/v) in the presence of a few drops of water. The lipid extract was dehydrated with anhydrous sodium sulphate, the solvents were evaporated and the residue was weighed after drying in a desiccator under nitrogen.

After preliminary comparison of several solvents the mixture of chloroform - - methanol (2:1, v/v) was chosen as the best solvent for extraction of the microsomal lipids. Table 1 shows that the amounts of total lipids and phospholipids extracted with this solvent were practically identical with those obtained during prolonged (2 - 3 days) extraction of microsomes with diethyl ether in a Soxhlet apparatus, followed by reextraction of the lipid material with petroleum ether - - chloroform (2:1, v/v).

Table 1

The yields of total lipids and phospholipids obtained from muscle microsomes by different kinds of extraction

The extraction with chloroform - methanol mixture was compared with the 2 - 3 days extraction with diethyl ether in Soxhlet apparatus

Heavy microsomes (mg. of dry weight in the sample)	Lipids extracted by		Phospholipids in lipids extracted by	
	$CHCl_3$ - CH_3OH	diethyl ether	$CHCl_3$ - CH_3OH	diethyl ether
	(mg.)	(mg.)	(mg.)	(mg.)
149	30.9	30.5	16.4	16.1
110	22.2	24.0	10.0	11.1
109	22.6	24.8	12.1	12.8
132	26.2	26.1	14.3	13.2

In some preparations of microsomes the extraction of lipids was performed with acetone containing about 10% of water, according to Green & Fleischer [13]. In this case the suspension of microsomes in 0.1 M-KCl with histidine or phosphate buffer was treated at 0° with 9 volumes of chilled acetone (–15°). After centrifugation at 0° acetone was evaporated from the supernatant and the crude lipids were reextracted from the residue with a mixture of chloroform - methanol (2:1, v/v). The obtained extract was treated as described above for purification of crude lipids.

For the determination of lipids in the final 100 000 *g* supernatant, trichloroacetic acid was added to the samples to 7% final concentration and the resulting precipitates were removed by centrifugation. After washing with water the lipids were extracted with a mixture of chloroform - methanol (2:1, v/v), and purified as described above.

Chromatographic analysis of lipids

Thin-layer chromatography was performed on glass plates (8.5×8.5 cm. or 20×20 cm.) covered with silica gel G (Merck, Darmstadt, Germany). Silicic acid was activated before use for 30 min. at 100°. Lipids were separated by thin-layer chromatography in a system: petroleum ether - ethyl ether - glacial acetic acid (6.0:4.0:0.2, by vol.) [29]. The separation of phospholipids was achieved by chromatography in a system: chloroform - methanol - water (6.5:2.5:0.4, by vol.) [42]. The spots were revealed by spraying with 10% phosphomolybdic acid in ethanol and subsequent heating for a few minutes at 80°.

Paper chromatography of phospholipids was performed on commercial silica gel paper (Schleicher - Schüll no. 287), in a solvent system: chloroform - methanol - - water (12:1.6:0.15, by vol.) (C. Michalec, private communication). The spots were revealed with the solution of acid fuchsine [19] or Rodamine B [30].

Column chromatography was performed according to Hanahan *et al.* [14] on silicic acid (Mallincrodt, USA) activated before use for 13 hr. at 110°. Usually about 100 mg. of lipids, dissolved in chloroform, were applied to a column 1 cm. in diameter, containing 8 g. of silicic acid. The flow rate was about 0.8 ml. per minute, achieved with the use of slight nitrogen pressure. Neutral lipids were eluted with chloroform and, afterwards, phospholipids with chloroform containing methanol in increasing concentrations. The collected fractions were rechromatographed with the use of the above chloroform - methanol mixtures. From the obtained fractions the solvents were evaporated, and the residue was weighed after drying under nitrogen in a desiccator. The homogeneity of each fraction was checked either directly with the use of thin-layer chromatography or after the mild alkaline hydrolysis. The mild alkaline hydrolysis of phospholipids was carried out according to Dawson [5] and the products of hydrolysis were separated by paper chromatography on Whatman no. 1 paper in the system: phenol, saturated with water - - glacial acetic acid - ethanol (100:10:12, by vol.) [5] and identified according to Hanes & Isherwood [15].

The following substances were used as standards: cardiolipins and phosphatidylcholine, both prepared according to Pangborn [35] and Macfarlane [27], sphingomyelin and phosphatidylethanolamine (Light, England), monoolein and oleic acid (Serva, Germany), diolein, triolein and cholesterol (La Roche, France).

Lipid phosphorus was determined according to Fiske & Subbarow [10], after combustion of the samples in concentrated HNO_3 and H_2SO_4 in the presence of few drops of 30% hydrogen peroxide. Phospholipid content was calculated by multiplying the amount of phosphorus by 25.

The content of total sterols was determined in extracted lipids according to Searcy *et al.* [37].

Determination of dry weight and protein content

The microsomal pellets were gently homogenized with a teflon homogenizer in 0.1 M-KCl with phosphate or histidine buffer and diluted to a known volume.

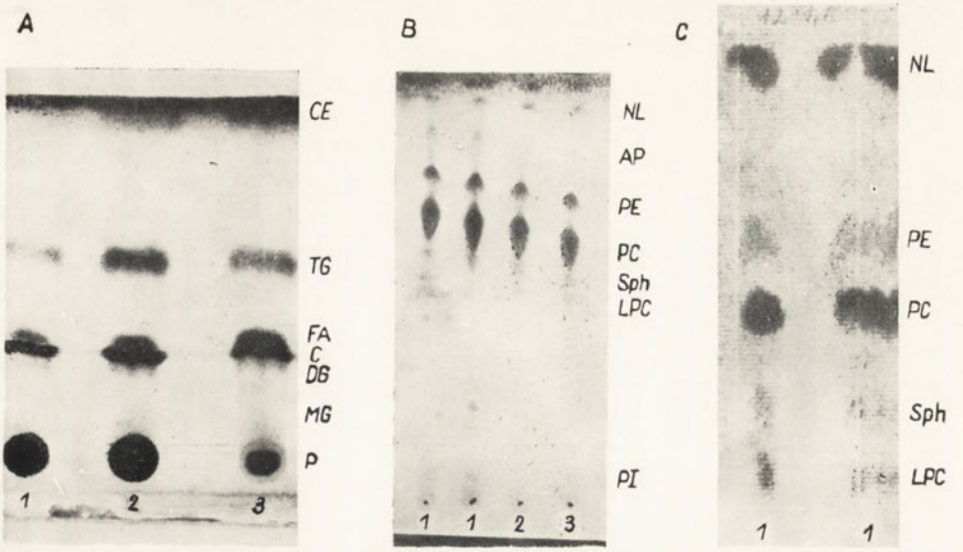

Fig. 1. Thin-layer chromatography and paper chromatography of total lipids and phospholipids in rabbit muscle homogenate fractions.

A. Thin-layer chromatogram of total lipids. Glass plate (8.5 × 8.5 cm.) covered with silica gel G. Solvent system: petroleum ether - diethyl ether - glacial acetic acid (6.0:4.0:0.2, by vol.) [29]. Each sample contained 200 μg. of lipid material. Developing time 8 min. Detection with phosphomolybdic acid.

B. Thin-layer chromatogram of phospholipids. Glass plate (20 × 20 cm.) covered with silica gel G. Solvent system: chloroform - methanol - H_2O (6.5:2.5:0.4, by vol.) [42]. Each sample contained 150 μg. of lipid material. Developing time 60 min. Detection with phosphomolybdic acid.

C. Paper chromatogram of phospholipids. Silica gel paper (Scheicher-Schüll, no. 287). Solvent system: chloroform - methanol - H_2O (12:1.6:0.15, by vol.) (Č. Michalec, private communication). Each sample contained 20 μg. of lipid material. Developing time 15 min. Detection with acid fuchsine [19].

Sample *1*, heavy microsomal fraction; sample *2*, light microsomal fraction; sample *3*, the final 100 000 *g* supernatant. *CE*, cholesterol esters; *TG*, triglycerides; *FA*, fatty acids; *C*, cholesterol; *DG*, diglycerides; *MG*, monoglycerides; *P*, phospholipids; *NL*, neutral lipids; *AP*, acidic phospholipids; *PE*, phosphatidylethanolamine; *PI*, phosphatidyl inositols; *PC*, phosphatidylcholine; *Sph*, sphingomyelin; *LPC*, lysophosphatidylcholine.

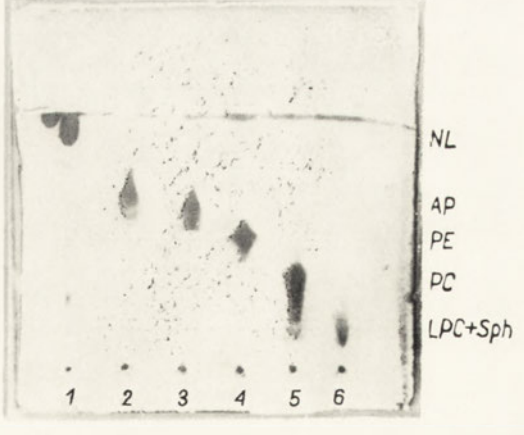

Fig. 2. Thin-layer chromatography of phospholipid fractions eluted from silicic acid column. Glass plate (8.5 × 8.5 cm.) covered with silica gel G. Each sample contained 50 μg. of lipid material. Solvent system: chloroform - methanol - H_2O (6.5:2.5:0.4, by vol.) [42]. Developing time 8 min. Detection with phosphomolybdic acid. Sample *1*, fraction eluted from the column with chloroform; samples *2* - *6*, fractions eluted with chloroform - methanol in the following ratios: *2*, 96 : 4; *3*, 90 : 10; *4*, 80:20; *5*, 40:60; *6*, 20:80 [14]. For abbreviations see legend to Fig. 1.

Protein was determined with the biuret method in a modification of Cleland & Slater [3], after precipitation with trichloroacetic acid. For the determination of dry weight the samples were evaporated at room temperature in a desiccator and dried at 105° to a constant weight. Protein determinations in the final supernatant were performed on the samples with the biuret reagent [12] and determination of the dry weight was carried out as in the microsomal fractions.

RESULTS

Table 2 presents the contents of protein, total lipids and phospholipids, expressed as percentages of dry weight in the heavy and in the light microsomal fractions as well as in the final supernatant. The percentage content of phospholipids in lipids is also presented. Heavy microsomes contain on the average about 54% of proteins and 22% of lipids. Phospholipids contribute to about 11% of dry weight, that is to about 50% of total lipids. Light microsomes contain on the average 50% of proteins and 20% of total lipids. Phospholipids are present in amounts corresponding to 9% of dry weight, that is to about 43% of total lipids. The average contents of both total lipids and phospholipids seem to be slightly lower in the light than in the heavy microsomes. As it can be seen in Table 2 the differences among the values obtained in the particular experiments for the light microsomes were much greater than those for the heavy microsomes. Since, however, the total material of light microsomal fraction corresponded only to about 15 - 25% of that of the heavy microsomal fraction the amounts of the material available for analysis were much smaller and therefore the error of estimation could be greater. The final supernatant contains about 66% of proteins and only small amounts of lipids; phospholipids are present only in traces. As it can be seen from the data in Table 2 the kind of buffer used for homogenization of the muscle and the presence or absence of oxalate have practically no influence on the contents of protein and lipids in all three investigated fractions.

The thin-layer and paper chromatograms, presented in Fig. 1, show the pattern of neutral lipids and phospholipids in the investigated fractions of muscle homogenate. The general pattern of lipids is very similar in the two microsomal fractions and in the final supernatant. The main components of neutral lipids are triglycerides, cholesterol and cholesterol esters. Free fatty acids are present in smaller amounts, whereas only traces of di- and monoglycerides are visible.

From the intensity of spots it can be judged that phosphatidylcholine is the main constituent of phospholipids in the investigated subcellular fractions, whereas phosphatidylethanolamine, acidic phosphatides (phosphatidic acids and cardiolipins), lysophosphatidylcholine, sphingomyelin and phosphatidyl inositides are present in much smaller amounts.

For quantitative determinations the phospholipids were separated on the silicic acid column with chloroform containing increasing concentrations of methanol [14]. As it can be judged from thin-layer chromatography (Fig. 2) the obtained fractions were satisfactorily homogeneous. The amounts of phospholipids in all fractions,

found by weight determination, were similar to those calculated on the basis of estimations of lipid phosphorus. Table 3 shows the percentage distribution of the particular fractions of phospholipids. The available amounts of material allowed to carry out five independent experiments for heavy microsomes but only one for the light microsomes, in the latter case done on the whole material collected. For the same reason it was not possible to separate the phospholipid fractions of the final supernatant.

As it can be seen from Table 3 there is practically no difference between the heavy and light microsomes in respect to quantitative composition of phospholipids. The main fraction, which forms about 75% of all phospholipids, was eluted

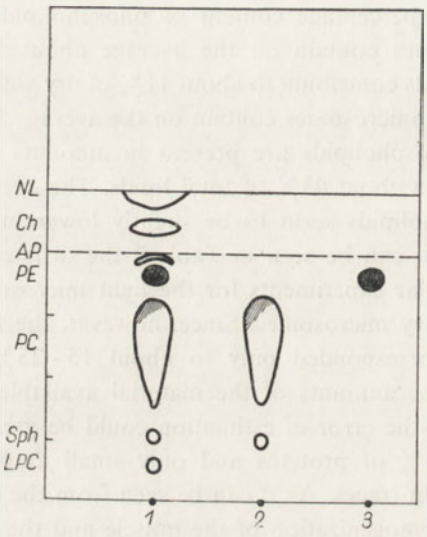

Fig. 3

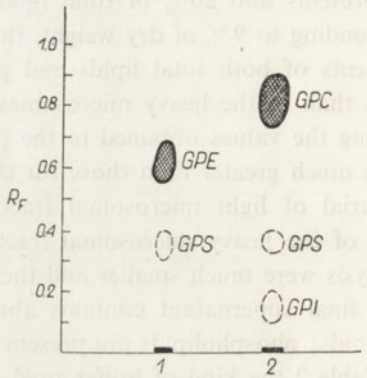

Fig. 4

Fig. 3. Thin-layer chromatography of microsomal phospholipids (localization of ninhydrin-positive spots). Glass plate (8.5 × 8.5 cm.) covered with silica gel G. Each sample contained 200 μg. of lipid material. Solvent system: chloroform - methanol - H_2O (6.5:2.5:0.4, by vol.) [42]. Developing time 8 min. Separated lipids were first localized by iodine vapours (these spots are visualized on the Figure by tracing). After evaporation of the iodine, the chromatoplate was sprayed with ninhydrin reagent. (The localization is shown as black spots on the Figure). Sample *1*, lipids of the heavy microsomes; sample *2*, phospholipid fraction eluted from the column with chloroform - methanol mixture (40:60, v/v); sample *3*, phospholipid fraction, eluted with chloroform - - methanol (80:20, v/v). For abbreviations see legend to Fig. 1.

Fig. 4. Paper chromatogram of the products of mild alkaline hydrolysis of phospholipids. Samples containing 6 mg. of phospholipid fractions were hydrolysed according to Dawson [5], and portions of the obtained products were chromatographed on Whatman no. 1 paper. Solvent system: phenol saturated with water - glacial acetic acid - ethanol (100:10:12, by vol.) [5]. Developing time 16 hr. Spots were localized on the chromatogram according to Hanes & Isherwood [15]. Sample *1*, products of the hydrolysis of phospholipids, eluted from silicic acid column with chloroform - methanol mixture (80:20, v/v); sample *2*, products of the hydrolysis of phospholipids, eluted from silicic acid column with chloroform - methanol (40:60, v/v). *GPE*, glycerylphosphorylethanolamine; *GPS*, glycerylphosphorylserine; *GPC*, glycerylphosphorylcholine; *GPI*, glycerylphosphorylinositol.

with the mixture of 40% of chloroform and 60% of methanol. It can be assumed that this fraction consists almost entirely of phosphatidylcholine, since as judged from the localization of ninhydrin-positive material (Fig. 3), as well as from the separation of the products of mild alkaline hydrolysis (Fig. 4), phosphatidylserine and phosphatidyl inositides are present in this fraction only in traces. Among the remaining phospholipids present in microsomes, phosphatidylethanolamine accounts for about 13.0%, lysophosphatidylcholine plus sphingomyelin for about 7% and acidic phosphatides for about 5% (Table 3).

Table 2

Contents of protein and lipids in the fractions of muscle homogenate

Rabbit muscles were homogenized in one of the following media: (A), 0.1 M-KCl, 5 mM-phosphate buffer, pH 7.0; (B), 0.1 M-KCl, 5 mM-phosphate buffer, pH 7.0, 5 mM-K-oxalate; (C), 0.1 M-KCl, 5 mM-histidine, pH 7.2; (D), 0.1 M-KCl, 5 mM-histidine, pH 7.2, 5 mM-K-oxalate. The homogenates were subsequently fractionated by centrifugation. For details of the procedure see Methods. Each value in the Table is the mean from 2 - 3 parallel determinations. The differences between separate determinations did not exceed 5%.

Fraction	Homogenization medium	Proteins	Total lipids	Phospholipids	Phospholipids (% in lipids)
		% of dry weight			
Heavy microsomes (fraction sedimented between 8000 and 28 000 *g*)	A	50.4	20.7	11.0	53.1
	A	54.5	20.2	9.1	45.0
	A	—	27.5	13.3	48.5
	B	69.7	20.6	11.2	54.5
	B	54.3	19.9	10.7	54.2
	C	—	24.7	12.0	48.5
	D	52.0	17.8	8.0	43.6
	D	45.5	24.2	12.1	52.0
	Average	54.3	22.0	10.9	49.5
Light microsomes (fraction sedimented between 28 000 and 100 000 *g*)	A	—	25.5	11.2	45.5
	B	—	—	—	46.3
	B	—	—	—	37.0
	C	—	29.6	14.5	48.5
	C	56.0	18.7	8.6	46.3
	D	44.5	11.6	4.5	38.3
	D	52.0	13.0	4.9	36.3
	Average	50.2	19.7	8.8	43.0
Final 100 000 *g* supernatant	A	—	0.15		
	C	—	0.23		
	C	66.0	0.16		
	D	68.0	0.22		
	D	65.0	0.18		
	Average	66.3	0.18		1.5*

* The determination of phosphorus content was performed on the material collected from all 5 experiments.

In the investigated subcellular fractions the content of sterols (Table 4) was the lowest in heavy microsomes (5 - 7%), somewhat higher in light microsomes, and the highest in the final supernatant (11 - 14%).

Table 3

The percentage distribution of phospholipid fractions in phospholipids from muscle microsomes separated by column chromatography

The sum of the phospholipid phosphorus found in the separate fractions was taken as 100.

Eluent (methanol to chloroform ratio, v/v)	Fraction of phospholipids	Heavy microsomes						Light microsomes
		Experiment					Average	
		1	2	3	4	5		
10:90	Acidic phosphatides	5	8	3	5	3	5	3
20:80	Phosphatidylethanolamine	12	11	14	14	15	13	16
60:40	Phosphatidylcholine	78	67	79	75	74	75	75
80:20	Lysophosphatidylcholine +sphingomyelin	5	14	4	6	8	7	6

Table 4

Content of total sterols in the fractions of muscle homogenate

Each value in the Table is the mean from 2 - 3 parallel determinations. The differences between separate determinations did not exceed 5%.

Fraction	Sterols (% of total lipids)
Heavy microsomes (8 000 - 28 000 *g*)	5.0
	7.3
	6.5
	6.1
Light microsomes (28 000 - 100 000 *g*)	9.0
	8.0
	8.5
Final 100 000 *g* supernatant	13.7
	14.1
	10.9

When the amounts of neutral lipids and phospholipids extracted from microsomes with the chloroform - methanol mixture were determined not as usually after exhaustive extraction but after a single treatment with this solvent it was found that about 99% of phospholipids had been extracted. Simultaneously

however, only about 48% of other lipid material was removed. Similarly, a single treatment of microsome suspension with 90% acetone removed practically all phospholipids, but only about 50% of other lipids. When the residue after acetone treatment was exhaustively extracted with chloroform - methanol the lipids in the obtained extract did not contain any phospholipids as judged by phosphorus determination and thin-layer chromatography. This observation seems to indicate that microsomal phospholipids are easier extractable than other lipids.

DISCUSSION

According to the presented results the heavy microsomal fraction contains about 54% of protein and about 22% of total lipids. Phospholipids correspond to about 50% of lipids. Similar values were found in the light microsomal fraction. When these results are compared with those of other authors (Table 5) it can be seen that the protein contents are similar. The comparison of the data for lipids is however rather difficult, since the corresponding values present in the literature are fragmentary. The lipid content found in the present paper is lower than that found by Lorand and coworkers [25, 26]. The content of phospholipids, although similar to the value presented by Martonosi [32], is lower than that recently reported by Inesi *et al.* [20]. These differences may be connected with the fact that in the present work special care has been taken to purify the extracted crude lipid material. Crude extracts could contain, in addition to lipids, some other material which might account for higher values found by other authors. On the other hand, our values for total lipids and phospholipids are much higher than those of Seraydarian & Mommaerts [38] [2].

The kind of buffer used for homogenization in the presence of potassium chloride, as well as the presence of oxalate, do not seem to have significant influence on the content of lipids found in microsomes. On the other hand, Seraydarian & Mommaerts [38] found that microsomes prepared in sucrose have higher lipid contents than those prepared in potassium chloride. In view of this observation one can assume that the higher phospholipid content found by Inesi *et al.* [20] might be also due to the use of bicarbonate for homogenization of muscle.

As far as the content of total lipids and phospholipids is concerned, muscle microsomes do not differ considerably from microsomes of other tissues, which also usually contain about 20% of total lipids, more than half of which consist of phospholipids [2, 4, 31].

The composition of phospholipids in the microsomes of skeletal muscle presented in this paper (Table 6) is in good agreement with that reported by Martonosi [32] and similar to that found by Waku & Nakazawa [43]. In all these papers phosphatidylcholine has been found to make up the main fraction, corresponding

[2] Dr. Mommaerts recently informed us that the low yields of lipids obtained by them were due to the treatment of microsomes with trichloroacetic acid prior to extraction of lipids. When this treatment was omitted the lipid content was also of the order of 30% (W. F. H. M. Mommaerts, personal communication).

Table 5

Lipid, phospholipid and protein content in muscle microsomes

Muscle	Homogenization medium	Analysed fraction of muscle homogenate (g. × 10^{-3})	Protein	Total lipids	Phospho-lipids	Total lipids	Phospho-lipids	Phospho-lipids (% in lipids)	Author
			% of dry weight			% related to protein			
Heavy microsomes									
Skeletal	0.15 M-KCl	12 - 40	50 - 60	35	—	—	—	—	Lorand *et al.* [25, 26]
Skeletal	0.1 M-KCl, 5 mM-buffer, pH 7.3	8 - 28	—	—	—	—	22 - 28	—	Martonosi [32]
Skeletal*			—	—	—	—	—	78	Waku & Nakazawa [43]
Skeletal	20 mM-$NaHCO_3$	10 - 22	59	—	18	—	—	—	Inesi *et al.* [20]
Skeletal	0.08 M-KCl, 20 mM-buffer, pH 7.4	15 - 41	—	—	—	10.8	7.6	81	Seraydarian & Mommaerts [38]
Skeletal	sucrose	15 - 41	—	—	—	14.9	10.0	78	Seraydarian & Mommaerts [38]
Skeletal	0.1 M-KCl, 5 mM-buffer, pH 7.0 - 7.2	8 - 28	54.3	22.0	10.9	40.5	20.0	49.5	this paper
Cardiac	sucrose	19.6 - 54	—	24.6	16.7	—	—	68	Marinetti *et al.* [31]
Cardiac	20 mM-$NaHCO_3$	10 - 22	57	—	21	—	—	—	Inesi *et al.* [20]
Light microsomes									
Skeletal	0.08 M-KCl, 20 mM-buffer, pH 7.4	41 - 105	—	—	—	5.7	1.8	37	Seraydarian & Mommaerts [38]
Skeletal	sucrose	41 - 105	—	—	—	7.2	2.8	44	Seraydarian & Mommaerts [38]
Skeletal	0.1 M-KCl, 5 mM-buffer, pH 7.0 - 7.2	28 - 100	50.2	19.7	8.8	39.0	17.4	43	this paper

* The details of the procedure were not given by the authors.

Table 6

The percentage distribution of various phospholipids in microsomes from different organs

Organ	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Sphingomyelin	Acidic phosphatides	Phosphatidylinositides	Other phosphatides	Author
Rat skeletal muscle	69	15.5	4.5	7	—	—	4	Martonosi [32]
Rabbit skeletal muscle	74	0	7.7	7.7	—	0	10.6	Waku & Nakazawa [43]
Rabbit skeletal muscle	75	13	traces	7*	5	traces	0	this paper
Rat liver	62	26		—	1	10	1	Macfarlane *et al.* [28]
Rat liver	37	16	11	8	—	—	28	Biezenski *et al.* [2]
Rat liver	68	18	—	—	2 - 5	12	—	Strickland *et al.* [41]
Rat kidney	44	14	11.5	19	—	—	11.5	Biezenski [2]
Rat uterus	47.5	12	20	8.6	—	—	11.9	Biezenski [2]
Pig heart	41.8	21.2	3.6	16.4	2	3.9	11.1	Marinetti [31]

* Containing some lysophosphatidylcholine.

to about three-fourths of total phospholipids. The fraction of phosphatidylethanolamine was found in this work to account for about 13%. Other phospholipids were present in rather small amounts.

The microsomes of other tissues [2, 28, 31, 40], especially those from cardiac and uterine muscles [2, 31], seem to contain less phosphatidylcholine than skeletal muscle microsomes. It is however difficult to decide at present whether this fact may be of some biological significance.

Other phospholipids seem to be present in microsomes of other tissues in slightly higher amounts, but the data obtained by different authors for the same material are divergent and hence difficult to compare.

The final 100 000 *g* supernatant was found in this work to contain only minute amounts of lipids (about 0.2% of dry weight). The corresponding values obtained by Seraydarian & Mommaerts for rabbit skeletal muscle [38] as well as by Marinetti for pig heart [31] are much higher (about 6 - 8%). Marinetti *et al.* [31], however, used lower speed for separation of microsomes (average 54 000 *g*) and hence their final supernatant could contain some small microsomes. The relatively high phospholipid content (46% of total lipids) found by these authors in the final supernatant might support this assumption.

On the basis of the results presented in this paper it seems that there is no essential difference between heavy and light microsomes in respect to total lipid and phospholipid content as well as to the composition of phospholipids. The speed used for sedimentation of heavy microsomes was the same as that previously shown by Nagai *et al.* [34] to sediment vesicles containing the whole relaxing factor activity. Hence it seems reasonable to assume that this activity cannot be attributed simply to the amount or particular composition of phospholipids in heavy microsomes.

On the other hand, the possibility cannot be excluded that the presence of large amounts of choline phospholipids in the fragments of sarcoplasmic reticulum may be of significance in respect to the biological function of the latter. Choline phosphatides, due to their structure, can form internal salts and thereby they can be fully ionized over a wide range of pH values. Both the high content of choline phosphatides and the presence of non-ionic lipids, as cholesterol, is supposed to be responsible for the low surface charge density of biomembranes [1].

Recently some authors have suggested that phospholipids play an essential role in the active transport of cations through various biological membranes [21]. The finding of Kimizuka & Koketsu [23] that phosphatidylcholine films can bind calcium is in favour of this supposition. The interaction of these two substances was also recently observed by Inesi *et al.* [20]. These authors concluded [20], however, that the strong binding of calcium by muscle microsomes could not be simply explained on this way.

The cholesterol content in skeletal muscle microsomes (5 - 7% of total lipids) is similar to that found by other authors in liver [40] and heart [31] microsomes. The final supernatants of liver [24, 40] or adrenal cortex [30] homogenate contain, however, less cholesterol than the corresponding microsomal fractions; on the other

hand, in the case of skeletal muscle, as found in the present work, and in heart muscle as reported by Marinetti *et al.* [31], the cholesterol content is higher in the supernatant than in the microsomal fractions.

The ratio of cholesterol to phospholipids in various membranes has been recently extensively investigated. Several authors observed that cholesterol allows a closer packing of the phosphorylcholine molecules in its monolayer films and suggested that a similar phenomenon takes place in various biomembranes [44]. The extent of this effect depends on the proportion of both compounds. One of the theoretically possible and favourable arrangements takes place when three phosphorylcholine molecules form a complex with one cholesterol molecule. This value is close to the molar ratio of phospholipids to cholesterol in muscle microsomes (3.2:1), calculated on the basis of the results of this work. Also this fact may be of some biological importance.

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REFERENCES

[1] Bangham A. D., in *Advances in Lipid Research* (R. Paoletti & D. Kritchewsky, eds.) vol. 1, p. 65, Academic Press, New York 1964.
[2] Biezenski J. J., Spaet T. H. & Gordon A. L. - *Biochim. Biophys. Acta* **70**, 75, 1963.
[3] Cleland K. W. & Slater E. C. - *Biochem. J.* **53**, 547, 1963.
[4] Cole B. T. - *Proc. Soc. Exp. Biol.* **114**, 731, 1963.
[5] Dawson R. M. C. - *Biochem. J.* **75**, 45, 1960.
[6] Dominas H. & Drabikowski W., *Summaries of Reports and Communications of Vth International Symposium of Histologists, Sofia, September 1963*, p. 20.
[7] Ebashi S. - *Arch. Biochem. Biophys.* **76**, 410, 1958.
[8] Ebashi S. - *J. Biochem., Tokyo* **50**, 236, 1961.
[9] Ebashi S. & Lipmann F. - *J. Cell. Biol.* **14**, 389, 1962.
[10] Fiske C. H. & Subbarow Y. - *J. Biol. Chem.* **66**, 375, 1925.
[11] Folch J., Less M. & Sloane Stanley G. H. - *J. Biol. Chem.* **226**, 497, 1957.
[12] Gornall A. G., Bardawill C. J. & David M. M. - *J. Biol. Chem.* **177**, 751, 1949.
[13] Green D. E. & Fleischer S. - *Biochim. Biophys. Acta* **70**, 554, 1963.
[14] Hanahan D. J., Dittmer J. G. & Warashina E. - *J. Biol. Chem.* **228**, 685, 1957.
[15] Hanes C. S. & Isherwood F. A. - *Nature* **164**, 1107, 1949.
[16] Hasselbach W. in *Progress in Biophysics and Molecular Biology* (Butler J. A. V. & Huxley H. E., eds.) p. 167, vol. 14, Pergamon Press, 1964.
[17] Hasselbach W. & Makinose M. - *Biochem. Z.* **333**, 518, 1961.
[18] Hasselbach W. & Makinose M. - *Biochem. Biophys. Res. Commun.* **7**, 132, 1962.
[19] Hoogwinkel G. J. M. & Hoogeven J. T. - *Proc. Kon. Ned. Acad. Wetensch., Series B*, **62**, 221, 1959.
[20] Inesi G., Ebashi S. & Watanabe S. - *Amer. J. Physiol.* **207**, 1339, 1964.
[21] Kavanau J. L., *Structure and Function in Biological Membranes*, vol. 1, p. 138, Holden - Day, Inc., San Francisco, London, Amsterdam, 1965.
[22] Kielley W. W. & Meyerhof O. - *J. Biol. Chem.* **183**, 391, 1950.

[23] Kimizuka H. & Koketsu K. - *Nature* **200**, 995, 1962.
[24] Kritchewsky D., Langan J. & Whitehouse M. W. - *Experientia* **16**, 452, 1960.
[25] Lorand L. & Molnar J., in *Muscle as a Tissue* (K. Rodahl & S. M. Horvath, eds.) p. 97. McGraw-Hill Book Co., New York 1962.
[26] Lorand L., Molnar J. & Moos C., in *Chemistry of Muscular Contraction*, p. 85, Igaku Shoin Ltd. Tokyo, 1957.
[27] Macfarlane M. G. - *Biochem. J.* **78**, 44, 1961.
[28] Macfarlane M. G., Gray G. M. & Wheeldon L. W. - *Biochem. J.* **77**, 626, 1960.
[29] Mangold H. K. & Malins D. C. - *J. Amer. Oil Chemists' Soc.* **37**, 383, 1960.
[30] Marinetti G. V., Erbland J. & Kochen J. - *Fed. Proc.* **16**, 837, 1957.
[31] Marinetti G. V., Erbland J. & Stotz E. - *J. Biol. Chem.* **233**, 562, 1958.
[32] Martonosi A. - *Fed. Proc.* **23**, 913, 1964.
[33] Martonosi A. & Feretos R. - *J. Biol. Chem.* **239**, 659, 1964.
[34] Nagai T., Makinose N. & Hasselbach W. - *Biochim. Biophys. Acta* **43**, 223, 1960.
[35] Pangborn M. C. - *J. Biol. Chem.* **161**, 71, 1945.
[36] Schultz R. L. & Meyer R. K. - *J. Biophys. Biochem. Cytol.* **4**, 23, 1958.
[37] Searcy R. G., Berquist Z.M. & Jung R. C. - *J. Lipid Res.* **1**, 349, 1960.
[38] Seraydarian K. & Mommaerts W. F. H. M. - *J. Cell. Biol.*, in press (private communication).
[39] Sikievitz P. - *Ann. Rev. Physiol.* **25**, 15, 1963.
[40] Spiro M. J. & Mckibbin J. M. - *J. Biol. Chem.* **219**, 643, 1956.
[41] Strickland E. H. & Benson A. A. - *Arch. Biochem. Biophys.* **88**, 344, 1960.
[42] Wagner H., Hörhammer L. & Wolf P. - *Biochem. Z.* **334**, 175, 1961.
[43] Waku K. & Nakazawa Y. - *J. Biochem., Tokyo* **56**, 95, 1964.
[44] Willmer E. N. - *Biol. Rev.* **36**, 368, 1961.

LIPIDY WE FRAKCJACH MIKROSOMALNYCH MIĘŚNI SZKIELETOWYCH KRÓLIKA

Streszczenie

1. Ciężkie mikrosomy mięśni szkieletowych królika (frakcja sedymentująca między 8 000 a 28 000 *g*) zawierają około 54% białek i 22% lipidów (odniesionych do suchej masy). Zawartość cholesterolu wynosi około 6% a fosfolipidów około 50% ogólnej ilości lipidów. Fosfatydylocholina stanowi 75% ogólnej ilości fosfolipidów, podczas gdy inne fosfolipidy występują w niewielkich ilościach.

2. Skład i zawartość lipidów w lekkich mikrosomach (frakcji sedymentującej między 28 000 a 100 000 *g*) jest podobny do ciężkich mikrosomów.

3. Końcowy 100 000 *g* supernatant zawiera około 66% białek i zaledwie 0,2% lipidów. Fosfolipidy występują w tej frakcji w ilościach śladowych, zawartość zaś cholesterolu wynosi około 12% ogólnej ilości lipidów.

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MARIA DYDYŃSKA

CHANGES IN PHOSPHORUS COMPOUNDS DURING DEHYDRATION AND REHYDRATION OF FROG SARTORIUS DRIED OVER SILICA GEL

Department of Biochemistry, Nencki Institute of Experimental Biology, Warszawa

1. It was found that dehydration of frog muscles over silica gel at 2° to a degree which does not exceed 75 - 80% of the water content did not damage the fibres and no special changes in properties of the muscle could be observed on rehydration. 2. If the dehydrated muscles lost more than 80% of fibre water, under rehydration a spontaneous contraction was observed accompanied by a complete splitting of phosphocreatine and partial dephosphorylation and deamination of adenine nucleotides. 3. It is suggested that all these changes are the results of a damage to muscle cell organization which may be due to the loss of structurally bound water.

Many investigations have been carried out on the influence of dehydration on the properties of different tissues, especially of the muscle. Several authors [7, 28, 16, 24] found that normal functions of the muscle were restored after rehydration so long as a certain degree of water loss had not been exceeded. In 1909 Hürthle [20] observed spontaneous contraction of *Hydrophilus* muscle fibres during rehydration after almost complete drying. More recently a similar phenomenon was described by Mueller & Szent-Györgyi [25], Hopkins [18] and Hunt & Matheson [19].

Biochemical analyses of the dried muscles have not been performed in these investigations; it appeared therefore interesting to study the changes in the content of labile phosphorus compounds, as they are known to be most important for muscular activity. In the present work these changes have been investigated on muscles dried over silica gel and rehydrated in Ringer's solution. Silica gel, which is a good absorbing substance and is also chemically relatively inert, seemed to be especially suitable for these experiments.

METHODS

The frogs, *Rana esculenta*, previously kept at 2°, were killed by decapitation, pithed, and the sartorius muscles were taken out. The muscles were weighed immediately and their length and width were measured in order to calculate the surface of the muscles.

The muscles were dried in 100 ml. glass vessels over about 2 g. of powdered silica gel (for chromatography, 100 - 200 mesh, imported by B. O. O. Gliwice) at 2°. The water loss of the muscles was determined by weighing. Rehydration of the muscles was carried out by soaking in Ringer's solution for 20 - 30 min. at 20°.

Extracellular space (sucrose space) in the fresh muscles as well as in the dehydrated ones was determined after Tasker *et al.* [30]. The muscles were immersed for 2 hr. at 2° in Ringer's solution containing 0.75% sucrose and then for 2 hr. in Ringer's solution without sucrose. The sucrose which had penetrated the extracellular space diffused out of the muscle into the medium and was determined by the anthrone method in the modification of Norman *et al.* [27]. From the amount of sucrose and the weight of the muscle (drained by pulling it slowly up the side of a glass beaker [1]) the extracellular space and the water content inside the muscle fibre were calculated according to Dydyńska & Wilkie [9].

For chemical analyses the tissue was ground in a mortar at 0° with 1 N-$HClO_4$ (2 ml. per 100 mg. of muscle tissue) and the acid-soluble compounds were separated by filtration. The filtrate was neutralized with NaOH and used for phosphate determination of: P_i, true inorganic, after precipitation with magnesium mixture; P_1 and P_{15}, corresponding to the amounts of orthophosphate which could be estimated after hydrolysis at 100° in 1 N-H_2SO_4 for 1 min. and 15 min., respectively. The phosphate groups of ATP and ADP (ΔP_{15}) could be calculated as the difference between P_{15} and P_1. Total phosphorus was determined after digestion of the whole muscles with conc. H_2SO_4 and HNO_3. All orthophosphate determinations were performed according to Fiske & Subbarow [14].

Table 1

Water partition and contents of phosphorus compounds and nucleotides in the symmetrical frog sartorii (R. esculenta)

Values per 100 mg. fresh tissue ± S. E. are given.

	No. of expts.	Right muscle	Left muscle
Total water content (µl.)	9	81.5 ± 0.63	82.1 ± 0.44
Extracellular water (µl.)	9	46.8 ± 1.57	47.4 ± 1.51
Fibre water (µl.)	9	34.8 ± 1.50	34.6 ± 1.82
P_i (µmoles)	11	1.18 ± 0.14	1.12 ± 0.11
Phosphocreatine (µmoles)	18	0.94 ± 0.10	1.01 ± 0.12
ΔP_{15} (µmoles)	18	0.82 ± 0.04	0.80 ± 0.05
P_{tot} (µmoles)	7	4.46 ± 0.22	4.21 ± 0.26
Adenine nucleotides (µmoles)	17	0.47 ± 0.02	0.46 ± 0.02
Inosine nucleotides (µmoles)	17	0.024 ± 0.006	0.024 ± 0.005

The nucleotide content was determined spectrophotometrically (Unicam 500) at two wavelengths 259 and 249 mµ and the concentrations of adenine and inosine nucleotides were calculated [2, 6]. Phosphocreatine was determined after Ennor

& Rosenberg [10]. The same analyses of phosphorus compounds were performed in the Ringer's solution in which the muscles had been rehydrated.

As the contents of the investigated compounds in the symmetrical muscles differed only slightly (Table 1), it was thought possible to use one of the paired muscles as control.

RESULTS

Phenomena observed during drying and rehydration of frog muscle

Water content of the sartorii of the frog appeared to be fairly stable; it was $82.2 \pm 1.6\%$ for smaller muscles (50 - 100 mg.) and $79.5 \pm 0.6\%$ for the larger ones (200 - 300 mg.). Drying of the muscles in the conditions used was influenced by the water vapour pressure at 2°, by the initial temperature of the muscle and its size (weight and surface). It can be seen from Fig. 1 that the surface of the muscles weighing 50 to 400 mg. increased linearly with the increase in weight.

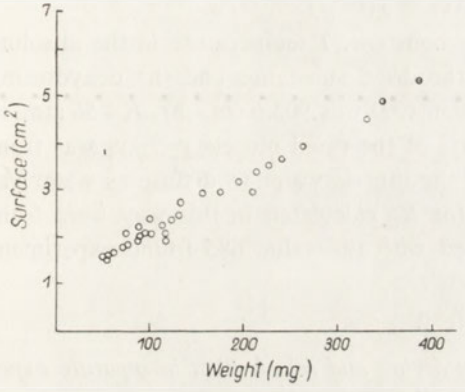

Fig. 1

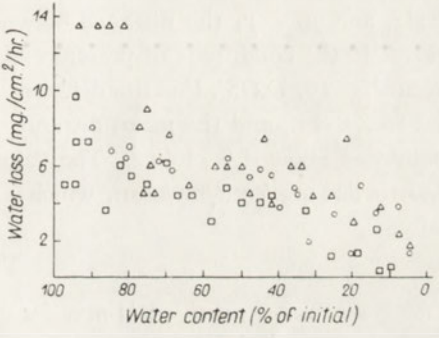

Fig. 2

Fig. 1. Relation between the weight of the muscles and their surface.

Fig. 2. Rate of evaporation of water from the muscle related to the actual moisture content. Weight of muscle: (○), 60 mg.; (△), 150 mg.; (□), 300 mg.

The loss of water by evaporation from a surface unit is proportional to the difference between water vapour pressures over the substance (P_s) and over the dehydrating agent (P_a) [5]:

$$\frac{dw}{dt} \times \frac{1}{A} = K_G (P_s - P_a)$$

where dw/dt is the loss of weight in a time unit, A the surface of the substance, K_G the mass transfer coefficient for diffusion of water vapour into air, and P_s and P_a are the corresponding water vapour pressures. The whole process of drying consists of two periods: the constant-rate period and the falling-rate period. The above equation permits the calculation of the constant rate of drying during the first period, which usually constitutes the main part of the process of desiccation.

The relation between the rate of water loss from a surface unit of the muscle and its actual water content is shown in Fig. 2. In the range of water content from about 70% of initial to about 20%, the rate of water loss was roughly stable and the mean value for 153 dried muscles was found to be 4.8 ± 2.1 mg./cm.^{2}/hr. Initially the rate of drying of the muscles was somewhat higher than during the constant-rate period, most probably because the temperature of the muscles which had been excised at 20° did not drop yet to 2°. The mean time necessary for the equilibration of temperature was about 1.5 hours.

Water vapour pressure over the muscle surface (P_s) at 2° was 7×10^{-3} atm. and over silica gel (P_a) it was 2.6×10^{-6} atm. The latter value, being extremely small, can be neglected and thus the calculation may be simplified:

$$4.8 \text{ mg./cm.}^2\text{/hr.} = K_G \times 7 \times 10^{-3} \text{ atm.}$$

K_G calculated from the present experimental data was 685 mg./cm.^{2}/hr./atm. The same mass transfer coefficient may be also calculated from the formula [5]:

$$K_G = \frac{D}{RT(y_2 - y_1)}$$

where D is the diffusion coefficient, R gas constant, T temperature in the absolute scale, and $y_2 - y_1$ the distance between the dried substance and the dehydrating agent. In the conditions of present experiments, D was 903.6 cm.^{2}/hr, R 4.56 atm.$\times$ $\times$cm.^{3}/g.$\times \theta$, T 275°. For the different parts of the dried muscle $y_2 - y_1$ was from 0.2 to 2.8 cm., and the mean distance for the muscle water to diffuse as water vapour was about 1.0 - 1.4 cm. The values for K_G calculated in this way were from 720 to 520 mg./cm.^{2}/hr./atm., which agreed with the value 685 found experimentally.

Table 2

Changes in water partition in muscles after drying and rehydration in separate experiments on paired muscles

The data are expressed as percentage of the initial water content in the control symmetrical fresh muscle. DR, muscle dried and rehydrated.

Control muscle		Muscle dried and rehydrated				Fibre water (DR to control ratio)
			Water partition after rehydration			
"Sucrose space"	Fibre water	Water content after drying	Total water	"Sucrose space"	Fibre water	
58.8	41.2	76.5	100.0	57.4	42.6	1.03
51.6	48.4	65.0	91.4	55.9	44.1	0.89
56.0	44.0	49.0	98.6	62.4	36.3	0.82
60.5	39.5	43.0	83.0	52.0	31.2	0.79
56.9	43.1	38.0	88.4	54.6	33.7	0.78
48.9	51.1	24.8	88.5	59.5	29.2	0.57
61.7	38.3	24.0	80.0	56.7	23.2	0.61
63.4	36.7	15.7	85.8	76.5	9.3	0.25
56.5	43.5	8.4	82.5	75.2	7.2	0.16
73.5	26.5	0.0	60.5	60.5	0.0	0.00

The duration of the constant-rate period depended on the weight of the muscles, varying from 3.5 to 7 hr. for muscles weighing from 50 to 300 mg. The falling-rate period lasted about 1.5 hr. for all the muscles. Therefore the time of complete desiccation of the muscles, including the initial equilibration of temperature, was from 6.5 to 10 hr.

Determinations of water partition in the fresh muscles and in muscles rehydrated after different degrees of dehydration (Table 2) indicate that if the muscle had lost not more than 80% of its initial water content, a good part of the fibre water can be restored during rehydration, attaining 60 - 90% of the amount present in the fresh muscle. Further drying apparently damaged the fibre membranes, resulting in an increase of the sucrose space and in the disappearance of the fibre water.

Phosphorus compounds in dehydrated muscles

Changes in the contents of phosphorus compounds brought about by drying (Table 3) were but slight and even after complete dehydration no significant changes took place. Minute quantities of deaminated nucleotides found in the dried muscles are not significant for the changes in the total amount of adenine nucleotides.

Phosphorus compounds in the rehydrated muscles and in the Ringer's solution used for rehydration

When fresh muscles were immersed in Ringer's solution for about 30 min. at 20° some changes in phosphorus compounds took place (Table 4). Although there was no dephosphorylation of labile compounds as phosphocreatine or ATP, an outflow of P_i into the medium and a small but significant deamination of adenine nucleotides could be observed. Many authors suggest that orthophosphate can pass through the sarcolemma only very slowly [11, 4, 15] and therefore it can be supposed that the P_i found in the Ringer's solution originated from the extracellular space.

The contents of various compounds in rehydrated muscles and in the solution used for rehydration are shown in Table 5. The amount of P_i found in the Ringer's solution did not exceed the value found after immersion of fresh muscle (cf. Table 4) if the loss of water on dehydration did not surpass about 80%. The content of phosphocreatine showed significant changes only in those muscles which had lost more than 80% of their water, when splitting of most of phosphocreatine occurred, accompanied by an outflow of P_i into the Ringer's solution. Dephosphorylation of adenine nucleotides took place during rehydration; it was more pronounced in those muscles which had lost 90% of water. Small amounts of ΔP_{15} found in the Ringer's solution were insignificant. Rehydration of the dried muscles led to deamination of about 30% of the total nucleotides, two thirds of which were found in the Ringer's solution.

Table 3

Changes in acid-soluble phosphorus compounds in the course of dehydration of the muscle

The data for the control muscle are expressed as μmoles/100 mg. of fresh muscle. Changes caused by dehydration are expressed as the difference between the values for the dehydrated muscle and control. Values quoted for *P* are based on *t* test [13].

Water loss in dried muscle (% of initial content)	P_{i-true}		Phosphocreatine		ΔP_{15}		Adenine nucleotides		Inosine nucleotides	
	Control	Difference	Control	Difference	Control	Difference	Control	Difference	Control	Difference
18.5	1.27	—0.36	0.90	+0.17	0.54	+0.03	0.49	—0.02	0.01	+0.01
30.0	0.95	—0.05	0.69	+0.21	0.71	—0.01	0.47	0.0	0.05	—0.01
40.0	1.66	—0.07	0.90	+0.24	0.75	—0.09	0.39	—0.01	0.03	+0.03
49.0	2.11	—0.39	0.98	+0.16	0.53	+0.05	0.55	0.0	0.0	0.0
60.0	2.02	+0.17	0.89	+0.02	1.23	—0.13	0.39	0.0	0.01	0.0
70.5	2.54	+0.34	0.67	—0.15	0.44	—0.02	0.42	+0.01	0.07	+0.01
78.0	1.81	—0.09	0.76	—0.06	0.55	—0.03	0.40	0.0	0.07	+0.03
85.0	1.50	+0.03	0.74	+0.01	0.77	+0.18	0.47	+0.06	0.03	+0.03
95.0	1.45	+0.23	0.77	+0.04	0.59	+0.07	0.55	—0.05	0.0	+0.04
Mean ± S. D.		—0.02±0.25		+0.07±0.13		+0.005±0.09		—0.001±0.03		+0.016±0.017
P		insignificant		0.1 - 0.2		insignificant		insignificant		0.01

Table 4

Changes in phosphorus compounds in the fresh resting muscle immersed for 30 min. in Ringer's solution at 20°

The changes are expressed as the difference between the values for the immersed muscle and the control. Mean values (μmoles/100 mg. of fresh muscle ± S.D.) are given, with the number of experiments in parentheses. Values quoted for *P* are based on *t* test [13].

Muscle	$P_{i\text{-true}}$		Phosphocreatine		ΔP_{15}		Adenine nucleotides		Inosine nucleotides	
Control, not immersed	1.31 ± 0.35		0.92 ± 0.07		0.63 ± 0.27		0.49 ± 0.04		0.01 ± 0.01	
After immersion for 30 min.	Immersed (difference)	Ringer	Immersed (difference)	Ringer	Immersed (difference)	Ringer	Immersed (difference)	Ringer	Immersed (difference)	Ringer
	(6)		(3)		(4)		(4)		(4)	
	−0.24 ± 0.26	0.49 ± 0.24	0.0 ± 0.19	0.0	+0.01 ± 0.18	0.0	−0.09 ± 0.04	0.03 ± 0.04	0.0 ± 0.01	0.06 ± 0.01
P	0.10	< 0.01	insignificant		insignificant		< 0.05	insignificant	insignificant	< 0.001

Table 5

Changes in the phosphorus compounds in the muscles which were dried and rehydrated in Ringer's solution

All data are expressed as μmoles/100 mg. of fresh muscle. Mean values are given when the experiments were repeated (number of experiments in parentheses). Values for the control muscle are given with S. D. (n = 8). Under heading: Muscle, is given the difference in the contents of investigated compounds between the rehydrated muscle and the control muscle; under Ringer, contents in the Ringer's solution after rehydration of the muscle.

	$P_{i\text{-true}}$		Phosphocreatine		ΔP_{15}		Adenine nucleotides		Inosine nucleotides		Total nucleotides	
Control	1.39 ± 0.26		0.86 ± 0.17		0.67 ± 0.26		0.44 ± 0.04		0.03 ± 0.03		0.47 ± 0.05	
Water loss (% of initial content)	Muscle	Ringer	Muscle	Ringer	Muscle	Ringer	Muscle	Ringer	Muscle	Ringer	Muscle	Ringer
20	—0.36 (1)	0.47	—0.05 (1)	0.0	—0.16 (1)	0.0	—0.16 (1)	0.02	—0.02 (1)	0.02	—0.14 (1)	0.04
30	—0.13 (3)	0.34	—0.25 (1)	0.25	—0.22 (3)	0.09	—0.06 (3)	0.01	—0.02 (3)	0.02	—0.08 (3)	0.03
45	—0.30 (3)	0.49	+0.04 (1)	0.0	—0.16 (3)	0.0	—0.07 (3)	0.02	+0.02 (3)	0.04	—0.05 (3)	0.06
67	—0.41 (3)	0.37	—0.18 (1)	0.26	—0.27 (3)	0.08	—0.02 (4)	0.02	—0.01 (3)	0.06	—0.03 (3)	0.08
75	—0.10 (1)	0.61	—0.28 (1)	0.0	—0.21 (1)	0.40	—0.01 (1)	0.0	+0.06 (1)	0.0	+0.05 (1)	0.0
84	—1.06 (3)	1.19	—0.46 (1)	0.0	—0.24 (3)	0.13	—0.20 (2)	0.06	+0.04 (2)	0.12	—0.16 (2)	0.17
90	—1.20 (3)	1.43	—0.59 (1)	0.0	—0.49 (3)	0.10	—0.11 (3)	0.12	+0.02 (2)	0.11	—0.12 (3)	0.23
94	—0.81 (2)	1.66	—0.86 (1)	0.0	—0.39 (2)	0.14	—0.25 (2)	0.07	+0.06 (2)	0.10	—0.18 (2)	0.18

Total phosphorus content and the amount of dry matter in muscles which were dried and rehydrated, were also investigated. The data obtained in these estimations related to the degree of water loss by the muscle are shown in Table 6. Dehydration and rehydration of the muscle led to progressive decrease both of solids and total phosphorus. The changes were especially pronounced in those muscles which had lost more than 80% of water.

Table 6

Changes in dry matter and total phosphorus contents in muscles which were dried and rehydrated

Results of separate experiments are given.

Water loss after drying (% of initial content)	Dry matter (mg./100 mg. fresh muscle)			P_{tot} (μmoles/100 mg. fresh muscle)		
	Control	Dried and rehydrated	Difference	Control	Dried and rehydrated	Difference
35	16.4	16.8	+0.2	4.19	4.02	—0.17
50	18.0	17.8	—0.2	4.77	4.14	—0.63
60	17.2	16.0	—1.2	4.77	3.64	—1.13
75	18.4	16.5	—1.9	4.82	3.32	—1.50
80	17.6	15.5	—2.1	5.07	2.19	—2.88
90	19.5	16.6	—2.9	5.11	1.46	—3.65
100	17.4	14.3	—3.1	4.77	1.02	—3.75

Shortening of the muscle during rehydration

Rehydration of the dried muscles had no effect on their length and shape if the muscles had lost less than 60% of water. After greater dehydration the muscles when immersed in Ringer's solution at 20° showed a slow spontaneous contraction. The shortening was especially pronounced in muscles which had lost 90% of water. In this case the reduction in length was about 30% of the initial value (Fig. 3).

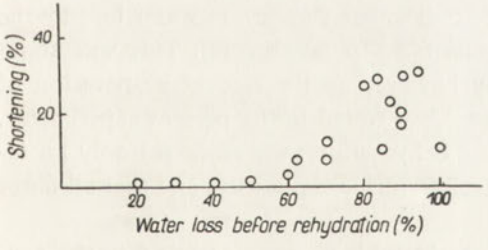

Fig. 3. Diminution of the length of muscle after rehydration.

DISCUSSION

The presented results show that during dehydration over silica gel the contents of examined muscle constituents remained almost constant and no significant breakdown

of ATP or phosphocreatine occurred. On the other hand, the changes observed after rehydration of the muscle indicate that at a certain degree of water loss the normal functions of the muscle are irreversibly destroyed. This is accompanied by splitting of ATP and phosphocreatine and an outward diffusion of some products of their breakdown. The tolerance for water loss found in our experiments seemed to be significantly higher than it was reported by Overton [28], Hill & Kupalov [16] or Fenn [12] who suggested that removal of about 50% of water from the muscles kept in hypertonic solution involves damage to the muscle fibres. Contrary to these earlier findings, Hodgkin & Horowicz [17], Pauschinger & Brecht [29] and Dydyńska & Wilkie [9] have shown that dehydration by the hypertonic medium did not seriously affect the muscles, provided that their fibres had lost not more than 80% of water. After rehydration the normal functions of the muscles were restored, probably because the properties of the cell membranes were maintained.

The results of extracellular space determinations in fresh and rehydrated muscles seem to throw some light on processes related to water loss by the muscle fibres. During drying of the muscle over silica gel the layer of water on the surface of the muscle amounting to 10% of the total water (Dydyńska, unpublished results) was the first to evaporate and only afterwards the water from the most superficial layers of muscle fibres came out. As the fibres were still able to gain water during rehydration, provided that the muscle had lost less than 80% of total water (Table 2), there are good reasons to assume that the fibres in the deeper layers of the muscle were undamaged and the properties of the membranes were unaffected. Calculations based on the diameter of a single muscle fibre (0.05 mm. [21, 9]) and the volume of the whole muscle, show that the sartorius of the frog consists of about 1600 fibres. The two most superficial layers contribute to approximately 20% of the muscle fibres, and these layers are most likely to be damaged at the onset of dehydration.

The dehydration of the muscles over silica gel seems to be limited by the rate of evaporation of water from the surface of the muscle. Experiments with the muscles kept in hypertonic solutions allowed to calculate the rate of water loss by the muscle through water movement from one fibre to another [9]. This was about 10 - 15 mg./cm^2./hr. which is significantly higher than the rate of evaporation of water from the muscle surface (4.8 mg./cm.^{2}/hr.) found in the present experiments. It can be, therefore, supposed that during dehydration over silica gel only an insignificant gradient of the water content exists inside the muscle and that all fibres contain the same percentage of water.

During rehydration of dried muscle considerable changes occurred in the contents of labile phosphorus compounds (Table 5) whereas in muscles which were dried but not rehydrated almost no changes were observed (Table 3). The value found for phosphocreatine in comparison to the data given by other workers and collected by Maréchal in his thesis [23] belongs to the lower ones. This may be due the to fact that the muscles excised from the pithed frogs were dried immediately without previous immersion in oxygenated Ringer's solution. This procedure was used to avoid changes in the content of water and other constituents; the elimi-

nation of initial rest in Ringer's solution could have prevented the restoration of phosphocreatine, the content of which was decreased due to stimulation caused by pithing and excision of the muscles.

Changes in phosphorus compounds occurring in the muscle during drying and rehydration summarized in Fig. 4 indicate that when the dehydration exceeds 80% of water loss the structure of the muscle becomes damaged. It has been shown previously [8] that frog heart submitted to dehydration over silica gel did not stop beating until it had lost over 70% of water content, which is in agreement with the present results.

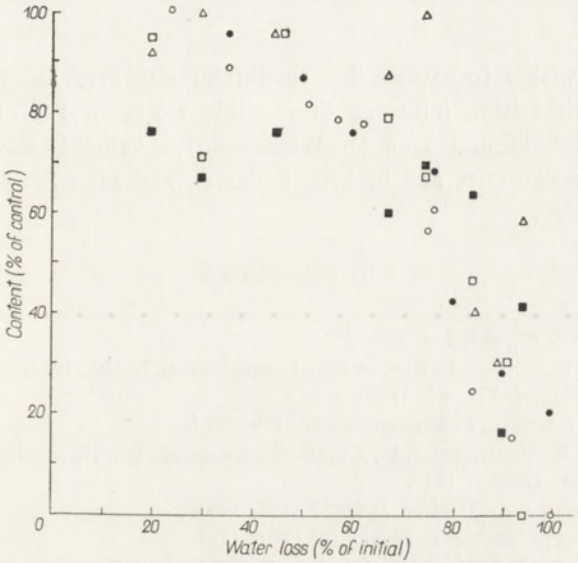

Fig. 4. Changes: (○), in fibre water content, and of (△), $P_{i\text{-true}}$; (□), phosphocreatine; (▦), ΔP_{15}; and (●), P_{tot}, in the muscles after drying and rehydration.

Recent experiments carried out on isolated muscle fibres kept in hypertonic or hypotonic solutions showed that only 66% (Blinks [3]) or 60% (Krolenko *et al.* [22]) of fibre volume was able to undergo changes according to the laws of osmosis. As from the remaining 33 or 40% of fibre volume only 20% may be accounted for solids, about 13 - 20% of fibre volume seems to consist of structurally bound water. On the other hand, the experiments on the whole muscle immersed in hypertonic solution seemed to show that the total amount of water in the fibre is osmotically active [9]. The present results indicate that a certain quantity of structurally bound water may be present in the muscle fibre; the loss of this water could possibly lead to various changes observed when more than 80% of the total amount of water had been evaporated.

The known phenomenon of spontaneous contraction of the dried muscle during rehydration [20, 25, 18, 26] was also observed in the present experiments and appeared to be connected with some special changes caused by drying. It seems that, although in such conditions the structure of the cell membrane is already damaged,

the contractile proteins remain relatively unaffected. Thus Hunt & Matheson [19] found that myosin extracted from the dried muscle showed ATPase activity. As almost normal quantities of labile phosphorus compounds have been found in dried muscle, the suggestion of Szent-Györgyi that the contraction of dried muscles on rehydration may involve chemical processes similar to those which occur in fresh muscles, seems fairly probable. The dehydrated muscles would be able to contract as soon as enough water had entered the muscle cells. The exact nature of the influence of water inflow on the contractile proteins and the explanation of the character of the observed contraction need, however, further investigations.

The author wishes to express her thankfulness to Prof. Dr. W. Niemierko for his help and stimulating influence during this work, to Prof. Dr. D. R. Wilkie, Doc. Dr. Drabikowski and Doc. Dr. Włodawer for valuable advice and discussions upon the manuscript and to Mrs. Bednarek and Miss Szwykowska for their technical assistance.

REFERENCES

[1] Adrian R. H. - *J. Physiol.* **133**, 631, 1956.
[2] Beaven G. H. & Holiday E. R. - *Advanc. Protein Chem.* **7**, 320, 1952.
[3] Blinks J. - *J. Physiol.* **177**, 42, 1965.
[4] Causey G. & Harris E. J - *Biochem. J.* **49**, 176, 1951.
[5] Coulson J. M. & Richardson J. F., *Chemical Engineering*, vol. II, p. 848 and vol. I, p. 234; Pergamon Press, London 1957.
[6] Drabikowski W. - *Acta Biochim. Polon.* **7**, 127, 1960.
[7] Durig A. - *Pflügers Arch. ges. Physiol.* **85**, 401, 1901.
[8] Dydyńska M. - *Bull. Acad. Polon. Sci. Ser. Biol.* **9**, 57, 1961.
[9] Dydyńska M. & Wilkie D. R. - *J. Physiol.* **169**, 312, 1963.
[10] Ennor A. H. & Rosenberg H. - *Biochem. J.* **51**, 607, 1952.
[11] Fenn O. W. - *Physiol. Rev.* **16**, 450, 1936.
[12] Fenn O. W. - *J. Cell. Comp. Physiol.* **9**, 93, 1936.
[13] Fischer R. A. & Yates F., *Statistical Tables*, Oliver & Boyd, London 1948.
[14] Fiske C. H. & Subbarow Y. - *J. Biol. Chem.* **66**, 375, 1925.
[15] Fleckenstein A., Gerlach E. & Janke J. - *Pflügers Arch. ges. Physiol.* **271**, 75, 1960.
[16] Hill A. V. & Kupalov P. S. - *Proc. Roy. Soc. Ser. B.* **106**, 445, 1930.
[17] Hodgkin A. L. & Horowicz P. - *J. Physiol.* **136**, 17P, 1957.
[18] Hopkins A. L. - *J. Cell. Comp. Physiol.* **51**, 67, 1958.
[19] Hunt M. V. & Matheson N. A. - *Nature* **181**, 472, 1958.
[20] Hürthle K. - *Pflügers Arch. ges. Physiol.* **126**, 1, 1909.
[21] Kono T. & Colowick S. P. - *Arch. Biochem. Biophys.* **93**, 520, 1961.
[22] Krolenko S. A., Adamian S. J., Winogradowa N. A. & Nikolskij N. N. - *Citologia* **7**, 173, 1965.
[23] Maréchal G., *Le métabolisme de la phosphorylcréatine et de l'adénosine triphosphate durant la contraction musculaire*. Ed. Arscia, Bruxelles 1964.
[24] Morozow B. D. - *Biol. Zentralblatt.* **51**, 307, 1931.
[25] Mueller H. & Szent-Györgyi A. - *Science* **126**, 970, 1957.
[26] Mueller H. - *Proc. Nat. Acad. Sci. U. S.* **44**, 235, 1958.
[27] Norman A., Rondell P. & Bohr F. - *Amer. J. Clin. Pathol.* **32**, 465, 1959.

[28] Overton E. - *Pflügers Arch. ges. Physiol.* **92**, 115, 1902.
[29] Pauschinger P. & Brecht K. - *Naturwissenschaften* **46**, 267, 1959.
[30] Tasker P., Simon S. E., Johnstone B. M., Shankley K. H. & Shaw F. H. - *J. gen. Physiol.* **43**, 39, 1959.

ZMIANY W ZAWARTOŚCI LABILNYCH ZWIĄZKÓW FOSFOROWYCH PODCZAS ODWADNIANIA I UWADNIANIA MIĘŚNI SUSZONYCH NAD SPROSZKOWANYM ŻELEM KRZEMIONKOWYM

Streszczenie

1. Wykazano, że o ile mięsień nie utracił więcej niż 75 - 80% wody nie obserwuje się znaczniejszych uszkodzeń włókien i po uwodnieniu mięsień powraca do stanu normalnego.

2. Jeżeli włókna mięśniowe utraciły więcej niż 80% wody, podczas uwadniania obserwuje się powolny spontaniczny skurcz mięśnia, któremu towarzyszy całkowity rozpad fosfokreatyny oraz częściowa defosforylacja i dezaminacja nukleotydów adenilowych.

3. Wysuwa się przypuszczenie, że obserwowane zjawiska są następstwem uszkodzenia organizacji włókna mięśniowego, być może z powodu utraty wody związanej strukturalnie.

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MAGDALENA FIKUS and D. SHUGAR

ALKALINE TRANSFORMATIONS OF THE PHOTOHYDRATES OF SOME 2,4-DIKETOPYRIMIDINES AND THEIR GLYCOSIDES

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

1. In aqueous alkaline medium the photohydrate of 1-methyluracil undergoes direct base-catalysed dehydration to regenerate quantitatively 1-methyluracil. 2. In aqueous *ammoniacal* medium the photohydrate of 1,6-dimethyluracil undergoes ring opening at the 1,6 bond, followed by dehydration and finally ring closure to quantitatively reform 1,6-dimethyluracil. If the medium is made alkaline with NaOH or KOH instead of ammonia, regeneration of 1,6-dimethyluracil is not fully quantitative. 3. In aqueous ammoniacal medium the photohydrate of uridine (and of a wide variety of other uracil glycosides) undergoes two simultaneous, competing, reactions: direct base-catalysed dehydration; and ring opening at the 1,6 bond, followed by dehydration and ring closure. These lead to quantitative regeneration of uridine. If the medium is made alkaline with NaOH or KOH, a third reaction intervenes, the extent of which is dependent on the pH, and which appears to involve opening of the ring at the 3,4 bond; this reaction does not lead to regeneration of uridine. However, addition of an excess of ammonia, following addition of NaOH or KOH, abolishes the effect of the latter, and again leads to quantitative regeneration of uridine. 4. In alkaline methanolic medium the photohydrates of 1,6-dimethyluracil, and of the uracil glycosides, undergo only one type of transformation: ring opening at the 1,6 bond, dehydration of the open chain compound, and ring closure to regenerate the parent compound. The presence of traces of water is necessary for this reaction sequence. 5. The difference in behaviour in alkaline medium between the photohydrates of 1-methyluracil on the one hand, and the uracil glycosides on the other, appears to be due to some influence of the glycosidic linkage, and not to any involvement of the carbohydrate hydroxyls. 6. The kinetics and activation energies for some of the reactions have been measured and calculated. Isotope effects in heavy water have also been measured, but these did not prove readily interpretable. 7. Attention is drawn to the significance of the results in pyrimidine syntheses, and for possible quantitative reversal of uracil hydration in irradiated poly-U and ribonucleic acids.

Ultraviolet irradiation of uracil glycosides in aqueous medium leads to the nucleophilic addition of water to the 5,6 double bond of the pyrimidine ring. In aqueous *frozen* medium the principal reaction is the formation of a photodimer in which the 5,6 bonds of two molecules combine to form a cyclobutane ring. In oligo- and poly-U, and in RNA, both of these reactions are known to occur, the

relative proportions of hydrates to dimers being partially a function of the irradiation wavelength (for review see ref. [10]).

The photohydrates are relatively stable at room temperature and neutral pH, but are readily subject to acid-base catalysed dehydration either at elevated temperatures at neutral pH or at room temperature in acid medium. The photodimers are fully stable in neutral and acid medium, even at elevated temperatures [19] but readily photodissociate to regenerate the original uracil rings. Photodissociation of dimers occurs more readily in alkaline medium due (a), to the higher absorption of the dimers under these conditions and, (b), because the dimerization reaction does not proceed at alkaline pH [18, 19].

In alkaline medium both uracil and thymine photodimers may photodissociate quantitatively to uracil and thymine [19]. This is not so for the photodimers of uridine and thymidine, or UpU and TpT, for all of which photodissociation in alkaline medium is accompanied by some side reaction(s) [18]. In the case of uridine photodimer, it was found that these side reactions were not due to the effects of radiation on the dimer itself; but that the uridine resulting from dimer photodissociation underwent photohydration in the alkaline medium, and subsequently underwent a complex series of alkali-catalysed reactions. The aim of the present investigation was to clarify the nature of the reactions undergone by uridine photohydrate in alkaline medium.

It had earlier been noted by Rapport *et al.* [12] that alkalization of irradiated uridine did not lead to quantitative reformation of the uridine spectrum, but that the resulting product could satisfy the growth requirements of the uridine dependent *Neurospora crassa*. It had also been noted by Sinsheimer [16] that some unknown product(s) were formed when irradiated uridine was treated with strong alkali. More recently Schuster [14] reported that when photohydrated uridylic acid is brought to 1 N-KOH, 65% dehydrates to uridylic acid, while 35% is transformed to a new compound resulting from scission of the 3,4 bond; this was applied to determination of the number of hydrated, as compared to dimerized, uracil residues in irradiated RNA.

MATERIALS AND METHODS

Uridine, deoxyuridine, uridylic acid were commercial preparations (Sigma, St. Louis, U.S.A., and Calbiochem., Los Angeles, U.S.A.).

Uridine and 2′,3′-*O*-isopropylideneuridine were acetylated as described by Visser *et al.* [20] and the products identified by paper chromatography. In water-saturated butanol the R_F values were: uridine, 0.17; isopropylideneuridine, 0.66; 2′,3′,5′-triacetyluridine, 0.72; 5′-acetyl-2′,3′-isopropylideneuridine, 0.80.

1,6-Dimethyluracil was obtained from 2,4-dimethoxy-6-methyluracil [11] by treatment with methyl iodide according to Hilbert & Johnson [7]. We are indebted to Mrs. K. Ziabicka for this preparation. 1,3,6-Trimethyluracil was prepared by treatment of 6-methyluracil with dimethylsulphate in alkaline medium [13]. Both of these compounds were hydrogenated to give the 5,6-dihydro derivatives, using a rhodium catalyst as elsewhere described [2, 6, 9].

1-Methyluracil was obtained as described by Hilbert & Johnson [7]. Uridine and thymidine were treated with diazomethane in anhydrous methanol to give the *N*-methyl derivatives [17]. Glucopyranosyluracil was prepared according to Hilbert & Johnson [8] and ribosethymine according to Fox *et al.* [5]. We are indebted to Dr. Barbara Żmudzka and Mr. M. Świerkowski for assistance in the preparation of several of these compounds.

Irradiations, and subsequent treatment of photoproducts, were carried out for the most part in 1- and 10-mm. quartz spectrophotometer cuvettes. The irradiation source was a 40-watt Phillips germicidal lamp (253.7 Å) encased in a quartz cylinder with an inner diameter 4 mm. greater than that of the lamp. This made it possible to surround the lamp with a 2-mm. layer of saturated sodium acetate to cut off all radiation below 230 mμ. The intensity of this source, in a direction perpendicular to the lamp axis, was of the order of 7×10^{-7} einsteins/min./cm.^{2} at the surface of the surrounding quartz cylinder. A 10^{-4} M aqueous neutral solution of uridine was employed as an actinometer for intensity measurements [23]. In several instances, where larger quantities of photoproduct were required, a specially constructed reactor [3] was employed.

Measurements and irradiations at temperatures other than ambient were carried out by locating the cuvettes to be irradiated in a spectrophotometer constant temperature block. Water or aqueous glycol was pumped through the block by means of an ultrathermostat and temperatures controlled by means of a thermistor in a dummy cuvette. All spectral measurements were carried out with a Beckman DU instrument.

RESULTS

Alkaline transformations of uridine photohydrate in ammoniacal medium

Irradiation of a neutral aqueous solution of uridine (10^{-4} - 10^{-3} M) results in almost quantitative conversion to 5-hydro-6-hydroxyuridine. The subsequent acid-catalysed elimination of the water molecule regenerates from 95 to 100% of the uridine [1].

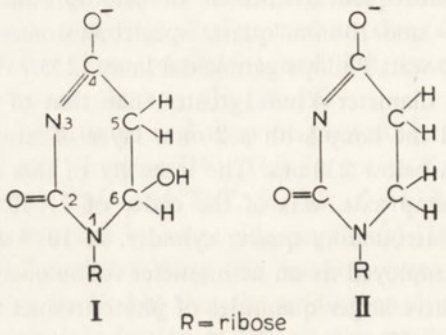
In neutral medium photohydrated uridine exhibits only end absorption in the quartz ultraviolet (Fig. 1) in expected accordance with saturation of the 5,6 bond (see Scheme 1). If the photoproduct solution is made alkaline (about pH 12) and spectral readings carried out *within a few seconds following addition of alkali*, curve *c* is obtained. In practice, curve *c* was recorded in the following manner: A larger volume of uridine photohydrate was prepared in a reactor. An aliquot was transferred to a spectral cuvette and concentrated ammonia added to give pH 11.8 - 12.0[2] with stirring. Within 3 - 5 sec. of the addition of ammonia, an extin-

[1] At a concentration of 10^{-3} M it has been shown that up to 5-6 % of uridine dimer may be formed under these conditions (E. Sztumpf-Kulikowska, Ph. D. thesis, Polish Academy of Sciences, 1966).

[2] The reason for the use of concentrated ammonia instead of NaOH or KOH will be explained below.

ction reading was made at one predetermined wavelength. A different aliquot of photoproduct was employed for each wavelength.

Curve *c* of Fig. 1 (I, E_{max} 232 mμ, $\varepsilon_{max} \sim 10^4$) is almost identical with that of the alkaline spectrum of 5,6-dihydrouridine (II) [9], and is due to dissociation of the N_3 proton with resultant conjugation of the $C_2{=}O$ and $N_3{=}C_4$ double bonds (see ref. [21] for full discussion of these structures); (Scheme 1).



Scheme 1

Within 2 - 3 min. at room temperature following addition of alkali, curve *c* disappears. Simultaneously a new peak appears at 290 mμ, the optical density of which attains a maximum within a time interval of about 3 - 4 min. When the spectra were recorded at 1 min. intervals, it was found that they pass through an isosbestic point at 245 mμ.

Table 1

Rate constants and energy of activation for disappearance of alkaline transformation product of uridine photohydrate (disappearance of absorption maximum at 290 mμ), to regenerate uridine, in ammoniacal aqueous medium pH *12*

t°C	*T*°K	k_r (sec.$^{-1}$)	*E* (kcal./M)
17.0	290.0	4.36×10^{-4}	
24.0	297.0	9.48×10^{-4}	18.5
27.5	300.5	13.4×10^{-4}	

About 3 - 4 minutes following addition of alkali, the peak at 290 mμ begins to decrease; simultaneously the absorption at 260 mμ increases (Fig. 2). These modifications are complete within about 1 hour. The ammonia was then partially removed from the solution with a stream of cold air and the spectrum of the solution, determined at acid and alkaline pH values, found to be identical with that of the initial uridine (Fig. 3). Note from Fig. 3 that regeneration of uridine was practically quantitative, notwithstanding that the processes involved were obviously different from the direct dehydration mechanisms at neutral or acid pH. If the ammoniacal solution is neutralized, in place of initial removal of some of the ammonia in a stream of air, regeneration of uridine is not quantitative.

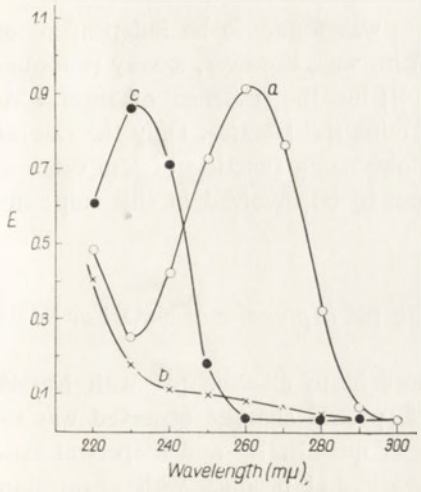


Fig. 1

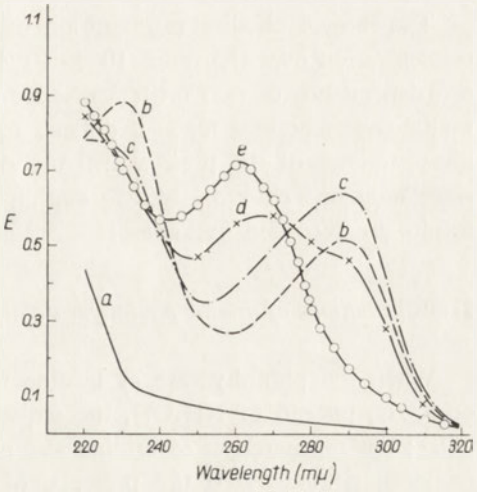


Fig. 2

Fig. 1. Absorption spectra of (*a*), uridine at neutral pH; (*b*), uridine photohydrate, 5-hydro-6--hydroxyuridine at neutral pH; (*c*), uridine photohydrate anion at pH 12 (Note: the anion is very labile in alkali; see text for method of obtaining this curve).

Fig. 2. Transformation at 20° of uridine photohydrate anion (10^{-3} - 10^{-4} M) in alkaline medium at pH 12 (50 μl. conc. NH_4OH in 2.5 ml. H_2O): (*a*), Spectrum of uridine photohydrate in neutral medium; (*b*), (*c*), (*d*) and (*e*) are spectra 2.5, 5, 16 and 49 min. respectively, after alkalization to pH 12.

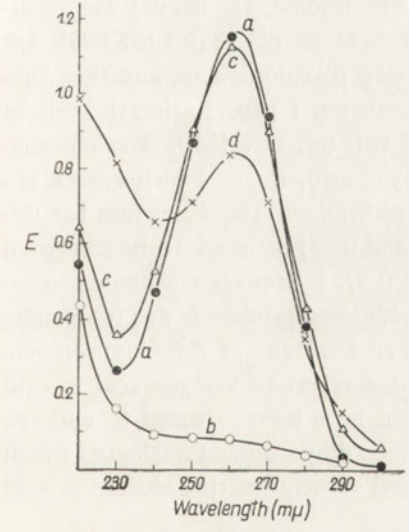


Fig. 3

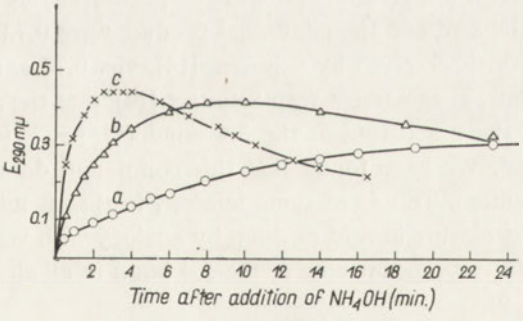


Fig. 4

Fig. 3. Illustrating quantitative regeneration of uridine from uridine photohydrate in ammoniacal aqueous medium at pH 12: (*a*), Spectrum of uridine prior to irradiation at neutral pH; (*b*), spectrum of uridine photohydrate at neutral pH; (*c*), spectrum following alkaline ammoniacal treatment of uridine photohydrate, removal of ammonia and neutralization to pH 7; (*d*), spectrum of regenerated uridine at pH 12.

Fig. 4. Influence of temperature on rate of transformation of uridine photohydrate in ammoniacal aqueous medium, pH 12, to product with absorption maximum at 290 mμ. (*a*) 4.4°, (*b*) 17.0°, (*c*) 27.5°.

The rate of alkaline regeneration of uridine was found to be independent of concentration over the range 10^{-4} - 10^{-3} M. There was, however, a very pronounced dependence of rate on temperature (Fig. 4); but the presence of several simultaneous reactions made it difficult to determine the kinetics. Only the rate of disappearance of the peak at 290 mμ was followed as a function of temperature, since only one chemical transformation appears to be involved in this step; the results are recorded in Table 1.

Transformations of uridine photohydrate at alkaline pH *in presence of* NaOH *or* KOH

When the photohydrate of uridine was brought to alkaline pH with NaOH or KOH, instead of NH_4OH, the sequence of spectral changes observed was similar; but regeneration of uridine was no longer quantitative and a spectral examination demonstrated the presence of an additional product with absorption in alkaline medium. The amount of this additional product was dependent on the pH to which the photoproduct was brought. If the uridine photohydrate was alkalized to pH 11 with NaOH or KOH, the final amount of regenerated uridine was 87%; for pH 12 it was 78%; and for pH 13, about 55%. These results differ somewhat from those of Schuster [14], who reported that the amount of uridylic acid regenerated at pH 14 was 65%.

A solution of uridylic acid (which behaves like uridine, see below) was transformed to the photohydrate by irradiation, brought to pH 13 (0.1 N-KOH), left for one hour at room temperature, neutralized with perchloric acid, and then subjected to ascending chromatography with Whatman no. 1 paper, using the solvent system *n*-butanol - ethanol - 5 N-HCl, 3:2:2, by vol. (cf. ref. [14]). The chromatogram showed, in addition to regenerated uridylic acid, a spot which gave a positive Fink reaction [4] without prior spraying with alkali. The R_F values for uridylic acid and the additional product were 0.72 and 0.59; these are to be compared with those given by Schuster [14], viz. 0.70 and 0.57. It appears reasonable to assume, in agreement with Schuster [14], that the additional product is due to opening of the uracil ring at the 3,4 bond [1], i.e. it is an analogue of β-ureidopropionic acid. We have found that this compound does not revert to uridylic acid in acid medium. This is of some interest, in that it might have been expected to undergo ring closure in acid medium by analogy with various dihydrouracil analogues which undergo ring opening at the 3,4 bond in alkali and subsequent ring closure in acid [1, 9].

Abolishment of effect of NaOH (*or* KOH) *with ammonia*

No immediate explanation is at hand for the presumed ring opening reaction at the 3,4 bond by KOH or NaOH. However, the following experiment demonstrated that this effect of the alkali cations could be fully reversed by the ammonium ion. The photohydrate of uridine was brought to pH 12 with NaOH and left at room temperature for 30 min.; under these conditions only 78% of the ori-

ginal uridine should have been regenerated (see above). At this point a threefold excess of NH_4OH was added and the solution then brought to neutrality. An examination now demonstrated that regeneration of uridine was quantitative.

Kinetics of photochemical transformation of uridine photohydrate in alkaline medium

When uridine is irradiated in alkaline medium, the primary photoproduct is ionized hydrated uridine (I) [3]. This also undergoes subsequent alkaline transformations as above. If irradiation, and spectral measurements, are conducted at 2°, the rate of photohydration is unchanged, but the subsequent dark reactions are slowed down considerably (see Fig. 5).

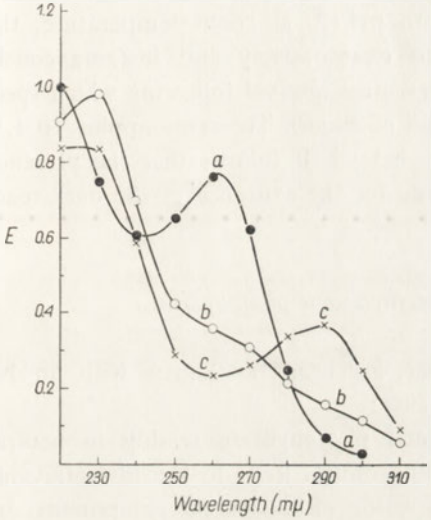


Fig. 5

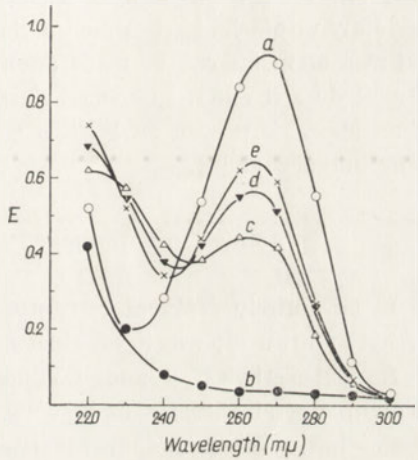


Fig. 6

Fig. 5. Photochemical transformation of uridine in aqueous medium at pH 12 (0.01 N-NaOH) at 0° with incident intensity (254 mμ) of 7×10^{-7} *E*/min./cm.^{2}: (*a*), Absorption spectrum prior to irradiation; (*b*), spectrum 6 min. after irradiation; (*c*), spectrum 12 min. after irradiation.

Fig. 6. Photohydration of 1-methyluracil and alkaline dehydration of photoproduct to regenerate 1-methyluracil at 0°: (*a*), Absorption spectrum of 1-methyluracil at neutral pH; (*b*), spectrum of 1-methyl-5-hydro-6-hydroxyuracil at neutral pH; (*c*), spectrum 5 min. after alkalization of photohydrate; (*d*), and (*e*) are spectra 10 and 25 min., respectively, after alkalization of photohydrate.

When photohydrated uridine was brought to pH 12, the rate constants for disappearance of the peak at 290 mμ were found to be 6.4×10^{-4} sec.$^{-1}$ in ordinary water and 3.6×10^{-4} sec.$^{-1}$ in heavy water, the resultant isotope effect $k_r(H_2O)/k_r(D_2O)$ being 6.4/3.6 or 1.8. No appreciable difference in rates of change of extinctions at 230 mμ or 260 mμ could be observed in light and heavy water.

[3] See ref. [21] for discussion of significance of this observation.

Behaviour of other uracil glycoside photohydrate analogues

The following compounds, after photohydration at neutral pH and subsequent alkalization to pH 12, behaved similarly to uridine: deoxyuridine, uridylic acid, 2′,3′-*O*-isopropylideneuridine, 5′-*O*-acetyl-2′,3′-isopropylideneuridine, 2′,3′,5′-tri-*O*--acetyluridine, glucopyranosyluracil, 2′,3′,4′,6′-tetraacetylglucopyranosyluracil. Irrespective of the modifications of the carbohydrate moiety, the rates and nature of the dark reactions resulting from alkalization were quantitatively the same as for uridine.

Behaviour of N_3*-substituted uracil analogues*

By contrast *N*-methyluridine exhibits none of the foregoing changes. When photohydrated *N*-methyluridine is brought to pH 12, at room temperature, the spectrum of *N*-methyluridine is regenerated quantitatively and instantaneously (certainly within 2 - 3 sec. which is the shortest time interval following which spectral measurements can be made after addition of alkali). The same applies to 1,3--dimethyluracil and 1,3,6-trimethyluracil (see below). It follows that the presence of an ionizable proton on N_3 is a prerequisite for the existence of the dark reactions outlined above.

Alkaline transformation of N_1*-methyluracil photohydrates*

In an entirely different category, however, are uracil analogues with an N_1 methyl substituent, which we shall now discuss:

Both 1-methyluracil and 1,3-dimethyluracil photohydrate readily in neutral medium. But alkalization of both these photoproducts leads to the instantaneous elimination of water and quantitative regeneration of the parent compounds. Irradiation of the parent substances in alkaline medium results only in irreversible modifications with low quantum yields.

In the case of 1-methyl-5-hydro-6-hydroxyuracil it is possible, by reducing the temperature to 2°, to observe the characteristic absorption maximum at about 230 mμ in alkaline medium (Fig. 6). At pH 12 under these conditions the rate of dehydration of the photoproduct in ordinary water was 24.7×10^{-4} sec.$^{-1}$, whereas the rate of elimination of a heavy water molecule in D_2O was 12.8. There is consequently an isotope effect $k_r(H_2O)/k_r(D_2O) = 1.9$. This is to be compared with the analogous isotope effect for dehydration of 1-methyluracil photohydrate in neutral medium, where the situation is reversed, i.e. elimination of a heavy water molecule in D_2O is 2 - 3 times more rapid than elimination of an ordinary water molecule in H_2O [15].

1,3,6-Trimethyluracil likewise photohydrates [24] and the resulting photoproduct undergoes rapid base-catalysed elimination of the water adduct, with quantitative regeneration of the parent compound. But 1,6-dimethyluracil exhibits quite a different behaviour, as we shall now show.

Irradiation of 1,6-dimethyluracil leads to a slow dissappearance of the characteristic absorption maximum, quantum yield 5.7×10^{-3}, the photoproduct exhibiting only end absorption in the ultraviolet. The reaction is first order, with a rate

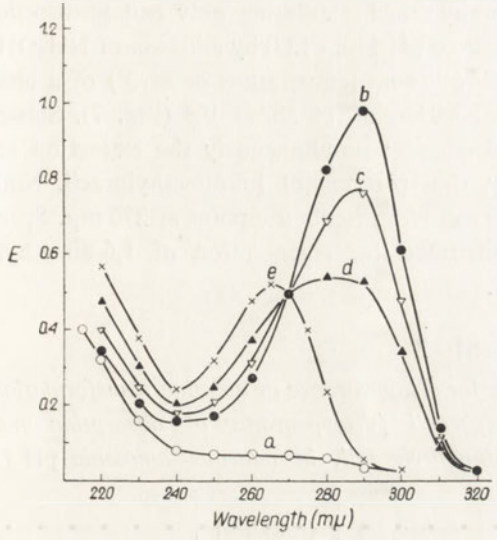


Fig. 7

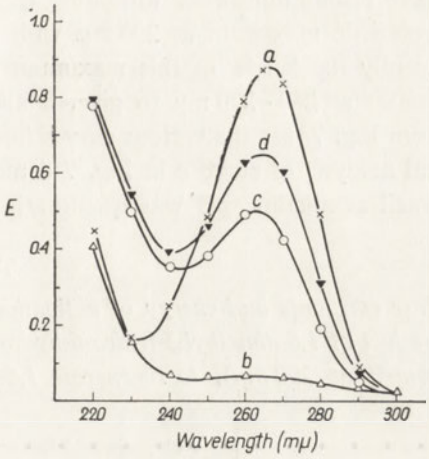


Fig. 8

Fig. 7. Transformation of photohydrate of 1,6-dimethyluracil in ammoniacal aqueous medium, pH 12, at room temperature to regenerate 1,6-dimethyluracil: (*a*), Absorption spectrum of photohydrate at neutral pH; (*b*), spectrum 2 min. after addition of ammonia; (*c*), (*d*) and (*e*) are spectra 6, 10 and 65 min., respectively, after addition of ammonia.

Fig. 8. Regeneration of 1-methyluracil from 1-methyl-5-hydro-6-hydroxyuracil in alkaline methanolic medium, 0.01 N-NaOH at 21°: (*a*), Absorption spectrum of 1-methyluracil in aqueous medium prior to irradiation; (*b*), spectrum of photohydrate in methanol; (*c*) and (*d*) are spectra in methanol 3 and 5 min., respectively, after addition of NaOH.

which is unaltered over a tenfold concentration range, 10^{-4} M to 10^{-3} M, and is also the same in light and heavy water, a rather unexpected finding (but see Discussion).

Acidification of the photoproduct of 1,6-dimethyluracil to 0.01 N-HCl at room temperature instantaneously regenerates the absorption spectrum of the parent substance; but regeneration is not quantitative, only 80% of the starting compound being reformed. Heating the photoproduct at neutral pH at 80° for 1 hr. likewise regenerates only 80% of the starting compound. The identity of the regenerated compound with the original was verified by paper chromatography (R_F 0.70 in water-saturated *sec.*-butanol) and by spectral analysis at various pH values. At neutral pH the rate constant at 76° for elimination of an ordinary water molecule in H_2O is 5.0×10^{-4} sec.$^{-1}$, and for elimination of a heavy water molecule in D_2O is 2.6×10^{-4} sec.$^{-1}$, so that the isotope effect is about 2.0. This is again rather surprising in that the photohydrates of uridine and 1-methyluracil eliminate a heavy water molecule in neutral D_2O more rapidly [15]. Notwithstanding these isotope effect anomalies, it is our opinion that the overall results, together with

the analogous behaviour of the photohydrates of 1,6-dimethyluracil and uridine in alkaline methanol (see next section), indicate that the photoproduct of 1,6-dimethyluracil is the hydrate, i.e. 1,6-dimethyl-5-hydro-6-hydroxyuracil.

When the photoproduct of 1,6-dimethyluracil, exhibiting only end absorption in the quartz ultraviolet (Fig. 7) is brought to pH 11.8 - 12.0 by addition of NH_4OH, there results immediate formation (both at room temperature or at 3°) of a characteristic maximum at 290 mμ with an extinction of about 10^4 (Fig. 7). Subsequently the height of this maximum decreases; simultaneously the extinction increases at 260 - 270 mμ to give, finally, the spectrum of 1,6-dimethyluracil. Note from Fig. 7 that the various curves intersect at an isosbestic point at 270 mμ. Spectral analysis of curve *c* in Fig. 7 demonstrated that regeneration of 1,6-dimethyluracil at alkaline pH was quantitative.

Table 2

Rate constants and energy of activation for disappearance of alkaline transformation product of 1,6-dimethyl-5-hydro-6-hydroxyuracil (disappearance of absorption maximum at 290 mμ), to regenerate 1,6-dimethyluracil, in aqueous ammonia pH *12*

t°C	T°K	k_r (sec.$^{-1}$)	E (kcal./M)
3.6	276.6	1.3×10^{-4}	
16.5	289.5	3.9×10^{-4}	17.8
23.0	296.0	8.1×10^{-4}	
27.6	300.6	13.4×10^{-4}	

The rate of disappearance of the peak at 290 mμ in alkali was found to follow a first-order reaction (Table 2) with an activation energy of 17.8 kcal./mole. The rate was found to be independent of concentration over the range 10^{-4} - 10^{-3} M, but exhibited a rather high isotope effect in light and heavy water,

$$\frac{k_r(H_2O)}{k_r(D_2O)} = \frac{9.36 \times 10^{-4}\ \text{sec.}^{-1}}{2.06 \times 10^{-4}\ \text{sec.}^{-1}} = 4.56.$$

Table 3

Rate constants and energy of activation for acid-catalysed regeneration of 1,6-dimethyluracil from alkali treated 1,6-dimethyl-5-hydro-6-hydroxyuracil

Solution of 1,6-dimethyl-5-hydro-6-hydroxyuracil brought to pH 12 with ammonia, and concentrated HCl added to bring to pH 1; solution then heated and extinction increase at 265 mμ followed.

t°C	T°K	k_r (sec.$^{-1}$)	E (kcal./M)
38.8	311.8	2.4×10^{-4}	
43.8	316.8	3.81×10^{-4}	15.0
50.0	323.0	5.35×10^{-4}	

When the photoproduct of 1,6-dimethyluracil is brought to alkaline pH with NaOH or KOH, the resulting transformations to the starting compound are similar to those described above; but regeneration of the starting compound is not quantitative and attains at most 75%.

When the compound with an absorption band at 290 mμ is acidified, the 290 mμ peak disappears. But subsequent alkalization restores the 290 mμ band. If the product is brought to about pH 1 and then warmed at 40 - 50° for about 2 hr., it reverts to 1,6-dimethyluracil; the kinetics for this reaction are presented in Table 3. This, of course, constitutes further evidence that the product with a 290 mμ maximum is no longer the primary photoproduct.

Reaction of photohydrates of uracil analogues in alkaline methanolic medium

An examination of the reactions undergone by the various photohydrates in alkaline methanolic medium provided additional data which proved useful in the interpretation of the reactions in aqueous alkaline medium.

These reactions were carried out in the following manner:

(1), A given compound was irradiated at a concentration of 10^{-3} M in a 1-mm. cuvette until photohydration was complete (disappearance of characteristic absorption maximum).

(2), The photoproduct solution was transferred to a 10-mm. cuvette and evaporated to dryness in a stream of cold air.

(3), The dried photoproduct was then dissolved in freshly distilled methanol, the resultant concentration being 10^{-4} M, and the cuvette stoppered. Aqueous 10 N-NaOH was then added to give a final concentration of 0.01 N-NaOH, and the resulting spectral modifications recorded, as follows.

Table 4

Rate constants and energy of activation for disappearance of alkaline transformation product of 1,6-dimethyl-5-hydro-6-hydroxyuracil (disappearance of absorption maximum at 290 mμ), to regenerate 1,6-dimethyluracil, in methanolic medium containing 0.01 N-NaOH

t°C	T°K	k_r (sec.$^{-1}$)	E (kcal./M)
42.5	315.5	2.8×10^{-4}	
47.8	320.8	4.2×10^{-4}	17.2
51.8	324.8	6.09×10^{-4}	

1-Methyl-5-hydro-6-hydroxyuracil. Dehydration of this photoproduct in methanol is appreciably slower than in aqueous 0.01 N-NaOH, so that the kinetics can be followed easily even at room temperature (Fig. 8), and there is no evidence from the spectral changes of any other reaction. At 0° the rate constants for dehydration in alkaline aqueous and methanolic media are, respectively, 2.47×10^{-3} sec.$^{-1}$ and 0.9×10^{-3} sec.$^{-1}$.

In alkaline *sec.*-butanol the dehydration reaction is slowed down still further, so that rates are measurable at temperatures of 35 - 40°. Here again there was no evidence of any reaction other than direct dehydration.

1,6-Dimethyl-5-hydro-6-hydroxyuracil. The behaviour of this photoproduct in alkaline methanol is identical with that in alkaline aqueous medium. Even at 0° there was no evidence of direct dehydration, but only instantaneous formation of a product with a peak at 290 mμ immediately following addition of alkali (cf. Fig. 7). However, this product (i.e. the product with a maximum at 290 mμ) was much more stable in alkaline methanol (cf. Tables 2 and 4). When the methanol solution was acidified with HCl this peak disappeared but was immediately restored on readdition of alkali.

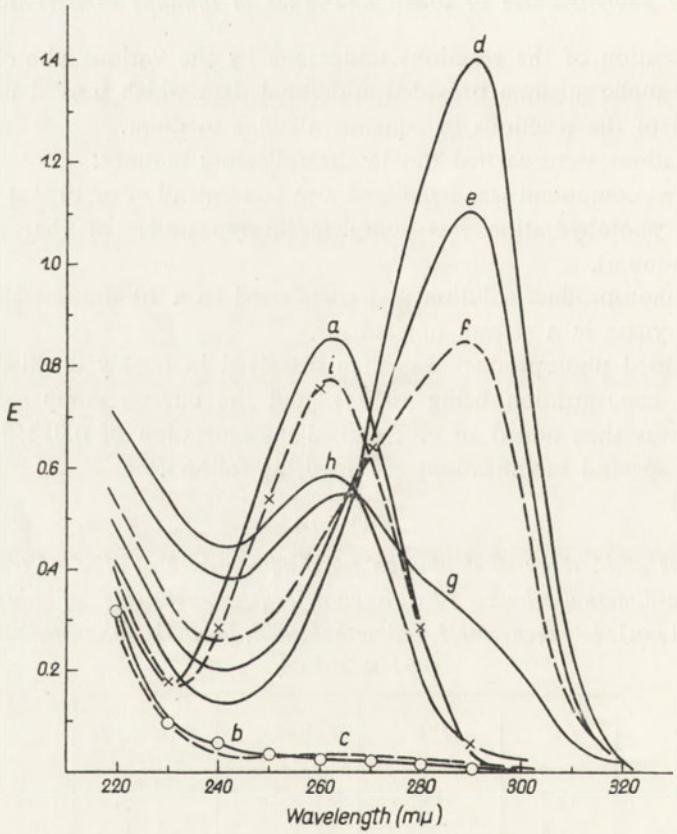


Fig. 9. Transformation of uridine photohydrate in alkaline methanol at room temperature leading to almost quantitative regeneration of uridine: (*a*), Absorption spectrum of initial aqueous solution of uridine, 10^{-3} M in 1-mm. cuvette at neutral pH; (*b*), spectrum of photohydrate following irradiation; (*c*), spectrum of photohydrate in methanol (10^{-4} M in 10-mm. cuvette); (*d*), spectrum 90 sec. after addition of alkali to concn. of 10^{-2} M; (*e*), (*f*) and (*g*) are spectra 18, 38 and 115 min, respectively, after addition of alkali to concn. of 10^{-2} M; (*h*), spectrum 18 hr. after addition of alkali to concn. of 10^{-2} M; (*i*), spectrum following addition of HCl to about 0.01 N. (Note: although extinction of curve *i* is about 8% below that of curve *a*, regeneration of original extinction is nearly quantitative when all manipulation errors are taken into account).

5-Hydro-6-hydroxyuridine. It should be recalled that in aqueous ammoniacal medium the behaviour of the photohydrate of uridine is intermediate between that of 1-methyluracil and 1,6-dimethyluracil, i.e. it undergoes simultaneously two reactions: *direct* dehydration, and formation of a product with an absorption maximum at 290 mμ which in turn reverts to uridine. In alkaline methanol, on the other hand, neither ionization of the photoproduct nor direct dehydration can be observed. From Fig. 9 it will be noted that uridine photohydrate in alkaline methanol reacts identically like the photohydrate of 1,6-dimethyluracil, i.e. forms a product with a maximum at 290 mμ which then reverts to uridine (see also Table 5). Regeneration of uridine at the end of the reaction is over 90% and is, in fact, probably quantitative when all manipulation errors are taken into account.

Table 5

Rate constants and energy of activation for disappearance of alkaline transformation product of uridine photohydrate (disappearance of absorption maximum at 290 mμ), to regenerate uridine, in methanol containing 0.01 N-NaOH *(2.5 μl. 10* N-NaOH *in 2.5 ml. methanol)*

t°C	T°K	k_T (sec.$^{-1}$)	E (kcal./M)
21.0	294.0	1.98×10^{-4}	
27.6	300.6	4.00×10^{-4}	16.1
33.2	306.2	6.02×10^{-4}	

The following experiment demonstrated that the reaction sequences in alkaline methanol involve participation of water, and that differences in behaviour from that in aqueous medium are due to the change in medium from water to methanol: 15 ml. of 10^{-3} M solution of photohydrate was freeze-dried and then dissolved in 0.15 ml. methanol containing 0.01 N-NaOH. The concentration of photoproduct in this solution was 0.1 M and the ratio of water (from the added NaOH solution) to photoproduct was 1000-fold lower than in the experiments outlined above. Under these conditions the photohydrates were fully stable and did not undergo any of the transformations described above.

DISCUSSION

It is clear from the overall results that alkalization of the photohydrates of 1-methyluracil, 1,3-dimethyluracil and 1,3,6-trimethyluracil leads to the rapid base-catalysed *direct* elimination of the water adduct, with quantitative regeneration of the parent compounds.

For 1,6-dimethyluracil and the various glycosides of uracil, the reactions are more complex. Before discussing these in detail, there are two points to which attention should be directed.

(a), The behaviour in alkali of all the uracil glycoside photoproducts is the same, irrespective of whether the carbohydrate moiety is pentose or hexose, or

whether the sugar hydroxyls are free or blocked. Consequently the difference in behaviour in alkali between the photoproducts of 1-methyluracil on the one hand, and the uracil glycosides on the other, is probably due to some influence of the glycosidic linkage. It is certainly not due to any interaction between the aglycon and carbohydrate hydroxyls, as in the case of cytosine glycosides where the rates of photohydration and subsequent dark dehydration are both influenced by the nature of the carbohydrate component [22]. A study of the behaviour of higher 1-alkyluracils may conceivably help to clarify the difference in behaviour between 1-methyluracil and uracil glycosides; we have, in fact, carried out preliminary experiments with 1-ethyluracil and found that its photohydrate undergoes in alkali (both in aqueous and methanolic media) only *direct* dehydration, at a rate much slower than that for 1-methyluracil, but with no evidence for formation of a product with an absorption maximum at 290 mμ.

(b), The alkaline transformations of the photohydrates of uridine (and all the other uracil glycosides), as well as of 1,6-dimethyluracil, are obviously linked with the dissociation of the N_3 proton. When this position is blocked, as in *N*-methyluridine or in 1,3,6-trimethyluracil, only direct base-catalysed dehydration occurs, rapidly.

Reverting now to the photohydrate of uridine, i.e. 5-hydro-6-hydroxyuridine, the spectral changes resulting from the alkalization of this photoproduct (Fig. 2) demonstrate that the ionized photoproduct with a maximum at 230 mμ (I) is partially or wholly transformed first to another product with an absorption maximum at 290 mμ. The location of this maximum and its high molar extinction (cf. Fig. 9) must, of necessity, be due to the appearance of an additional conjugated double bond resulting from elimination of the water adduct. But this must be preceded by opening of the pyrimidine ring, for otherwise we would observe only the direct regeneration of uridine with a E_{max} at 262 mμ. The resulting open chain compound, following elimination of the water molecule, in turn undergoes ring closure to regenerate the parent uridine.

As regards 1,6-dimethyluracil, there is very little doubt but that its photoproduct is also the hydrate, i.e. 1,6-dimethyl-5-hydro-6-hydroxyuracil. Photoproduct formation is first order, with a rate unchanged over a 10-fold concentration range; the photoproduct exhibits only end absorption in the ultraviolet, and reverses to the parent compound in neutral and acid medium directly, and in alkaline medium partly, like uridine. It is somewhat surprising that photohydration of 1,6-dimethyluracil does not exhibit the expected isotope effect in heavy water [15]; but it should be recalled that photohydration of 5-fluorouracil analogues is accompanied by only a small isotope effect [3]. It is even more surprising that the isotope effect for the dehydration reaction of the photohydrate of 1,6-dimethyluracil is so different from that of other uracil analogues, but this is obviously a problem which requires additional study; our data are inadequate to clarify this anomaly.

While the ionized photoproduct of 1,6-dimethyluracil is extremely labile in alkaline medium, in that it is transformed very rapidly to the product with an absorption maximum at 290 mμ, the subsequent ring closure reaction is identical to that described for uridine. The reaction is first order, exhibits a high isotope

effect in heavy water, and the energy of activation is similar to that for uridine (cf. Tables 1 and 2). The ring closure reaction may also be observed in acid medium; this reaction is easily differentiated from the *direct* acid-catalysed dehydration of the photohydrate of 1,6-dimethyluracil, which is very rapid; some kinetic data for the ring closure reaction in acid medium are presented in Table 3.

The overall results may be summarized as follows.

(a), For the photohydrate of 1-methyluracil the base-catalysed dehydration reaction is rapid and ring opening does not occur.

(b), For the photohydrate of 1,6-dimethyluracil, in alkaline aqueous or methanolic media, ring opening first occurs, followed by dehydration, and finally ring closure to regenerate 1,6-dimethyluracil.

(c), In alkaline methanolic medium the photohydrate of uridine undergoes the same reaction sequence as 1,6-dimethyluracil.

But in aqueous alkaline medium uridine photohydrate undergoes three reactions simultaneously: (1) direct base-catalysed dehydration; (2) ring opening and dehydration, followed by ring closure; (3) ring opening, probably at the 3,4 bond,

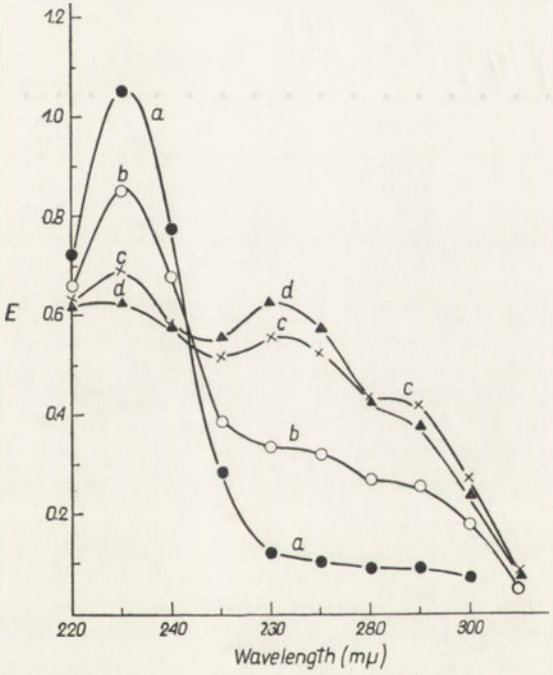
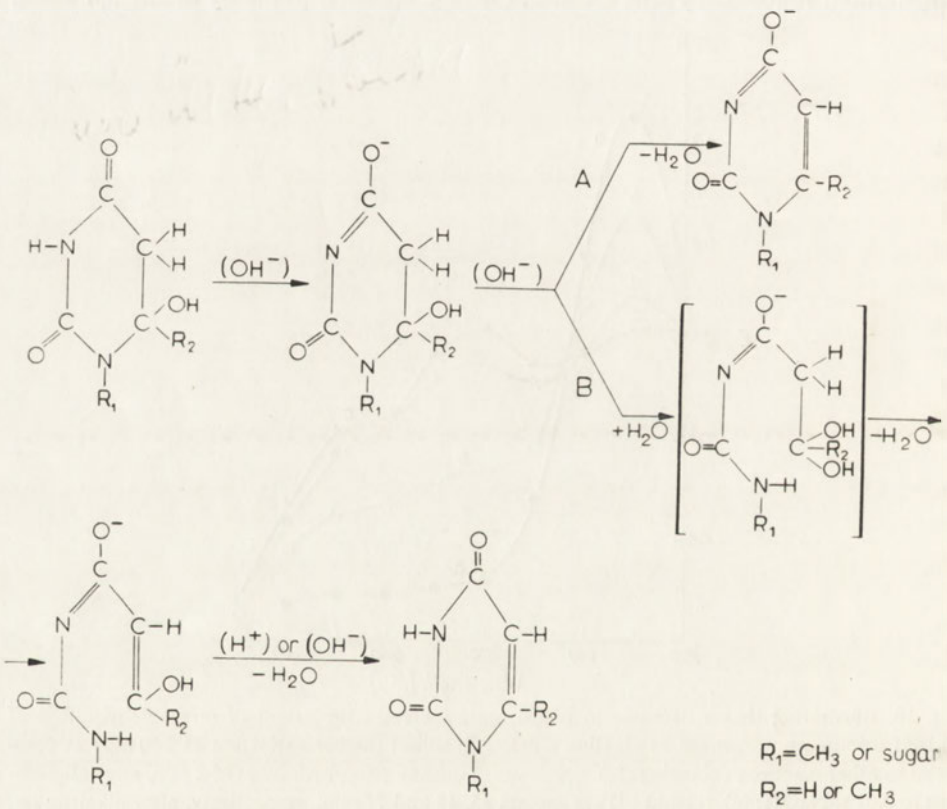


Fig. 10. Illustrating that a decrease in temperature increases the extent of direct dehydration of ionized uridine photohydrate in alkaline aqueous medium (higher extinction at 260 mμ) as compared to other reactions occurring: (*a*), Spectrum of uridine photohydrate at 0° 2 min. after alkalization to 0.01 N-NaOH; (*b*), (*c*) and (*d*) are spectra 15, 48 and 77 min., respectively, after alkalization.

to give a product which does not revert to uridine. The extent to which reactions (1) and (2) predominate may be modified by changing the temperature; this is shown in Fig. 10, where a decrease in temperature decreases the extent of ring opening more than that of direct dehydration.

It remains to establish which bond in the photohydrated uracil ring undergoes scission in the reaction sequence leading to regeneration of the ring. Four possibilities may be envisaged, viz. N_1-C_2, N_3-C_2, N_3-C_4 and N_1-C_6. The first two of these may be excluded on theoretical grounds in that they would lead to formation of the extremely labile groupings $>N-C\begin{matrix}OH\\O\end{matrix}$, which are unknown and the derivatives of which are very unstable, particularly in acid medium; whereas the product of ring opening and dehydration of the photohydrate of 1,6-dimethyluracil is stable even at pH 1. Ring opening at N_3-C_2 may also be excluded from spectral considerations, since the resulting chain compound could not possibly contain three conjugated double bonds. The same argument may be used to exclude the N_3-C_4 bond, and this is supported by the fact that the products with an absorption maximum at 290 mμ do not give the Fink reaction with *p*-dimethylaminobenzaldehyde.



Scheme 2

On the basis of the experimental data and the foregoing considerations, the two reaction sequences for the alkaline transformations of uracil analogue photohydrates to the parent compounds may be represented as shown in scheme 2.

Reaction path A is that observed for the dehydration of 1-methyl-5-hydro-6-hydroxyuracil and is also one of the transformation pathways of 5-hydro-6-hydroxyuridine to regenerate uridine.

Reaction sequence B is that observed for the transformation of 1,6-dimethyl-5-hydro-6-hydroxyuracil to 1,6-dimethyluracil and is one of the transformation pathways whereby 5-hydro-6-hydroxyuridine is converted to uridine.

It remains to emphasize that the above two reaction sequences lead to the quantitative regeneration of uracil glycosides from their photohydrates *only* in ammoniacal medium. This should be of practical importance in experiments on the alkaline photodissociation of uracil glycoside photodimers. Why the presence of sodium or potassium cations leads to partial opening of the 3,4 bond, with the consequent reduction in the amount of uracil glycoside regenerated, is still to be clarified. Finally, it should be emphasized that these results will undoubtedly prove of value in pyrimidine chemistry, since many syntheses of the pyrimidine ring are based on ring closure to form the 1,6 bond.

REFERENCES

[1] Batt R. D., Martin J. K., Ploeser J. M. & Murray J. - *J. Amer. Chem. Soc.* **76**, 3663, 1954.
[2] Cohn W. E. & Doherty D. G. - *J. Amer. Chem. Soc.* **78**, 2863, 1956.
[3] Fikus M., Wierzchowski K. L. & Shugar D. - *Photochem. Photobiol.* **4**, 521, 1965.
[4] Fink R. M., Cline R. E., McGaughey C. & Fink K. - *Anal. Chem.* **28**, 4, 1956.
[5] Fox J. J., Yung N., Davoll J., Brown G. B. - *J. Amer. Chem. Soc.* **78**, 2117, 1956.
[6] Green M. & Cohen S. S. - *J. Biol. Chem.* **225**, 397, 1957.
[7] Hilbert G. E. & Johnson T. B. - *J. Amer. Chem. Soc.* **52**, 2001, 1930.
[8] Hilbert G. E. & Johnson T. B. - *J. Amer. Chem. Soc.* **52**, 4489, 1930.
[9] Janion C. & Shugar D. - *Acta Biochim. Polon.* **7**, 309, 1960.
[10] McLaren A. D. & Shugar D., *Photochemistry of Proteins and Nucleic Acids*, Pergamon Press, Oxford 1964.
[11] McOmie J. F. W., Sayer E. R. & Chesterfield J. - *J. Chem. Soc.* 1830, 1957.
[12] Rapport D., Canzanelli A. & Sossen R., - *Fed. Proc.* **14**, 386, 1955.
[13] Scannell J. P., Crestfield A. M. & Allen F. W. - *Biochim. Biophys. Acta* **32**, 406, 1959.
[14] Schuster H. - *Z. Naturf.* 815, 1964.
[15] Shugar D. & Wierzchowski K. L. - *Biochim. Biophys. Acta* **23**, 657, 1957.
[16] Sinsheimer R. L. - *Radiat. Res.* **1**, 505, 1954.
[17] Szer W. & Shugar D. - *Acta Biochim. Polon.* **7**, 491, 1960.
[18] Sztumpf E. & Shugar D. - *Biochim. Biophys. Acta* **61**, 555, 1962.
[19] Śmietanowska A. & Shugar D. - *Bull. Acad. Pol. Sci. Ser. Biol.* **9**, 375, 1961.
[20] Visser D. W., Barron G. & Beltz R. - *J. Amer. Chem. Soc.* **75**, 2017, 1953.
[21] Wierzchowski K. L., Litońska E. & Shugar D. - *J. Amer. Chem. Soc.* **87**, 4621, 1965.
[22] Wierzchowski K. L. & Shugar D. - *Biochim. Biophys. Acta* **25**, 355, 1957.
[23] Wierzchowski K. L. & Shugar D. - *Acta Biochim. Polon.* **6**, 313, 1959.
[24] Wierzchowski K. L. & Shugar D. - *Acta Biochim. Polon.* **7**, 63, 1960.

PRZEKSZTAŁCENIA NIEKTÓRYCH FOTOCHEMICZNIE UWODNIONYCH 2,4-DWUKETOPIRYMIDYN I ICH GLIKOZYDÓW W ŚRODOWISKU ALKALICZNYM

Streszczenie

1. W wodnym, alkalicznym środowisku produkt fotolitycznego uwodnienia 1-metylouracylu ulega reakcji odwodnienia katalizowanej zasadami, z utworzeniem ze 100% wydajnością 1-metylouracylu.

2. 1,6-Dwumetylo-5-hydro-6-hydroksyuracyl w roztworze wodnym, alkalizowanym amoniakiem, ulega reakcji hydrolitycznego pęknięcia wiązania 1,6. Z tak powstałego produktu następuje wydzielenie wody i zamknięcie pierścienia. W wyniku tych przekształceń uzyskuje się, ze 100% wydajnością, 1,6-dwumetylouracyl. W obecności NaOH lub KOH reakcje te przebiegają z niższą wydajnością (ok. 75%).

3. W wodnym amoniakalnym środowisku uwodniona fotolitycznie urydyna (oraz inne glikozydy uracylu) ulega dwom, przebiegającym jednocześnie, konkurującym reakcjom: (a), katalizowanemu zasadami wydzieleniu wody i utworzeniu urydyny oraz (b), reakcji otwarcia pierścienia wskutek pęknięcia wiązania 1,6. Produkt otwarcia pierścienia ulega z kolei odwodnieniu i ponownemu zamknięciu pierścienia. W wyniku tych przekształceń tworzy się również urydyna. W środowisku alkalicznym uzyskanym przez dodanie NaOH lub KOH pojawia się trzecia reakcja, polegająca prawdopodobnie na otwarciu pierścienia fotoproduktu w wiązaniu 3,4. Reakcja ta nie prowadzi do utworzenia urydyny. Dodanie wodnego roztworu amoniaku już po alkalizacji roztworu fotoproduktu przy pomocy NaOH lub KOH przesuwa równowagę między omawianymi procesami w kierunku ilościowego odtworzenia urydyny z jej fotoproduktu.

4. W alkalizowanym roztworze metanolu uwodnione 1,6-dwumetylouracyl i glikozydy uracylu ulegają jedynie reakcji otwarcia wiązania 1,6 i następującym po nim odwodnieniu i zamknięciu pierścienia. Warunkiem przebiegu tych reakcji jest obecność śladowych ilości wody.

5. O różnicach w reakcjach, którym ulegają w środowisku alkalicznym uwodnione: 1-metylouracyl z jednej, a glikozydy uracylu z drugiej strony decyduje prawdopodobnie wiązanie glikozydowe, nie zaś udział w tych reakcjach grup wodorotlenowych cukru.

6. Zbadano kinetykę i energię aktywacji niektórych z omówionych reakcji. Wykonano również pomiary efektów izotopowych, jednakże wyniki te wymagają dalszych uzupełnień.

7. Autorzy pragną zwrócić uwagę na znaczenie opisanych wyników w syntezie pirymidyn oraz wynikające z nich możliwości odwrócenia uwodnienia uracylu w naświetlanym promieniowaniem nadfioletowym kwasie poliurydylowym oraz kwasach rybonukleinowych.

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WANDA MEJBAUM-KATZENELLENBOGEN and ZOFIA KUDREWICZ-HUBICKA

APPLICATION OF UREA, FERRIC AMMONIUM SULPHATE AND CASEIN FOR DETERMINATION OF TANNING SUBSTANCES IN PLANTS

Department of Biochemistry, University, and Department of Physiological Chemistry, Faculty of Pharmacy, Medical School, Wrocław

1. In plants, tannins are present in the form of insoluble complexes with protein or alkaloids, which are soluble in urea solution. 2. Ferric ammonium sulphate gives a violet colour with gallic acid derivatives and a green colour with catechin derivatives. This reaction in the presence of acetate ion, urea and a protective colloid, was utilized for colorimetric determination of 40 - 400 μg. of tannins in a 5 ml. sample. 3. From protein-free water extracts, tannins can be precipitated by casein, and determined colorimetrically after being dissolved in urea. 4. Paper electrophoresis facilitates identification of tannins and their derivatives.

It has been demonstrated earlier [6] that pentadigalloyloglucose (chinese tannin) forms insoluble compounds with protein and alkaloids, depending upon the concentration of both reactants and the pH of the medium. The complexes with protein are formed when the pH of the medium is lower than the P. I. of the protein, and at the optimum pH for complex formation the weight ratio of protein to tannin should be 1:1. From protein-tannin complexes the protein can be regenerated with caffeine according to the following scheme:

$$\text{Protein} + \text{tannin} \rightarrow \text{Protein-tannin}$$

$$\text{Protein-tannin} + \text{caffeine} \rightarrow \text{Caffeine-tannin} + \text{protein}$$

Insoluble protein-tannin and caffeine-tannin complexes do not form, or become dissociated, in the presence of urea, acetone, alcohol, or in an alkaline medium.

In plants the tannins are presumably bound with protein or alkaloid and only the excess of one of the compounds can be extracted with water. When alkaline extraction is applied, the protein-tannin complexes are dissociated, and the protein passes to the solution, but at the same time the tannins are partially oxidized to compounds possessing no tanning properties. Acetone and alcohol, although they are good solvents for tannins, cause precipitation of protein. It was found that successful extraction can be obtained with urea solution. Urea releases the tannins

from complexes with protein or alkaloids without affecting their tanning properties, and both tannin and protein pass to the solution.

In 1905 Stiasny has applied ferric ammonium sulphate for determination of tannins and their derivatives in plants. In the previous work [6] this reaction was applied for colorimetric determination of 40 - 400 μg. of gallic acid or tannin. It was demonstrated that the addition of urea does not affect the sensitivity of the reaction, and moreover it makes possible the determination of tannins and other polyhydroxyphenols in the presence of protein. In the present work an attempt was made to apply ferric ammonium sulphate for determination of dihydroxyphenols and catechin tannin, and of trihydroxyphenols and gallotannins.

Determination of tannins in the presence of non-tanning phenol derivatives requires their isolation. For isolation of the tanning substances, powder from specially processed hide is commonly used. In this work, hide was replaced by casein which at pH 4.7 is insoluble and has the best binding properties. Complexes of casein with tannins were dissolved in urea and the tannins were determined colorimetrically by the reaction with the ferric-ammonium reagent, without removing the protein. Instead of casein, plant proteins were also used.

The procedure elaborated on standard compounds was applied both to plant material rich in tanning substances (bark of trees) and to material rich in protein and fat (seeds). Moreover, the tanning properties and purity of some commercial preparations of tannin were determined.

MATERIAL AND METHODS

The following compounds were used as standards: from the group of galloyl compounds, chinese tannin (pentadigalloyloglucose), gallic acid (Ce-Farm. Kraków), and pyrogallol (F. O. Ch. Gliwice, Poland); and from the group of catechin derivatives, pyrocatechol (U.S.S.R. product).

Pyrogallol and pyrocatechol were purified by sublimation. Commercial preparations of tannin (products of Politechnika Śląska, Gliwice, Poland, and Mallinckrodt U.S.P. XII, St. Louis, Mo., U.S.A.) were purified by extraction to ethyl acetate according to Armitage *et al.* [1]. A 10% solution of tannin in 0.5 M-Na_2HPO_4 was adjusted to pH 6.8 with 0.5 N-NaOH and extracted five times to half its volume of ethyl acetate. The extracts were pooled, washed several times with small portions of phosphate buffer and filtered. The filtrate was evaporated to dryness under reduced pressure on a water bath at 35°.

All the standards were dried and stored in a vacuum desiccator over $CaCl_2$.

For precipitation of the tanning substances, fat-free casein (Inw. Sp. Chem. Pokój, Kraków, Poland) was used.

Plant material: bark of oak (*Quercus robur*) and willow (*Salix alba*) were commercial products (Herbapol, Kutno, Poland); bark of catechu (*Acacia catechu*) and quebracho were obtained from the collection of the Department of Pharmacognosis of the Medical School in Wrocław, through courtesy of Prof. Dr. Tadeusz Bodalski. The seeds of cultivated plants were obtained from the Department of

Biology and Storage of Seeds of the Institute of Cultivation and Acclimatization of Plants in Wrocław.

Protein was determined by the micromethod of Mejbaum-Katzenellenbogen [5]. The urea extracts were diluted to a concentration of urea lower than 1% because higher concentration interfered with the determination of protein.

The content of glucose in tannin was determined by the anthrone method according to Björnesjö [3].

Paper chromatography was carried out according to Barton, Evans & Gardner [2]. Paper electrophoresis was carried out on Whatman no. 1 paper (4×28 cm.) in 0.1 M-acetate buffer, pH 4.7; time 4 hr., potential 200 v. A sample of 0.02 - 0.05 ml. of a solution containing 100 - 500 mg. of the polyhydroxyphenol studied was placed on the middle of the strip.

RESULTS

Determination of tannins and polyhydroxyphenols by the ferric-ammonium reagent

In the previous work [6] determinations of gallic acid and tannin have been made using the ferric-ammonium reagent containing 0.05% ferric ammonium sulphate and 0.1% arabic gum in 1 M-acetate buffer, pH 4.7. Gallic acid and other trihydroxyphenols gave violet compounds with an absorption maximum at 550 mμ. Changes in the concentration of the buffer and addition of urea did not affect the intensity of the colour and absorption spectra.

With pyrocatechol this reagent gave a green colour, with a maximum of absorption at 700 mμ. The extinction at 700 mμ for equal amounts of pyrocatechol and pyrogallol was always higher for pyrogallol despite the fact that the maximum of absorption for gallic acid derivatives is at 550 mμ. Therefore attempts were made to modify the composition of the reagent so as to obtain equal extinctions for trihydroxy- and dihydroxyphenols corresponding to gallic and catechin tannins.

It was found that lowering of the concentration of the acetate ion from 1 M to 0.1 M results in intensification of the green colour of pyrocatechol, and a further increase in the sensitivity of the reaction was attained by introducing urea (Fig. 1). The extinctions were proportional to the amount of pyrocatechol at each concentration of urea studied, and the presence of 50% urea resulted in a twofold increase of extinction. By applying the colour reagent containing 0.1 M-acetate buffer and 50% of urea, it was possible to obtain equal extinctions at 680 mμ for both groups of standard substances.

The absorption spectra at 500 - 700 mμ for pyrogallol and pyrocatechol are shown in Fig. 2. The minimum of absorption of the pyrocatechol reaction product and the maximum for that of pyrogallol are both at 550 mμ and the two curves intersect at 680 mμ. Using the modified ferric-ammonium reagent, in spite of differences in the colour formed, the same extinction at 680 mμ was obtained both for pyrogallol and pyrocatechol when these were applied in equal amounts over the range

of 40 - 400 μg. per sample. In Fig. 3 the standard curve for pyrogallol and pyrocatechol is given. The readings were made in a Coleman Junior spectrophotometer at 680 mμ and Pulfrich photocolorimeter with the S/66 filter. Lower extinctions

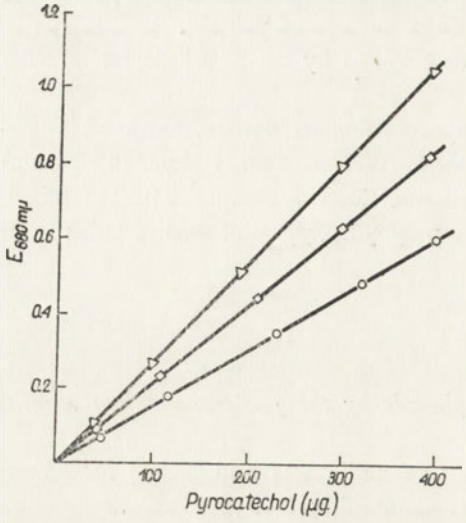

Fig. 1. The effect of urea on the colour intensity of the reaction product of pyrocatechol and ferric-ammonium sulphate. (○), Without urea; (□), with 20% urea; (△), with 50% urea solution.

for catechin derivatives and higher for gallic acid derivatives were obtained with other red filters, e.g. in the photocolorimeter KF-II (produced by Warszawskie Zakłady Aparatury Laboratoryjnej, Poland).

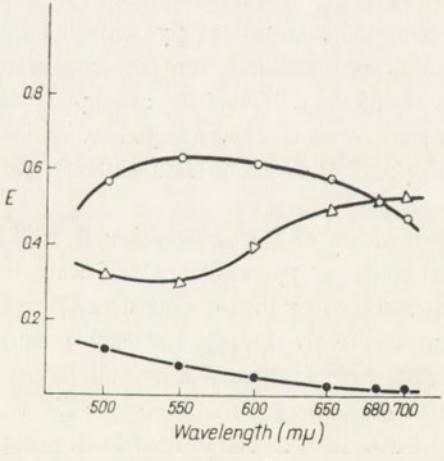

Fig. 2

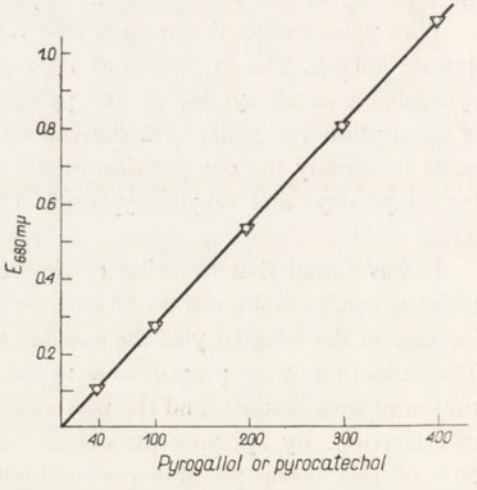

Fig. 3

Fig. 2. Absorption spectra of the coloured derivatives of (△), pyrocatechol; (○), pyrogallol; and (●), the reagent blank. To 1 ml. of a solution containing 200 μg. of the standard, or 1 ml. of water, 4 ml. of the modified ferric-ammonium reagent was added and the extinction was read in the Coleman Junior spectrophotometer in tubes 12 mm. in diameter.

Fig. 3. Standard curve for pyrogallol and pyrocatechol with the modified ferric-ammonium reagent. To 1 ml. of the sample, 4 ml. of the reagent was added and extinction was read in the Coleman Junior spectrophotometer in tubes 12 mm. in diameter.

The results presented in Table 1 illustrate the reproducibility and sensitivity of the method. The average extinction calculated per 200 μg. for the three standard compounds studied was 0.52, the error of the method being 4 - 6%.

Table 1

Reproducibility and sensitivity of the method for determination of polyhydroxyphenols by the modified ferric-ammonium reagent

Extinctions for different concentrations of standard substances ranging from 40 to 400 μg./ml. were read in the Coleman Junior spectrophotometer at 680 mμ and calculated per 200 μg. Mean values ± S. D. are given.

Standard	No. of determ.	$E_{680\ m\mu}$
Pyrogallol	50	0.52 ± 0.02
Pyrocatechol	50	0.52 ± 0.02
Gallic acid	35	0.52 ± 0.03

The intensity of the colour of the reaction products of both groups of compounds depends on the pH value and ferric ammonium sulphate concentration. At pH values lower than 4.4 and higher than 5.2 the colour was distinctly changed and the extinction decreased. Changes in the concentration of ferric ammonium sulphate to below 0.025% or above 0.1% affect the sensitivity and proportionality of the method.

The modified ferric-ammonium reagent is prepared by mixing, just before use, 9 parts of 50% urea solution in 0.1 M-acetate buffer, pH 4.7, 1 part of 1% solution of arabic gum, and 0.1 part of 5% solution of ferric ammonium sulphate ($NH_4Fe(SO_4)_2 \cdot 12H_2O$).

For colorimetric determinations, to 1 ml. of the sample 4 ml. of the ferric-ammonium reagent are added, mixed, and the extinction read at 680 mμ against a reagent blank. The colour is stable up to two hours.

The standard solutions are prepared by boiling 400 mg. of pyrogallol (pyrocatechol, gallic acid or tannin) in a small amount of water, and after cooling adjusted to 100 ml. Before use, appropriate dilutions are done.

Paper chromatography and electrophoresis of polyhydroxyphenols and tannins

The polyhydroxyphenols were detected on the chromatograms and electrophoretograms by spraying with the modified ferric-ammonium reagent. The mixture of ferric chloride and potassium ferricyanide used by Barton *et al.* [2] stained blue all polyhydroxyphenols; the ferric-ammonium reagent gives a violet colour with gallic acid derivatives and a green colour with catechin derivatives. The well visible difference of the two colours facilitates identification, and moreover the ferric-ammonium reagent gives no colour reaction with phenol, phenylalanine, resorcinol, phloroglucinol and vanilin.

The electrophoretograms of the standard substances run at pH 4.7 are shown in Fig. 4. Purified tannin remained at the point of application, gallic acid migrated

towards the anode and pyrogallol and pyrocatechol, both with the same velocity, towards the cathode. From the non-stained electrophoretograms the individual fractions were eluted with 50% solution of urea in 0.1 M-acetate buffer, pH 4.7, and after 1 hr. the amount of polyhydroxyphenol was determined. The recovery amounted to 96%.

Application of casein, caffeine and urea for determination of tannins in the presence of other polyhydroxyphenols

To 0.4% solution of standard substances, casein was added in tenfold weight ratio in relation to the standards used, and the pH was adjusted to 4.7. The mixture was ground in a porcelain mortar for 15 min., cooled in a refrigerator and centrifuged at 6000 rev./min. In the supernatant fluid the phenol derivatives not bound to protein were determined; the sediment, which contained casein and casein-bound tannins, was dissolved in 50% solution of urea in 0.1 M-acetate buffer, pH 4.7, and the amount of tannins was determined. It was found that under these conditions all purified tannin was bound to casein whereas gallic acid, pyrogallol and pyrocatechol remained in the supernatant. The electrophoretograms of tannin before and after precipitation with casein and extraction from the complex were identical, one band at the point of application being found.

When the purified tannin was precipitated by an equal or fivefold amount of caffeine, mixed for 15 min., then cooled to 2° and centrifuged, the supernatant fluid gave no reaction with the ferric-ammonium reagent indicating that the precipitation of tannin by caffeine was complete.

To examine the ability of plant proteins to bind tannin, 1 ml. of 1% tannin solution was added to 500 mg. of finely ground seeds of pea or lupine. The mixture was ground for 15 min. in a porcelain mortar and left for 1 hr. in the refrigerator, then adjusted to a volume of 10 ml. with water or 50% solution of urea in 0.1 M-acetate buffer, pH 4.7, and shaken mechanically for 1 hr. at room temperature. After centrifugation at 6000 rev./min., tannin was determined colorimetrically in the supernatant fluid. It was found that all the added tannin was bound to proteins of the seeds and could not be extracted with water, whereas with 50% urea solution it was extracted completely.

Analysis of commercial preparations of tannin

In the course of working on protein it has been observed that not all commercial preparations of tannin are suitable for determination and purification of protein. Therefore different preparations were analysed by paper electrophoresis and the content of sugar compounds was determined (Table 2). The results were compared with those obtained with a tannin preparation purified by ethyl acetate extraction. The electrophoretograms of the purified tannin and two commercial products are presented in Fig. 5. On the electrophoretograms of the tannin produced by Politechnika Śląska three fractions were obtained: two anodic ones, *F-1* cor-

responding to gallic acid and a non-identified *F-2*, and a third fraction (*F-3*) which remained at the point of application and corresponded to tannin (pentadigalloylglucose). The preparation of "aqueous tannin" contained an additional cathodic fraction (*F-4*) having the mobility of pyrogallol. The products of Mallinckrodt, Politechnika Śląska and Ce-Farm, Kraków (Table 2) contained 8.3 - 9.8% of glucose and 65 - 85% of the fraction *F-3* corresponding to tannin. The remainder consisted of fraction *F-1* having the mobility of gallic acid, and the non-identified fraction *F-2*. On the other hand, the preparation of Ce-Farm, Wrocław contained only 47% of the fraction *F-3* and as much as 41% of the fraction *F-2*; the content of glucose in the preparation was 4.7% but in relation to the tannin fraction its content amounted to 10%. "Aqueous tannin" supplied by B. O. Chem., Gliwice (the producer not stated) contained as much as 28% of glucose and separated into four fractions.

Table 2

Content of glucose and distribution of electrophoretic fractions in some commercial tannin preparations

Glucose was de termined with anthrone according to Björnesjö [3]. Paper electrophoresis on Whatman no. 1 paper was carried out in 0.1 M-acetate buffer, pH 4.7; 4 hr., 200 v.

No.	Tannin preparation	Glucose (%)	Distribution of fractions (%)			
			F-1	*F-2*	*F-3*	*F-4*
1	Hurt.Farm. Wrocław 793/61	4.7*	12	41	47	—
2	Aqueous tannin, Gliwice 299/53	28.5	4	11	72	13
3	Ce-Farm Kraków 1025/56	8.9	14	10	76	—
4	Mallinckrodt U. S. P. XII	9.8	15	9	76	—
5	Politechnika Śląska 9/To/59	8.3	8	7	85	—
6	Politechnika Śląska 9/To/60	8.9	11	7	82	—
7	Politechnika Śląska 1/3/61	9.3	11	8	81	—
8	Politechnika Śląska 7/62	9.1	9	6	85	—
9	Politechnika Śląska T/1/65	9.8	17	18	65	—
10	Purified by extraction to ethyl acetate	10.5	—	—	100	—

* Glucose formed 10 percent of the fraction *F-3* which corresponds to tannin.

The electrophoretograms shown in Fig. 6 illustrate the formation of insoluble complexes with casein by the individual fractions of commercial tannin (preparation of Politechnika Śląska). Fraction *F-1* did not bind with protein and after the addition of casein remained in solution, whereas *F-2* and *F-3* were completely precipitated by casein. Extraction with 50% urea solution at room temperature released fraction *F-3* (pentadigalloylglucose); on the other hand, fraction *F-2*, which also possessed tanning properties, could be released only by extraction with hot urea. This treatment, however, although it did not interfere with colorimetric determination of fraction *F-2*, resulted in its destruction, and on the electrophoretogram a diffuse pattern was obtained.

Figure 7 illustrates the binding of the fractions by caffeine. *F-3* was the only fraction that formed complexes with caffeine whereas not only fraction *F-1* but also fraction *F-2* remained in the supernatant fluid.

The ability of plant proteins to bind commercial tannin preparations was examined on pea and lupine seeds using the preparation of Politechnika Śląska T/1/65 (Table 2, no. 9). One ml. of 1% tannin solution was treated with 500 mg. of finely ground seeds. From the total colorimetrically determined polyhydroxyphenols, about 17% was extracted with water, this value corresponding to the content of fraction *F-1*; 82% of the material was extracted with urea at room temperature (fractions *F-1* and *F-3*) and the whole of the added material (including fraction *F-2*, 18%) by extraction with hot urea. In this experiment it appeared that seed proteins, similarly as casein, bind the tanning substances from the polyhydroxyphenol mixture.

Natural tannins

The worked-out procedure has been applied to determine the tanning substances in plant seeds and in the bark of trees. Protein and polyhydroxyphenols were determined in aqueous cold and hot urea extracts, and the results are summarized in Table 3.

Among the six kinds of seeds studied, only pea and lupine seeds did not contain phenol derivatives reacting with the ferric-ammonium reagent; in other seeds such substances were found to be present. The blue-violet colour of the reaction products of the lettuce-seed extract indicated the presence of gallic acid derivatives, the green colour in the clover-seed extract, the presence of catechin derivatives. Extracts from the celery and parsley seeds gave an intermediate colour which suggests the presence of both groups of polyhydroxyphenols. Seeds of the celery, lettuce and clover contained some non-tanning polyhydroxyphenols, as aqueous extracts contained protein as well. The seeds studied, except pea and lupine, contained urea-extractable compounds, more tanning substances being extracted with hot urea solution than with urea at room temperature.

The amount of gallotannins from willow and oak bark, and of mixed catechin-galloyl compounds from catechu and quebracho bark, extracted with urea solution was twice that extracted with water. However, no protein was found either in water or urea extracts.

The electrophoretograms of oak-bark extracts made with water, urea and acetone, are shown in Fig. 8. In all the extracts the same two fractions were found to be present.

The amount of tanning substances present in the bark was estimated in aqueous extracts by precipitation with casein (Fig. 9). In the willow-bark extract, 92% of substances giving a colour reaction with the ferric-ammonium reagent was precipitated by casein, whereas the fractions corresponding to gallic acid and pyrogallol remained in the supernatant; their amount was very small and on electrophoresis they gave weak bands. In the oak-bark aqueous extracts 95% of polyhydroxyphenols was precipitated by casein and the rest corresponded to gallic

Fig. 4

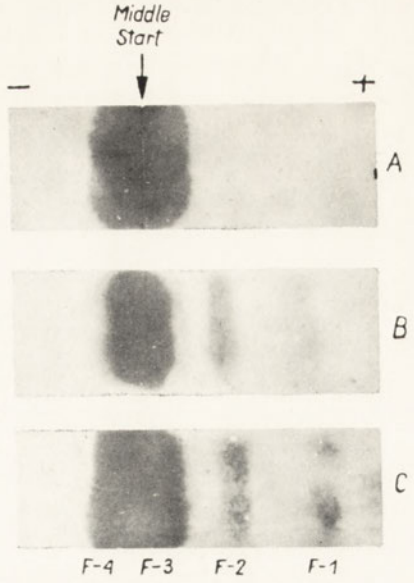

Fig. 5

Fig. 4. Paper electrophoresis of standard substances in 0.1 M-acetate buffer, pH 4.7. The polyhydroxyphenols were located with the modified ferric-ammonium reagent. (*A*), Gallic acid; (*B*), tannin; and (*C*), pyrogallol, violet colour; (*D*), pyrocatechol, green colour.

Fig. 5. Electrophoresis: (*A*), of tannin purified by extraction with ethyl acetate, and of commercial products: (*B*), of Politechnika Śląska; (*C*), "aqueous tannin".

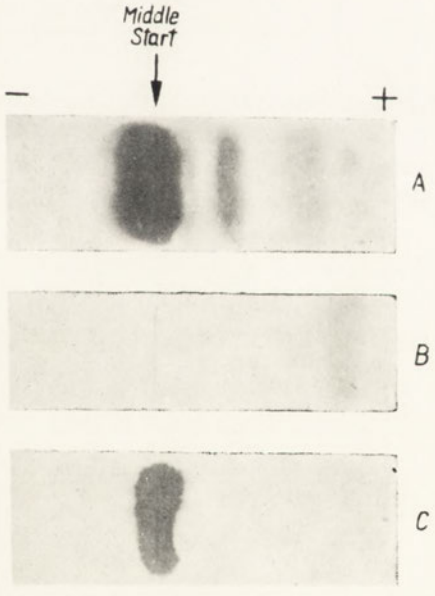

Fig. 6

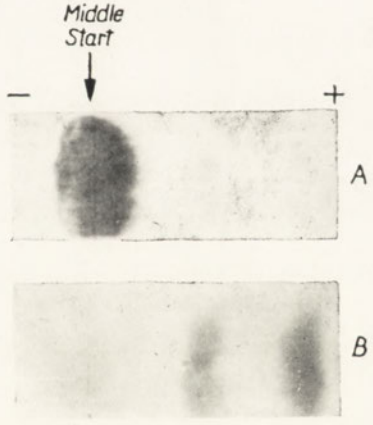

Fig. 7

Fig. 6. Binding of protein by the tannin preparation produced by Politechnika Śląska. (*A*), Starting solution; (*B*), supernatant fluid after precipitation of the tanning substances by casein; (*C*), urea extract of the precipitate.

Fig. 7. Binding of caffeine by the tannin preparation produced by Politechnika Śląska. (*A*), Star-

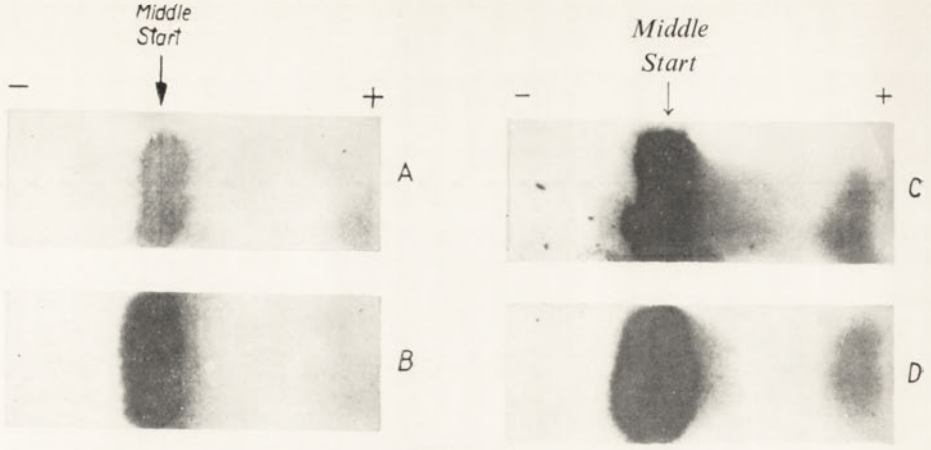

Fig. 8

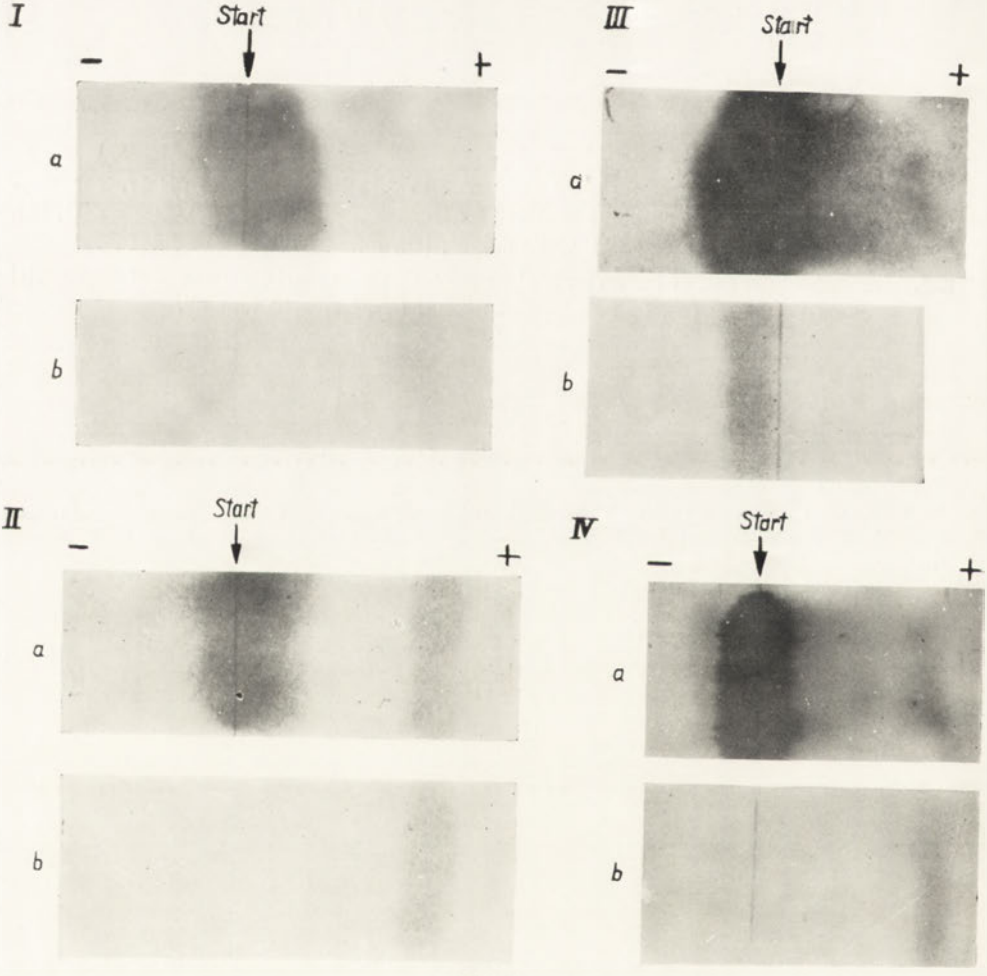

Fig. 9

Table 3

Tanning substances, polyhydroxyphenols and protein in aqueous and urea extracts from seeds and tree-bark

To 500 mg. of finely ground seeds and to 50 mg. of bark, water or 50% urea solution in 1 M-acetate buffer, pH 4.7, was added to the volume of 10 ml. The samples were mechanically shaken at room temperature for 1 hr., or for hot extraction immersed in a boiling water bath for 10 min. Then the sample was centrifuged at 6000 rev./min. and in the clear extract the tanning substances were determined in 1 ml. by the modified ferric-ammonium reagent, and protein in 0.01 ml. by the tannin micromethod [5]. The values are percentages of fresh tissue weight.

Plant material	Tanning substances			Protein		
	Aqueous extract	Urea extract		Aqueous extract	Urea extract	
	Room temp. (%)	Room temp. (%)	Boiling water bath (%)	Room temp. (%)	Room temp. (%)	Boiling water bath (%)
I. Seeds						
Pea "Szlachetna Perła" *Pisum sativum* L.	0	0	0	3.8	5.8	16.0
Yellow lupine *Lupinus luteus* L.	0	<0.08	<0.08	5.0	16.5	18.5
Parsley "Berlińska" *Petroselinum sativum* L.	<0.08	0.22	0.40	1.5	4.1	5.8
Celery "Jabłkowy" *Apium graveolens* L.	0.13	0.34	0.54	3.3	7.2	11.6
Red clover *Trifolium pratense* L.	0.20	0.62	0.73	2.2	5.1	7.2
Lettuce "Rakowicka" *Lectuca sativa* L.	0.29	0.31	1.48	8.2	8.0	18.8
II. Barks						
Willow *Salix alba* L.	2.36	5.12	5.72	0	<0.1	<0.1
Oak *Quercus robur* L.	4.60	8.11	8.36	0	0	<0.1
Catechu *Acacia catechu*	10.4	21.00	20.00	0	0	<0.1
Quebracho	11.2	17.00	22.00	0	0	<0.1

acid. The catechu-bark extract contained, in addition to the fraction bound by casein (94%), a fraction corresponding to pyrocatechol. On the other hand, the quebracho-bark extract contained 97% of substances precipitable by casein, whereas the fraction remaining in the supernatant and migrating towards the anode gave a green colour with the ferric-ammonium reagent.

Fig. 8. Electrophoresis of tanning substances from oak bark extracted with: (*A*), water; (*B*), 50% urea solution; (*C*), 40% acetone; (*D*), 100% acetone.

Fig. 9. Paper electrophoresis of (*a*), aqueous extracts from tree bark, and (*b*), supernatants after precipitation of tanning substances with casein. Extracts from the bark of: (*I*), willow; (*II*), oak; (*III*), catechu; (*IV*), quebracho. All the samples were applied on the middle of the paper strips.

DISCUSSION

Up till now, no method was available for determination of tanning substances in the presence of protein and alkaloids, or of phenol derivatives commonly found in plants. The first successful step in this direction was the elaboration of a micromethod permitting to determine simultaneously both gallic acid and catechin derivatives.

The natural tanning substances form coloured derivatives with metal ions. The reaction of tannin with iron has been one of the first reactions used for detecting iron salts, and it had been utilized in the antiquity for production of ink. Its specificity is, however, insufficient for determination of tannins in the presence of other phenol compounds.

In the elaborated procedure, Fe^{3+} of the ferric ammonium sulphate, in the presence of the acetate ion, acts as a sensitive and selective reagent towards polyhydroxyphenols, giving green-coloured derivatives with dihydroxyphenols and a violet colour with trihydroxyphenols, while with monohydroxyphenols it gives no colour reaction.

The adaptation of this reaction for colorimetric determination of polyhydroxyphenols was greatly assisted by the introduction of the protective colloid which maintains in solution the coloured reaction products. However, when the amount of polyhydroxyphenol surpasses 400 μg. per sample, a precipitate is formed even in the presence of the colloid. The addition of urea to the ferric ammonium reagent resulted, rather unexpectedly, in increased sensitivity towards the catechin derivatives, making the reagent equally sensitive towards the galloyl compounds and the catechin derivatives.

Identification of tannins in a mixture of polyhydroxyphenols was based on the reaction with casein. It was found that at appropriate pH values casein and also plant proteins are able to bind tanning substances. The commonly used methods for the determination of tannins are based on the estimation of weight after adsorption on hide powder or after precipitation by cupric salts [4]. In the presented procedure the rather inaccurate and tedious gravimetric methods have been replaced by a colorimetric micromethod. Protein-bound tannins are dissolved in a solution of urea and determined colorimetrically. The presence of urea permits to determine the tanning substances in the presence not only of protein but also of alkaloids.

This method, when applied to plants, was found to be suitable for determination of tanning substances both in material rich in protein and containing but traces of tannins (seeds) and in material rich in tannins and containing no protein (bark of trees). Aqueous extracts of the studied seeds contained rather high amounts of protein compared to slight amounts of compounds giving a colour reaction with the ferric-ammonium reagent. It can be assumed that in aqueous extracts containing soluble protein, only the non-tanning phenol derivatives are present (probably they consist of some intermediate metabolites of synthesis or degradation of tannins), whereas the tanning substances, together with the bound protein and alkaloids, can be extracted with a good yield only by urea solution. The dif-

ference between the polyhydroxyphenols extracted by hot urea and by water at room temperature gives the amount of total tanning substances. In this way it was demonstrated that the seeds of some cultivated plants contain slight amounts of protein-bound tannins.

Since aqueous extracts from protein-free plant material (bark) may contain both tanning and non-tanning phenol derivatives, for the determination of the tanning substances it is necessary to add exogenous protein. The tannins are determined either indirectly from the difference of colorimetric determinations before and after precipitation with casein, or directly in the precipitated casein-tannin complexes. This procedure can be applied equally well to tanning substances of the catechin group and of the gallic acid group.

The utilization of chinese tannin (pentadigalloyloglucose) for concentration and purification of protein by the tannin-caffeine procedure [6] makes necessary standardization of preparations of tannin. It appeared that the commercial preparations are non-homogeneous in paper electrophoresis and contain some non-tanning fractions as well as a fraction which forms insoluble compounds with protein but does not react with caffeine. Such contaminations are harmless in the tanning process and in medicine but they do limit the usefulness of the respective products for the analytical methods. Therefore commercial tannin preparations before use should be checked by electrophoresis and purified with ethyl acetate according to Armitage *et al.* [1].

REFERENCES

[1] Armitage R., Bayliss G. S., Gramshaw J. W., Haslam E., Haworth R. D., Jones K., Rogers H. J. & Searle T. - *J. Chem. Soc.* 1842, 1961.
[2] Barton G. M., Evans R. S. & Gardner J. A. F. - *Nature* **170**, 249, 1952.
[3] Björnesjö K. B. - *Scand. J. Clin. Labor. Invest.* **4**, 244, 1952.
[4] Bodalski T., *Prace Kom. Nauk Farm.* **2**, 33, 1950.
[5] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **2**, 279, 1955.
[6] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **6**, 385, 1959.

ZASTOSOWANIE MOCZNIKA, AŁUNU ŻELAZOWO-AMONOWEGO I KAZEINY DO OZNACZANIA GARBNIKÓW ROŚLINNYCH

Streszczenie

1. W roślinach garbniki występują w formie dobrze rozpuszczalnych w moczniku kompleksów z białkami i alkaloidami.

2. Ałun żelazowo-amonowy daje fioletowe zabarwienie z pochodnymi galusowymi i zielone z pochodnymi katechinowymi. W obecności jonu octanowego, mocznika i koloidu chroniącego reakcja może być wykorzystana do kolorymetrycznego oznaczania 40 - 400 μg tych substancji w 5 ml próby.

3. Garbniki można wytrącać z wyciągów nie zawierających białka kazeiną i po rozpuszczeniu w moczniku oznaczać kolorymetrycznie.

4. Elektroforeza bibułowa ułatwia identyfikację garbników i ich pochodnych.

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WANDA MEJBAUM-KATZENELLENBOGEN and ELEONORA WIECZOREK

SULPHOSALICYLIC ACID-SOLUBLE PROTEINS FROM KIDNEYS OF SOME ANIMALS

Department of Physiological Chemistry, Faculty of Pharmacy, Medical School; and Department of Biochemistry, University, Wrocław

1. In animals belonging to the same species, small differences in the amount of SSA-soluble proteins were observed. 2. In rodents, calf and hen, the SSA-soluble protein was found to contain very small amounts of hexose (2 - 7%). Only in frog kidney protein, 57 - 71% of sugars was found. 3. On paper electrophoresis in citrate - phosphate buffer, similar fractions were obtained from SSA-soluble proteins of different animals.

In the blood serum, after deproteinization with sulphosalicylic or perchloric acids, there remains a small amount of proteins, about 80 mg.%. These proteins, possessing a high content of sugar, are called seromucoid. In pathological sera the concentration of seromucoid increases up to several times and this increase is supposed to be due to mucoproteins derived from pathologically changed tissues. Mejbaum-Katzenellenbogen and coworkers applied the tannin-caffeine procedure to isolate the proteins soluble in sulphosalicylic acid (SSA) from normal [13, 10, 15] and pathological [15, 12] sera, from other body fluids [13, 1] and plant and animal tissues [13, 11, 5]. In all the materials studied, small amounts of the SSA-soluble protein reacting with tannin were found to be present. In most animal tissues the concentration of this protein was higher than in the serum; it was the lowest in muscles and white brain matter and the highest in the pituitary and thyroid glands. The nature and function of the SSA-soluble proteins are unknown.

Rzeczycki *et al.* [17] demonstrated that the basic protein from hog kidney mitochondria can be extracted with SSA. Rzeczycki [16] reported also that the mitochondrial basic protein inhibits the activity of cytochrome oxidase and prevents the swelling of mitochondria (Hillar & Rzeczycki [3]).

The aim of the present work was to isolate and compare by paper electrophoresis the sulphosalicylic acid-soluble proteins from kidney of some animals belonging to different taxonomic classes.

MATERIAL AND METHODS

Animals. The experiments were carried out on kidneys of the calf, rabbit, guinea pig, white mouse, white rat, Leghorn hen and frogs: *Rana temporaria* caught in autumn and kept in a tank, and *Rana esculenta* caught in spring. The animals were killed by exsanguination or by ether (frogs, guinea pigs and white mice), the kidneys removed immediately and freed of fat and connective tissue.

Sulphosalicylic acid extracts. Kidneys were cut into slices about 0.5 cm. thick and homogenized in a porcelain mortar with a tenfold volume of 0.15 M-sulphosalicylic acid (SSA), left for 10 - 15 min., then centrifuged, and the clear supernatant fluid was used for experiments. In the case of frogs, guinea pigs and white mice, the extracts were made from kidneys of several animals.

Table 1

Concentration by the tannin-caffeine procedure of sulphosalicylic acid-soluble proteins from rabbit kidney

SSA-soluble protein		Degree of concentration
in SSA-extract (g./100 ml.)	after concentration (g./100 ml.)	
0.024	2.15	90
0.016	1.80	112
0.020	1.95	97
0.017	1.85	108
0.017	1.60	100
0.022	1.35	61
0.022	1.75	80
0.019	2.05	107
0.020	1.05	52
0.020	1.55	77
0.020	2.25	112
0.019	1.45	76
0.020	1.65	82

The extracts from rabbit-kidney nuclei were prepared according to Hogeboom [4]. The kidney was washed with water until free of blood, then cut into slices, and 1 g. was homogenized with 9 ml. of 0.25 M-sucrose - 1.8 mM-$CaCl_2$, and filtered through gauze. The homogenate was layered with 0.34 M-sucrose - 0.18 mM-$CaCl_2$ and centrifuged for 10 min. at 700 *g*. The sedimented nuclei were homogenized with 5 ml. of 0.25 M-sucrose - 0.18 mM-$CaCl_2$. Then 10 ml. of 0.34 M-sucrose - 0.18 mM-$CaCl_2$ was introduced beneath the suspension of nuclei and centrifuged at 700 *g*. This procedure was repeated twice. To the nuclei pellet, 0.15 M-SSA was added to the volume of 10 ml.

Concentration of the SSA extracts. The SSA-soluble protein was concentrated by the tannin-caffeine procedure [8]. To the extract, 20-fold amount of tannin in relation to protein, was added. The sediment formed was washed with water,

mixed with a small amount of the veronal buffer of Michaelis [14] of pH 2.6, and then caffeine was added, mixed thoroughly and left overnight in a refrigerator. Then the sample was centrifuged at 3000 rev./min. The supernatant containing the SSA-soluble proteins was of a yellow colour; some samples were opalescent. The efficiency of the procedure is presented in Table 1 on rabbit kidney SSA-soluble protein.

Paper electrophoresis. The following buffers were used: citrate - phosphate, 0.15 M, pH 4.4 [6]; citrate - HCl, 0.15 M, pH 4.6; and veronal - acetate, 0.1 M, pH 9 [9]. On the middle of Schleicher-Schüll no. 2043a paper (28×3 cm.) 0.02 ml. of about 1% solution of the concentrated protein was applied and the separation was carried out for 5 - 6 hr. at 150 v. From the electrophoretograms stained with bromophenol blue according to Mejbaum-Katzenellenbogen & Dobryszycka [9] the separate fractions were eluted with 0.1 N-NaOH and protein was determined by the tannin method [7]. To detect sugars, the electrophoretograms were stained with Schiff's reagent according to Wunderly & Piller [19].

Determination of protein-bound sugars. SSA-soluble proteins were precipitated with a 30-fold amount of tannin and the samples left overnight at 4°. The protein--tannin sediment was washed several times with acetone until tannin-free. The protein sediment was dissolved in 0.1 N-NaOH, then protein was determined by the tannin method [7] and hexoses with anthrone according to Graff & Groenspan [2].

RESULTS

In rabbit kidney the average content of SSA-soluble proteins amounted to 204±34 mg. per 100 g. of fresh tissue weight. The content of hexoses in these proteins was about 2.6%.

It was found that the procedure applied for isolation of the nuclear fraction did not affect the concentration of SSA-soluble protein; only traces of this protein were present in the nuclei (3.5% of the content of SSA-soluble protein in kidney homogenate).

To examine more closely the SSA-soluble proteins, the concentrated protein solutions were submitted to electrophoresis (Fig. 1a). In the veronal - acetate buffer, pH 9, used for separation of serum proteins, the SSA-soluble protein migrated towards the cathode but no distinct resolution could be obtained. In the citrate - - HCl buffer, pH 4.6, the SSA-soluble protein separated into three cathodic fractions (Fig. 1b). The distribution of the individual fractions was determined on 10 rabbits. The slower fraction (K_1) accounted for 60±3.2%, the second fraction (K_2) for 10±2.4%, and the most mobile fraction (K_3) for 30±2.9%. In the citrate - phosphate buffer, pH 4.4, as many as five fractions were obtained (Fig. 1c), four of which displayed cathodic mobility and one anodic mobility. In no fraction any sugars could be detected by the Schiff's reagent [19].

In guinea pig kidney, the average content of SSA-soluble proteins in a group of 20 animals, was 166±13 mg. per 100 g. of fresh weight. The SSA-soluble pro-

teins contained on the average 2.8% of hexoses, and from the complex with tannin they were regenerated by caffeine with a yield of 77%. On paper electrophoresis

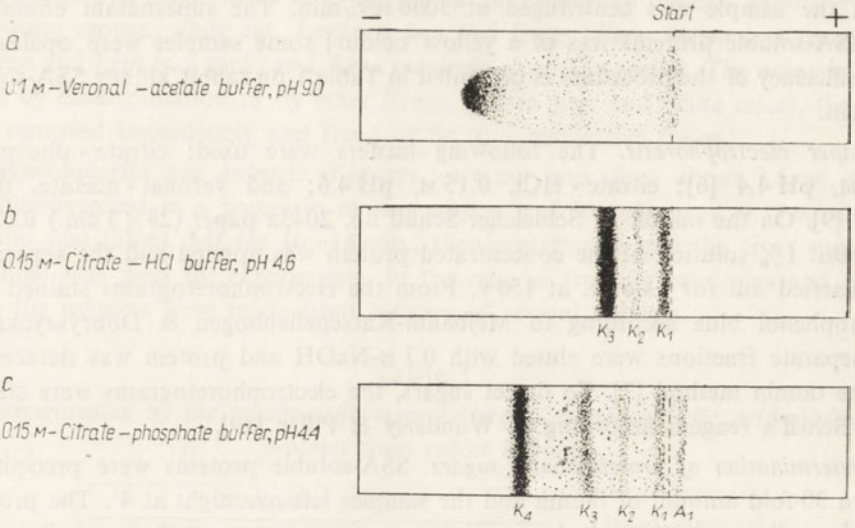

Fig. 1. Paper electrophoresis of sulphosalicylic acid-soluble proteins from rabbit kidney. Proteins were detected by staining with bromophenol blue dissolved in the tannin reagent; 0.02 ml. samples containing 500 μg. of protein were applied.

in citrate - phosphate buffer they separated into six, and sometimes eight, cathodic fractions (Fig. 2a). Although the number of fractions differed from one preparation to another, the characteristic pattern of four groups of fractions was always

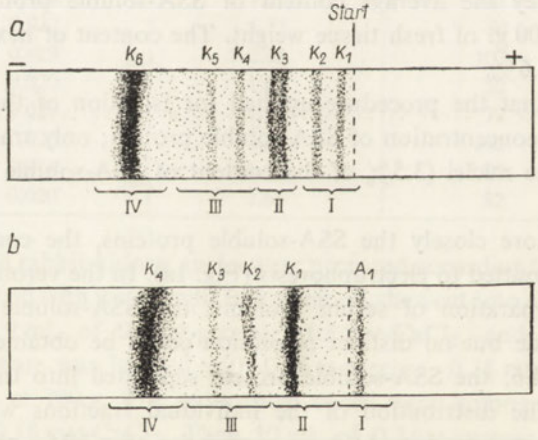

Fig. 2. Paper electrophoresis of sulphosalicylic acid-soluble proteins from the kidney of: (*a*), guinea pig, and (*b*), white mouse, in 0.15 M-citrate - phosphate buffer, pH 4.4. Proteins were detected by staining with bromophenol blue dissolved in the tannin reagent.

observed and could be compared with the four fractions of rabbit kidney (Fig. 1c). The first group consisted usually of two slow-moving fractions (K_1 and K_2). Fraction K_3 which distinctly stained with bromophenol blue and sometimes had a ten-

dency to further separation, was taken as the second group. The third group consisted of two fractions, K_4 and K_5. Fraction K_6, corresponding to the fourth group, had the greatest mobility and was most intensively stained; sometimes it separated into two fractions. The distribution of SSA-soluble proteins from guinea pig kidney is shown in Table 2.

Table 2

Distribution of sulphosalicylic acid-soluble protein fractions from guinea pig kidney, separated by paper electrophoresis

The electrophoresis was carried out in citrate - phosphate buffer, pH 4.4 (Fig. 2a). In the fractions eluted with 1 N-NaOH the protein was determined.

Group	Fraction	% of the total			Average	
		Expt. I	Expt. II	Expt. III		
I	K_1	27.6	23.2	25.4	25.4	40
	K_2	12.4	14.2	18.0	15	
II	K_3	15.2	27.6	25.4	22.7	22.7
III	K_4	12.2	11.6	9.0	11.0	19
	K_5	9.1	8.9	6.0	8.0	
IV	K_6	23.5	14.6	16.2	18.0	18

In white mouse kidney the content of SSA-soluble proteins amounted to 167± ±40 mg. per 100 g. of fresh tissue. The extracts were concentrated, and separated by electrophoresis in citrate - phosphate buffer, pH 4.4 (Fig. 2b). Four cathodic fractions and one anodic fraction were present, similarly as in rabbit kidney. The mobility and the intensity of staining of the cathodic fractions corresponded to the fractions K_3 - K_6 of guinea pig kidney.

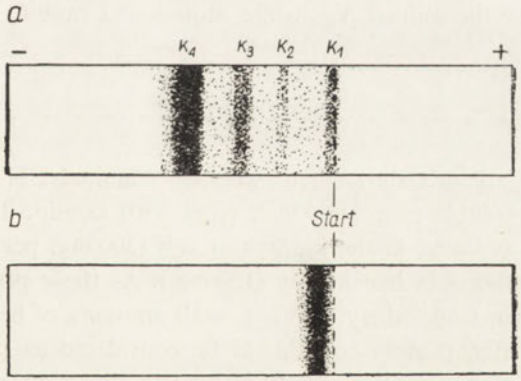

Fig. 3. Paper electrophoresis of sulphosalicylic acid-soluble proteins from frog kidney (*Rana temporaria*) in 0.15 M-citrate - phosphate buffer, pH 4.4. (*a*), Electrophoretogram stained with bromophenol blue dissolved in the tannin reagent; (*b*), electrophoretogram stained with the Schiff's reagent.

In frog kidney in winter (*Rana temporaria*) the content of SSA-soluble protein amounted to 262±11 mg. per 100 g. of fresh tissue, and in spring (*Rana esculenta*)

to 286±10 mg. The frog SSA-soluble proteins differed from those of other animals studied in that their content of hexoses was very high (57% in winter and 71% in spring). On paper electrophoresis in citrate - phosphate buffer, pH 4.4, four cathodic fractions were obtained (Fig. 3a) and the sugar was found to be present only in the slowest-moving fraction K_1 (Fig. 3b).

Table 3

Sulphosalicylic acid-soluble proteins from the kidney of different animals

Protein was determined by the tannin method [7], hexoses by the anthrone method [2]. Mean values ± S. D. are given; in parentheses the number of animals.

Animal	SSA-soluble protein (mg./100 g. fresh wt.)	Hexoses in the protein (%)
Calf (10)	298 ± 11.6	3.8 ± 1.3
Rabbit (20)	204 ± 34	2.6 ± 0.6
Guinea pig (20)	166 ± 13	2.8 ± 0.7
White rat (10)	234 ± 14.9	3.9 ± 0.8
White mouse (45)	167 ± 40	—
Leghorn hen (10)	122 ± 10	7.1 ± 0.1
Frog, *Rana esculenta.* in spring (10)	286 ± 10	71.5 ± 2.4
Frog, *Rana temporaria,* in winter (10)	262 ± 11	57.0 ± 5.3

In the kidney of the rat, calf, hen and frogs only the content of SSA-soluble proteins and of sugar bound with these proteins, were determined. These results as well as those for the guinea pig, white mouse and rabbit, are summarized in Table 3.

DISCUSSION

The kidneys of all animals studied, whether mammals, birds or amphibians, contain some SSA-soluble proteins which react with tannin. The highest content of these proteins was found in the kidneys of calf (300 mg. per 100 g. of fresh tissue) and frog, the lowest in hen kidney (120 mg.). As these proteins, with the exception of those from frog kidney, contain small amounts of hexoses and no sialic acid, they are not mucoprotein and should be considered as protein or polypeptide. The presence of polypeptides could be due to digestion of tissue protein by active proteolytic enzymes; however, even after several hours of autolysis of the kidney at room temperature, the content and electrophoretic pattern of the SSA-soluble protein was not affected.

In SSA-soluble proteins from frog kidney the content of hexoses was very high. On paper electrophoresis it appeared, however, that only one, the slowest-moving protein fraction was intensively stained for sugars. The content of hexoses was

lower for the frogs kept in winter in tanks than for frogs that had passed the winter in natural conditions. This difference could be due to environmental conditions, or be species-dependent.

The electrophoretic patterns of SSA-soluble proteins were similar for all animals studied, four cathodic fractions being always present. In the citrate - phosphate buffer, pH 4.4, some fractions underwent further separation. The fractions with the greatest cathodic mobility displayed a special affinity for bromophenol blue, giving an intensively blue band. Rzeczycki [17] reported that the fast-moving fraction accounts for 35% of the SSA-soluble proteins isolated from hog kidney. In the present work, the most intensively staining, fast-moving fraction accounted also for 30% of the SSA-soluble protein, whereas fraction K_1, which on the electrophoretogram gave a weakly stained band (Fig. 1a), accounted for 60%. Strong binding of the dye to the fast-moving fraction is probably due to the high content of lysine which according to Rzeczycki amounted to 25%.

In a few cases when it was possible to examine human kidney, it was observed that in some pathological conditions (tumour, nephrosis) the electrophoretic patterns were similar to those found in animals but differed from those for blood seromucoid.

The presence of SSA-soluble proteins in the kidney of all animal species studied and the similarities in the electrophoretic patterns seem to indicate that these proteins form an essential component of the cell and may have an important role in the structure and activity of the kidney.

REFERENCES

[1] Dobryszycka W. - *Acta Biochim. Polon.* **10**, 345, 1963.
[2] Graff M. M. & Groenspan S. M. - *J. Lab. Clin. Med.* **37**, 736, 1951.
[3] Hillar M. & Rzeczycki W. - *Acta Biochim. Polon.* **12**, 133, 1965.
[4] Hogeboom G. H., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) Vol. 1, p. 18, Academic Press, New York 1955.
[5] Kubicz A. & Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **11**, 395, 1964.
[6] McIlvaine T. C. - *J. Biol. Chem.* **49**, 183, 1921.
[7] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **2**, 279, 1955.
[8] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **6**, 375 and 385, 1959.
[9] Mejbaum-Katzenellenbogen W. & Dobryszycka W. - *Clin. Chim. Acta* **4**, 515, 1959.
[10] Mejbaum-Katzenellenbogen W., Dobryszycka W. & Morawiecka B. - *Nature* **192**, 262, 1961.
[11] Mejbaum-Katzenellenbogen W., Dobryszycka W. & Morawiecka B. - *Proc. of the Intern. Union of Physiol. Science, XXIII Intern. Congr. Leyden* **2**, 568, 1962.
[12] Mejbaum-Katzenellenbogen W., Dzioba A. & Hubicka Z. - *Zbl. Gynäk.* **87**, 285, 1965.
[13] Mejbaum-Katzenellenbogen W., Morawiecka B. & Dobryszycka W. - *Acta Biochim. Polon.* **7**, 401, 1960.
[14] Michaelis L. - *Biochem. Z.* **139**, 234, 1931.
[15] Morawiecka B. & Mejbaum-Katzenellenbogen W. - *Clin. Chim. Acta* **7**, 722, 1962.
[16] Rzeczycki W. - *Acta Biochim. Polon.* **10**, 279, 1963.
[17] Rzeczycki W., Grudzińska A. & Hillar M. - *Acta Biochim. Polon.* **9**, 295, 1962.
[18] Rzeczycki W., Grudzińska A., Hillar M. & Wszelaki-Lass E. - *Acta Biochim. Polon.* **10**, 49, 1963.
[19] Wunderly Ch. & Piller S. - *Klin. Woch.* **32**, 425, 1954.

BIAŁKA WYCIĄGÓW SULFOSALICYLOWYCH NEREK ZWIERZĘCYCH

Streszczenie

1. Stężenie białek rozpuszczalnych w kwasie sulfosalicylowym ulega nieznacznym wahaniom i jest charakterystyczne dla przedstawicieli poszczególnych gromad.

2. U gryzoni, cieląt i kur białka rozpuszczalne w kwasie sulfosalicylowym zawierały małe ilości heksoz (2 - 7%). Jedynie nerki żab zawierały 57 - 71% heksoz.

3. W elektroforezie bibułowej w moderatorze cytrynianowo-fosforanowym uzyskano podobne frakcje dla białek rozpuszczalnych w kwasie sulfosalicylowym, pochodzących z nerek różnych zwierząt.

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WANDA MEJBAUM-KATZENELLENBOGEN and IRENA LORENC-KUBIS

THE INSULIN-TANNIN COMPLEXES

Department of Biochemistry, University; and Department of Physiological Chemistry, Faculty of Pharmacy, Medical School, Wrocław

1. It was demonstrated that insulin forms with tannin primary complexes as well as saturated complexes. From the primary complexes the insulin can be regenerated by caffeine, its biological activity remaining unaffected. 2. The behaviour towards tannin of the polypeptide chains A and B obtained by reduction of insulin by sulphite, is similar to that of insulin. 3. When the polypeptide chains are obtained by oxidation of insulin with performic acid, only the phenylalanyl chain forms complexes with tannin.

In the previous studies on the application of tannin and caffeine for precipitation and regeneration of protein [7, 5], it has been found that binding of protein with tannin is a steicheiometric reaction, as formation of the product depends on the weight ratio of the reactants. Certain suggestions concerning the mechanism of this reaction have been advanced but they required further confirmation.

The reaction of protein with tannin proceeds through a number of steps. First, a primary insoluble complex is formed which is able to react with caffeine or metal ions, or to form further complexes with fresh portions of tannin, the formation of these complexes being revealed by increasing turbidity.

The complexes formed in the first step, containing the smallest amount of tannin, have been utilized for isolation and concentration of protein [7]. The tannin-bound protein does not undergo denaturation, and after regeneration maintains its biological activity [9, 10, 5]. The complexes saturated with tannin formed in further steps of the reaction, have been applied for protein determination by the turbidimetric micromethod [6].

According to the hitherto made observations, the micromethod worked out on three standard proteins, lysozyme, gelatin and serum protein, permits to determine the protein directly. The turbidity formed is proportional to the weight of the protein in the sample. This is in agreement with the initial assumption of the steicheiometric reaction. In this respect it is possible to compare this method with the gravimetric method of determination of proteins and polypeptides.

Taking into account the molecular weight of tannin (1800) and the results of analysis of the primary protein-tannin complexes, it was assumed that 10 amino

acid residues of the peptide chain are bound with 1 mole of tannin. Therefore the peptides with a molecular weight higher than 1500 could be expected to form complexes from which tannin would be easily removed with caffeine, and to be determined directly in the turbidimetric method on weight basis. To support this assumption it was thought necessary to study in more detail the reaction of tannin with insulin and with the polypeptide chains A and B, obtained by oxidation or reduction of disulphide bonds. The glycyl chain A (mol. wt. 2400) and the phenylalanyl chain B (mol. wt. 3600) differ from each other not only in the number of amino acid residues but also in the number and position of the sulphur-containing amino acids. Moreover, the glycyl chain contains an internal disulphide bond between residues 6 and 11. Such S—S bonds are found as a characteristic feature of the structure of many biologically active proteins.

MATERIALS AND METHODS

Insulin "Polfa" from Tarchomińskie Zakłady Farmaceutyczne (Warszawa, Poland) and insulin from Wytwórnia Surowic i Szczepionek (Warszawa, Poland) were used for experiments. Samples of insulin solution prepared for medical use contained 40 or 80 international units per 1 ml., and 0.1% of phenol.

For oxidation of insulin, performic acid was used according to Sanger [14]. The oxidized glycyl chain A and phenylalanyl chain B were separated by DEAE-Sephadex A 25 column chromatography after Fittkau [4] or according to Sanger [14] by precipitation at pH 6.5 of the poorly soluble phenylalanyl chain; the glycyl chain remained in the solution.

Insulin was reduced to *S*-sulphonated products by the method of Bailey & Cole [1]. Insulin, 25 mg., was dissolved in 1.25 ml. of 8 M-urea solution in 0.2 M-tris buffer, pH 7.4, placed in a water bath at 38°, and added successively, at 20 min. intervals, with: 0.15 ml. of 1 M-sodium sulphite, 0.31 ml. of 0.5 M-sodium tetrathionate, and again 0.15 ml. of sodium sulphite. This treatment was repeated three times. Then the mixture was dialysed for one hour against 0.01 M-sodium sulphite and placed on a Dowex 50 X 2 column that had been equilibrated with 8 M-urea in 0.1 M-citrate buffer, pH 2.2. The glycyl chain was eluted with 8 M-urea in citrate buffer, pH 2.2, and the phenylalanyl chain with 8 M-urea solution, pH 7.6.

Insulin, and the A and B chains were concentrated by the tannin-caffeine procedure [7].

Biological activity of insulin was determined on rabbits according to Polish Pharmacopoea III [3]. Sugar in the blood was determined according to Nelson [12], 0.1 ml. of blood being taken from the marginal vein of rabbit ear.

Protein was determined spectrophotometrically in the Zeiss-VUS 1 spectrophotometer at 278 mμ, by the tannin micromethod [6] and by the Kjeldahl method. Selenium oxide was used as catalyst and for insulin the coefficient 6.43 was applied.

Paper electrophoresis was carried out in 0.1 M-phosphate buffer, pH 7.3, at 280 v, for 5 hr., about 300 μg. of protein being placed on Schleicher-Schüll 2403a

paper (28×3 cm.). The electrophoretograms were stained with bromophenol blue dissolved in the tannin reagent [8].

Reagents: Tannin was a product of Politechnika Śląska (Gliwice, Poland), no. contr. 3-TO/60, and was nitrogen-free. Sodium tetrathionate was prepared as described by Supniewski [15]. Other reagents were of analytical grade.

RESULTS

Application of the tannin micromethod for determination of insulin

The amount of protein present in the solution of the "Polfa" insulin was determined by the Kjeldahl method after precipitation with trichloroacetic acid. The solutions of insulin containing a known amount of protein were estimated by the tannin micromethod and the extinctions were compared with those for the standard gelatin solutions (Fig. 1). The same extinction readings were obtained

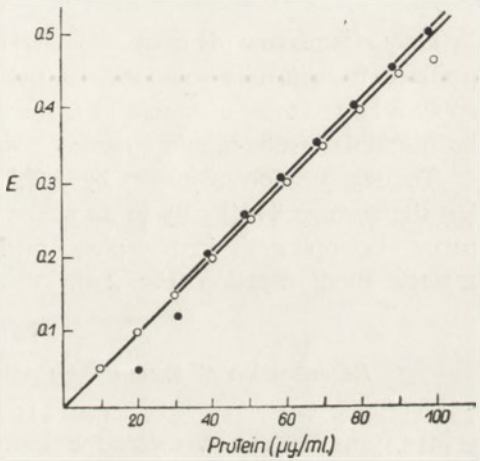


Fig. 1. Standard curve for gelatin and insulin in the tannin micromethod. Photocolorimeter KF, red filter. (○), Gelatin, 18% of nitrogen; (●), insulin, 15.9% of nitrogen.

for the same amounts of protein at concentrations higher than 30 μg. per 1 ml. In some preparations of insulin, the determinations of total nitrogen and nitrogen present in the tannin-protein sediment were compared with the amount of protein determined by the tannin method (Table 1). In separate commercial samples the total nitrogen content was practically identical, amounting on the average to 0.416 mg. per 1 ml. Despite the constant total nitrogen content, in the material precipitated by tannin the nitrogen content varied markedly, from 50 to 80% of the total nitrogen. In parallel determinations, the content of protein in the solution determined by the tannin micromethod was in agreement with the values obtained by the Kjeldahl method for the protein-tannin precipitate. This indicated that the nitrogen compound which was not precipitated by tannin, corresponds to a non-protein contamination. Therefore in further work protein was determined by the tannin method.

Table 1

Determination of protein in the samples of "Polfa" insulin by the Kjeldahl and tannin methods

The data pertain to separate samples of the indicated series. Mean values from three parallel determinations are given; the differences did not exceed 5%.

No. of series	Total N (mg./ml.)	Protein-tannin complex N		Protein	
		(mg./ml.)	(% of total N)	N in complex × 6.43 (mg./ml.)	tannin method (mg./ml.)
700660	0.418	0.342	81.8	2.20	2.20
700660	0.419	0.273	65.1	1.75	1.76
700660	0.411	0.273	66.6	1.75	1.76
270361	0.420	0.240	59.3	1.54	1.55
270361	0.413	0.208	50.3	1.34	1.34

Application of tannin and caffeine for isolation of insulin from solutions

Only at a narrow pH range, from 4.0 to 5.3, the protein was quantitatively precipitated from insulin solutions. At pH values lower than 4 it was necessary to apply a large excess of tannin in relation to protein. In neutral or alkaline media no insoluble insulin-tannin complexes were formed.

The regeneration of insulin by caffeine from the complexes gave a good yield (on the average 84%) only in an acidic medium, at pH approx. 2 (Table 2). On paper electrophoresis in phosphate buffer, pH 7.3, the regenerated insulin gave a single anodic fraction (Fig. 2 *a*).

Table 2

Regeneration of insulin from protein-tannin complexes by caffeine

To solutions of "Polfa" insulin, tannin was added in an amount equal to the weight of the protein, at pH 5.3; the precipitate was washed with acetate buffer, pH 5.3. The protein-tannin sediment was added with a twofold amount of caffeine (in relation to protein weight) and with 0.5 N-HCl in such an amount as to obtain an about 1% solution of the regenerated protein. Protein was determined by the tannin method.

No. of series	Protein				Yield (%)
	in the starting material		after concentration		
	(g./100 ml.)	(g./sample)	(g./100 ml.)	recovered (g.)	
620561	0.750	0.075	1.90	0.066	87
620561	0.060	0.006	0.46	0.003	77
620561	0.060	0.005	0.44	0.004	80
720662	0.210	0.069	0.46	0.046	74
720662	0.180	0.022	1.14	0.020	92
720662	0.190	0.950	1.44	0.778	82
720662	0.190	0.840	1.44	0.670	77
710361	0.210	0.002	1.34	0.002	83
710361	0.200	0.016	0.78	0.016	97

The activity of the regenerated insulin is presented in Table 3. The sample of insulin "Polfa" used, contained in 1 ml. 80 i.u. and 1.6 mg. of protein reacting with tannin. As after precipitation of the protein by tannin about 14% of the total

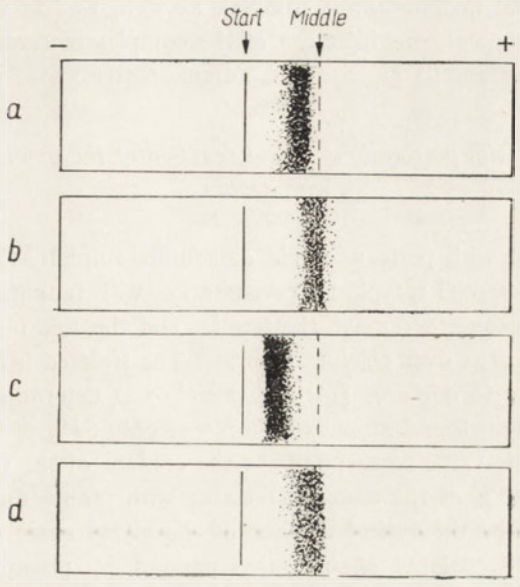


Fig. 2. Paper electrophoresis of insulin and chains A and B precipitated by tannin and regenerated by caffeine. (*a*), Insulin; (*b*), oxidized phenylalanyl chain; (*c*), *S*-sulphoglycyl chain; (*d*), *S*-sulphophenylalanyl chain. Conditions of electrophoresis as described in Methods.

nitrogen remained in the supernatant fluid, it could be expected that also 14% of the activity (11 i.u.) would remain in the supernatant. However, the whole activity was found in the insulin regenerated from the protein-tannin sediment. The

Table 3

Activity of insulin regenerated from the complex with tannin

For the experiments, samples of "Polfa" insulin, series no. 710361, containing in 1 ml. 80 i.u. and 1.6 mg. of protein, were used. The activity of preparations was assayed by subcutaneous administration to rabbits weighing 2 - 3 kg., starved for 24 hr. Group I obtained 1 i.u. (20 μg. of protein) per 2 kg. body weight, of the starting sample. Group II obtained 20 μg. per 2 kg. body weight, of insulin regenerated from the insulin-tannin complex. Group III obtained the whole supernatant fluid remaining from 1 ml. of the "Polfa" insulin after precipitation with tannin, and containing presumably 11 i.u. Glucose was determined according to Nelson [12].

Group	Preparation	Blood glucose (mg.%)			
		at zero time	after		
			1 hr.	2 hr.	3 hr.
I	Insulin "Polfa" standard	97.0	40.3	37.2	60.5
II	Regenerated insulin	97.0	46.1	39.2	51.3
III	Supernatant fluid after precipitation of the protein-tannin complex	99.0	90.0	88.0	83.0

supernatant fluid presumably corresponding to 11 i.u. administered to rabbits showed virtually no insulin activity.

In further experiments it was found that not only the insulin regenerated from the complex, but the insulin-tannin complex as well, was biologically active. In some cases, after subcutaneous injection of the complex to rabbits, a heavy hypoglycaemic shock resistant to glucose and adrenalin treatment, was observed.

Oxidation of insulin with performic acid and reaction of the oxidized chains A and B with tannin

Before treatment with performic acid or sodium sulphite, the insulin was isolated from the commercial sample by precipitation with tannin, then the complex was treated with acetone to remove the tannin, and the free insulin was air-dried and kept in a desiccator over calcium chloride. The isolated insulin was dissolved and oxidized with performic acid [14] and protein was determined in the solution. The amount of protein found after oxidation was only 58% of that present in the starting material; this value corresponds to the content of the phenylalanyl chain. The loss of about 40% of the material reacting with tannin due to the oxidation agrees closely with the theoretical content of the glycyl chain.

The oxidized phenylalanyl chain was separated by precipitation at pH 6.5, and the sediment dissolved in 0.1 N-HCl. In the sediment the total amount of protein found in the solution of oxidized insulin was recovered, and the supernatant fluid, which contained the glycyl chain, gave no reaction with tannin.

Table 4

Separation by column chromatography and determination of the products of insulin oxidation by performic acid

From the "Polfa" preparation, insulin was precipitated by tannin and from the complex the tannin was removed with acetone. Insulin, 25 mg., was oxidized with performic acid and placed on a DEAE-Sephadex A 25 column; then the phenylalanyl chain was eluted with 20% formic acid, and the glycyl chain with 1 N-HCl [4]. In both fractions, protein was determined by the tannin method and spectrophotometrically. In the tannin method, the content of protein was read from the standard gelatin curve. From extinction readings the content of protein was calculated taking $E_{278}^{0.1\%} = 1$. Mean values of three experiments are given; the differences did not exceed 5%.

	Protein		
	Tannin method (mg.)	Spectrophotometric method	
		(mg.)	(%)
Glycyl chain A	0.0	8.6	35
Phenylalanyl chain B	13.2	13.5	61
Total	13.2	22.1	100

Oxidized insulin was submitted to column chromatography on DEAE-Sephadex A 25 [4], and in the obtained two fractions protein was determined by the tannin method and spectrophotometrically (Table 4). The same results were obtained

by the two methods for the oxidized phenylalanyl chain; 13.3 mg. of protein was found, which corresponded to 61% of the chromatographed insulin. In the fraction containing the glycyl chain, the tannin method did not show the presence of protein whereas spectrophotometrically 8.6 mg. was found, which corresponded to 39% of the chromatographed material.

The phenylalanyl chain, similarly as insulin, was precipitated by tannin at pH 5.3, and could be regenerated with caffeine only in an acidic medium; on paper electrophoresis a single fraction was obtained (Fig. 2 *b*).

Reduction of insulin with sodium sulphite and reaction of the S-*sulphonated products of the chains A and B with tannin*

Insulin, after sulphite-tetrathionate treatment, was submitted to chromatography on a Dowex 50 X 2 column [1]. The two fractions were dialysed until urea was removed, then protein was determined (Table 5). Both the glycyl and the phenylalanyl chain could be determined by the tannin method; 63% of the material recovered from the column corresponded to the phenylalanyl chain and 36% to the

Table 5

Separation and determination of the products obtained by treatment of insulin with sodium sulphite

From the "Polfa" preparation, insulin was precipitated by tannin and from the complex the tannin was removed with acetone. Insulin, 25 mg., was reduced and chromatographed on Dowex 50 X 2 column according to Bailey & Cole [1]. In the eluates, protein was determined by the tannin method. Mean values of three separate experiments are given; the differences did not exceed 5%.

	Protein	
	(mg.)	(%)
Glycyl chain A	6.5	34.6
Phenylalanyl chain B	12.3	65.4
Total	18.8	100.0

glycyl chain. From the dialysed eluates, chains A and B were concentrated by the tannin-caffeine procedure. The *S*-sulphophenylalanyl chain, similarly as that obtained after oxidation of insulin, was quantitatively precipitated by tannin at pH 5.3 and regenerated by caffeine at pH 2. The *S*-sulphoglycyl chain was also precipitated by tannin at pH 5.3, but for the regeneration a neutral medium was required. The differences in the electrophoretic mobility of the two chains were but slight (Fig. 2 *c* and *d*).

DISCUSSION

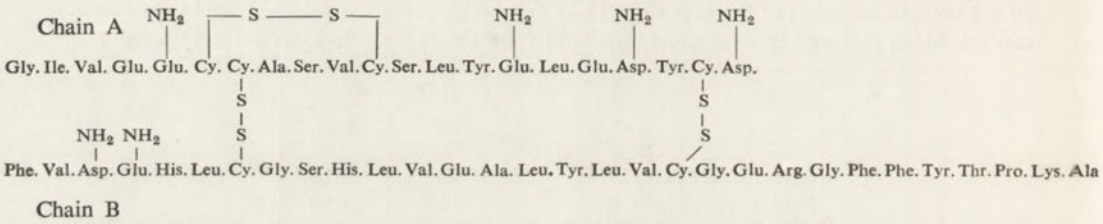
Insulin, like other hitherto examined proteins, forms with tannin primary complexes in which it maintains its full biological activity, and final complexes which are utilized for quantitative determination of protein.

The observation that the insulin-tannin complexes, when administered subcutaneously, exhibit prolonged activity may be of practical importance. Presumably the tannin-bound insulin is resistant to enzymic proteolysis. In experiments not presented in this paper it was found that tannin-bound insulin is not digested by pepsin. Therefore it seems that it should be possible to utilize tannin for isolation of insulin from the pancreas, as a factor protecting the hormone against proteolysis. The *S*-sulphonated products of the glycyl and phenylalanyl chains react with tannin similarly as insulin. On the other hand, after oxidation of insulin only the phenylalanyl chain forms insoluble complexes with tannin, whereas the oxidized glycyl chain gives no reaction; this indicates that the length of the peptide chain is not the only factor in the formation of complexes with tannin.

Oxidation of insulin with performic acid splits the S-S bonds, and the cysteine residues are oxidized to cysteinic acid

$$R_1S\cdot SR_2 \xrightarrow{\text{performic acid}} R_1SO_3H + R_2SO_3H$$

The glycyl chain contains more cysteine residues than the phenylalanyl chain (see Scheme 1), and after oxidation the four strongly ionized groups SO_3^{2-} can act as a steric hindrance preventing the formation of hydrogen bonds with tannin. The formation of hydrogen bonds has been previously suggested [7] and Morawiecki [11] demonstrated that in the polylysine-tannin complexes, between the carbonyl groups of the polypeptide and the phenol hydroxy groups of gallic acid, hydrogen bonds are formed.

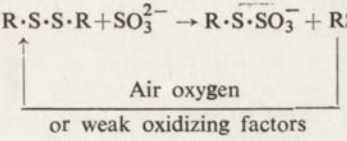


Scheme 1. Structure of insulin according to Ryle, Sanger, Smith & Kitai [13].

In the glycyl chain, fragments consisting of 5, 3, 8 and 1 amino acid(s) are present between the half-cystine residues. The largest 8-amino acid fragment with a molecular weight of 1029 is probably too small for stable binding of tannin after oxidation of cysteine to cysteinic acid. In the phenylalanyl chain, fragments consisting of 6, 11 and 11 amino acids are present. In this case, after oxidation two SO_3^{2-} groups do not interfere with the binding of tannin. Probably two tannin molecules are bound to the phenylalanyl chain, each to one 11-amino acid fragment.

Interesting results were obtained by splitting the S-S bonds by sulphite treatment in the presence of urea according to Bailey & Cole [1]. In this method, by successive oxido-reduction cycles, all S-S linkages are broken forming *S*-sulphonated products. According to the authors, the reaction proceeds as shown in Scheme 2.

In the first step, one sulphur of the split disulphide becomes reduced. Its oxidation by air oxygen is accelerated by tetrathionate. After three cycles of oxido-reduction all half-cystine residues of insulin are *S*-sulphonated. As demonstrated by Cecil & Loening [2], in the absence of urea in the medium the intra-chain S-S bond is not split.



Scheme 2.

In our experiments, by the procedure of Bailey & Cole the chains A and B were obtained from insulin in the appropriate proportion and with a good yield. Both chains reacted with tannin. Thus it appears that the presence of *S*-sulphocysteine residues does not interfere with binding of tannin.

Insulin and the oxidized or *S*-sulphonated phenylalanyl chain react with tannin. They form primary complexes and can be isolated by the tannin-caffeine procedure. They form also complexes fully saturated with tannin and can be determined by the tannin method. A close agreement was obtained between the results of determinations obtained in this way and by the Kjeldahl and spectrophotometric methods.

The oxidized glycyl chain does not react with tannin but in the sulphonated form it is bound by tannin and then it can be determined directly; it can be also concentrated by the tannin-caffeine procedure.

Rather unexpected behaviour of nitrogen compounds was observed in "Polfa" insulin (Tarchomińskie Zakłady Farmaceutyczne). In some samples the content of nitrogen of compounds not reacting with tannin amounted even to 50% of the total nitrogen. One of the possible explanations is that the insulin was oxidized and in the sample the oxidized glycyl chain was present. This, however, did not affect the activity of the preparations, and no attempt was made to identify these compounds.

REFERENCES

[1] Bailey J. L. & Cole R. D. - *J. Biol. Chem.* **234**, 1733, 1959.
[2] Cecil R. & Loening U. E. - *Biochem. J.* **76**, 147, 1960.
[3] *Farmakopea Polska III*, 759, 1954.
[4] Fittkau S. - *Naturwissenschaften* **15**, 522, 1963.
[5] Kubicz A. & Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **11**, 395, 1964.
[6] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **2**, 279, 1955.
[7] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **6**, 375 and 385, 1959.
[8] Mejbaum-Katzenellenbogen W. & Dobryszycka W. - *Clin. Chim. Acta* **4**, 515, 1959.
[9] Mejbaum-Katzenellenbogen W. & Morawiecka B. - *Acta Biochim. Polon.* **6**, 453, 1959.
[10] Mejbaum-Katzenellenbogen W., Dobryszycka W. & Morawiecka B. - *Nature* **192**, 262, 1961.
[11] Morawiecki A. - *Arch. Immun. Therap. Experim.* **11**, 133, 1963.

[12] Nelson N. - *J. Biol. Chem.* **153**, 375, 1944.
[13] Ryle A. P., Sanger F., Smith L. F. & Kitai R. - *Biochem. J.* **60**, 541, 1955.
[14] Sanger F. - *Biochem. J.* **44**, 126, 1949.
[15] Supniewski J., *Preparatyka nieorganiczna*, p. 419, PWN, Warszawa 1958.

KOMPLEKSY INSULINY Z TANINĄ

Streszczenie

1. Stwierdzono, że insulina tworzy z taniną kompleksy pierwotne i maksymalnie wysycone. Z kompleksów pierwotnych można insulinę regenerować przy pomocy kofeiny, przy czym zachowuje ona pełną aktywność biologiczną.

2. Uzyskane po redukcji insuliny łańcuchy polipeptydowe A i B wykazują podobne własności w stosunku do taniny jak insulina.

3. Z łańcuchów polipeptydowych utlenionych przy pomocy kwasu nadmrówkowego jedynie łańcuch feniloalanylowy tworzy kompleksy z taniną.

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WANDA MEJBAUM-KATZENELLENBOGEN, T. WILUSZ and A. POLANOWSKI

DETERMINATION AND PREPARATION OF THE PRODUCTS OF ENZYMIC DEGRADATION OF PROTEIN

Department of Physiological Chemistry, Faculty of Pharmacy, Medical School; and Department of Biochemistry, University, Wrocław

1. Tannin was applied for studying the course of enzymic proteolysis and for isolation of end polypeptide products. 2. The course of digestion of casein, gelatin and fibrin by trypsin and pepsin was followed by estimating the decrease of protein, and the formation of polypeptides and of peptides. The polypeptides were isolated by the tannin-caffeine procedure. 3. On the basis of the obtained results, a rapid and simple micromethod for determination of trypsin and pepsin activity was elaborated.

The possibility of using tannin for studying the course of proteolysis appeared when it has been realized that tannin reacts not only with protein but also with a wide group of peptides formed during the enzymic reaction.

In acidic medium, tannin forms insoluble complexes with all proteins and with peptides of molecular weight higher than 2000 [7]. When the amount of protein is small, after addition of the tannin reagent a fine suspension is formed which can be stabilized by applying a protective colloid. The turbidity formed is proportional to the amount of protein over the range of 10 - 100 μg. per sample [4]. Without the addition of the protective colloid and at appropriate tannin concentration and pH value, the protein-tannin complex precipitates, and in this way the protein and polypeptides can be removed from the solution. By treating the sedimented protein-tannin complex with caffeine, pure protein can be recovered [5].

Both these methods were applied for studying the course of the degradation of protein by trypsin and pepsin, and for analysis by paper electrophoresis of the resistant (core) polypeptides.

Enzymic degradation of protein was studied by the tannin method in three tests: (1), Determination of the decrease in the content of protein precipitated by sulphosalicylic acid. (2), Direct determination in the incubation mixture of the compounds reacting with tannin, which are a mixture of non-digested protein and polypeptides. The decrease of the compounds reacting with tannin corresponds to the formation of the low-molecular peptides. This procedure is called the peptide test. (3), Determination of compounds reacting with tannin, present in the

filtrate after precipitation of protein with sulphosalicylic acid, called the polypeptide test.

The course of degradation of casein (2 mg./ml.) incubated for 5 min. with varying amounts of trypsin, tested by the three methods is shown in Fig. 1. Curve *1* represents the decrease of casein precipitated by 0.15 M-sulphosalicylic acid. At a trypsin concentration of 100 μg./ml., the whole casein was degraded to sulphosalicylic acid-soluble products.

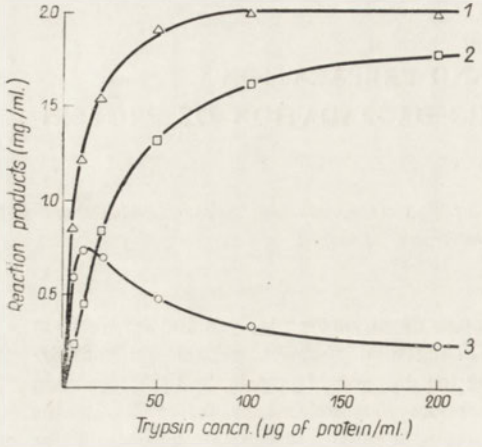

Fig. 1

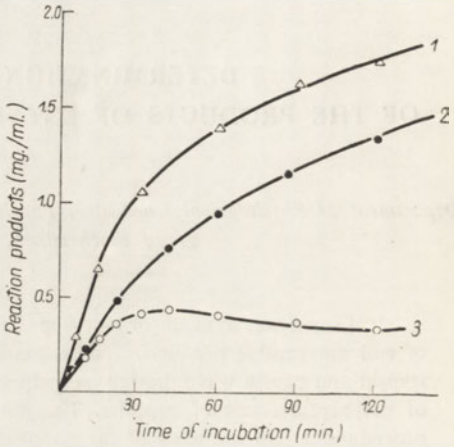

Fig. 2

Fig. 1. The effect of trypsin concentration on the rate of proteolysis of casein, estimated by three tests. (*1*), Decrease of casein; (*2*), the peptide test; (*3*), the polypeptide test. One ml. of the incubation mixture contained 2 mg. of casein in 0.1 M-phosphate buffer, pH 7.6, and increasing amounts of trypsin protein. Incubation 5 min. at 25°. For the peptide test, to 1 ml. of the incubation mixture, 9 ml. of 0.3 N-HCl was added and protein was determined (curve *2*). For casein determination and the polypeptide test, an equal amount of 0.3 M-sulphosalicylic acid was added to the incubation mixture. After 30 min. the sample was centrifuged. Protein was determined in the sediment dissolved in 0.1 N-NaOH and in the supernatant.

Fig. 2. Time-course of casein proteolysis by trypsin, estimated by three tests. (*1*), Decrease of casein; (*2*), the peptide test; (*3*), the polypeptide test. Conditions as in Fig. 1.

Curve *2* (the peptide test) represents the decrease of the compounds reacting with tannin in the non-deproteinized incubation mixture. At high enzyme concentrations, 90% of casein was digested to low-molecular peptides and 10% remained as polypeptides.

Curve *3* (the polypeptide test) represents the formation of sulphosalicylic acid -soluble peptides reacting with tannin. The maximum amount of polypeptides was found at low trypsin concentrations. With increasing enzyme concentration, the amount of polypeptides diminished indicating that the polypeptides formed during proteolysis are further degraded by trypsin to low-molecular peptides. At high enzyme concentrations, all compounds reacting with tannin present in the incubation mixture, are found in the sulphosalicylic acid filtrate; they amount to 10% of the starting protein and consist of a fraction containing bonds resistant to trypsin.

From the course of curve *3* it can be seen that the reaction rate determined for, e.g., 5 and 30 μg. of trypsin, is expressed by the same increase of polypeptides. Therefore, to determine the trypsin activity by the polypeptide test it is necessary to make two assays at different enzyme concentrations and to perform the determinations of activity under conditions which assure the linearity of the ascending part of the curve. The results of determinations of trypsin activity by the polypeptide test are presented in Table 1. In the range of 2 - 12 μg. of enzyme protein, 31 ± 2.1 μg. of polypeptides were formed per 1 μg. of trypsin.

Table 1

Determination of trypsin activity by the polypeptide test

One ml. of the incubation mixture contained 2.5 mg. of casein in 0.1 M-phosphate buffer, pH 7.6, and the amounts of trypsin protein indicated in the Table. Incubation 5 min. at 25°. The reaction was stopped by addition of an equal volume of 0.3 M-sulphosalicylic acid. After 30 min. the sample was centrifuged and in the supernatant the polypeptides were assayed by the tannin micromethod.

Trypsin (μg./ml.)	Polypeptides formed	
	(μg./ml.)	(μg./μg. of trypsin)
1.75	46	26.2
2.20	66	30.0
3.00	84	28.0
4.00	132	33.0
5.00	165	33.0
6.00	198	33.0
7.00	225	32.2
8.00	243	30.4
9.00	285	31.7
11.00	346	31.5
12.00	363	30.3
13.00	402	30.9
		Average 31.0 ± 2.1

The time-course of casein digestion with 2.5 μg. of trypsin was also studied by all three tests. The results presented in Fig. 2 indicate that for estimation of the initial reaction rate at low enzyme concentration, the polypeptide test is the most convenient one, whereas the determination of protein is charged with great experimental error due to a great excess of protein.

Similar determinations were made to study the course of digestion of casein by pepsin. It was found that the polypeptides formed are not determined proportionally by the tannin method. However, the activity of pepsin can be estimated by measuring the decrease of protein precipitated by sulphosalicylic acid or, in the initial period of the reaction, by determining in the incubation mixture the decrease of total compounds reacting with tannin. In either test, a proportional relation between the concentration of pepsin and the reaction rate was obtained.

In addition to casein, which is commonly used as substrate for studying the activity of endopeptidases, e.g. in the method of Kunitz [3], the elaborated pro-

cedure was also tested on other substrates: fibrin, which is used in clinical methods, and gelatin, applied in physico-chemical tests. These substrates differ very much from each other. Casein is soluble in water and is precipitated by trichloroacetic and sulphosalicylic acids; fibrin is insoluble in water; and gelatin, which is soluble in water, is not precipitated by the acids used for deproteinization.

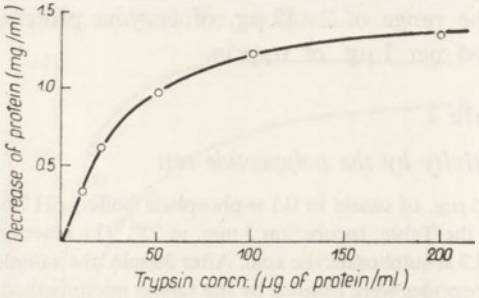

Fig. 3. The effect of trypsin concentration on the rate of proteolysis of gelatin, estimated by the peptide test. One ml. of the incubation mixture contained 2.5 mg. of gelatin in 0.1 M-phosphate buffer, pH 7.6, and increasing amounts of trypsin protein. Incubation 5 min. at 25°. The reaction was stopped by the addition of 9 ml. of 0.3 N-HCl.

With gelatin as substrate, the activity of the proteolytic enzymes can be determined by the peptide test. Figure 3 presents the course of digestion of gelatin by trypsin. At high enzyme concentrations, 60% of gelatin underwent degradation to low-molecular peptides, and even after prolonged incubation 40% remained in the form of polypeptides reacting with tannin.

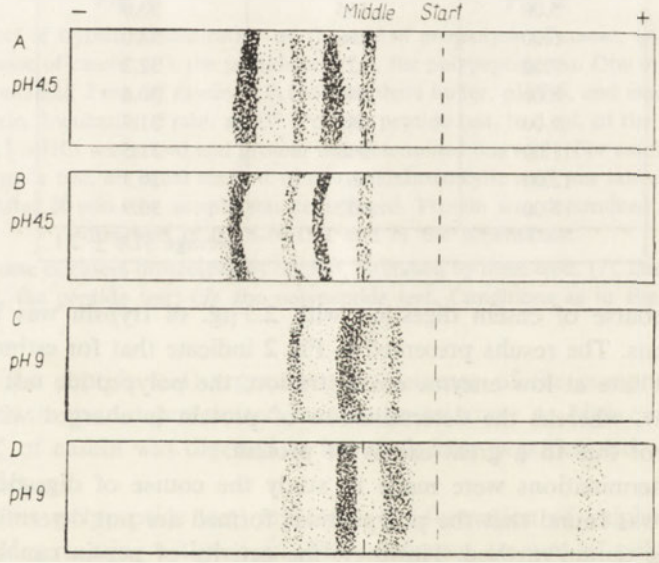

Fig. 4. Electrophoresis of end polypeptides of casein digestion by (*A*, *C*), trypsin, and (*B*, *D*), pepsin. The polypeptides were concentrated by the tannin-caffeine procedure and submitted to electrophoresis as described in Methods. Samples (*A*) and (*C*) contained in 1 ml. of the incubation mixture 2.5 mg. of casein in 0.1 M-phosphate buffer, pH 7.6, and 15 μg. of trypsin protein. Samples (*B*) and (*D*) contained in 1 ml. of the incubation mixture 2.5 mg. of casein in 0.06 N-HCl and 30 μg. of pepsin protein. After 6 hr. of incubation at 25°, the content of the end polypeptides amounted to 0.25 mg./ml.

The degradation of the insoluble fibrin by a proteolytic enzyme is expressed by its dissolution, and the digestion products can be determined by the tannin method. Trypsin digested fibrin slowly to give products reacting with the tannin reagent. Pepsin solubilized fibrin rapidly, and the polypeptides formed were further degraded to low-molecular peptides, but 20% of the initial amount of fibrin remained in the form of polypeptides resistant to pepsin. The difference between the products formed from fibrin under the action of trypsin and of pepsin suggests that fibrin could be used for differentiation of the specificity of the two enzymes.

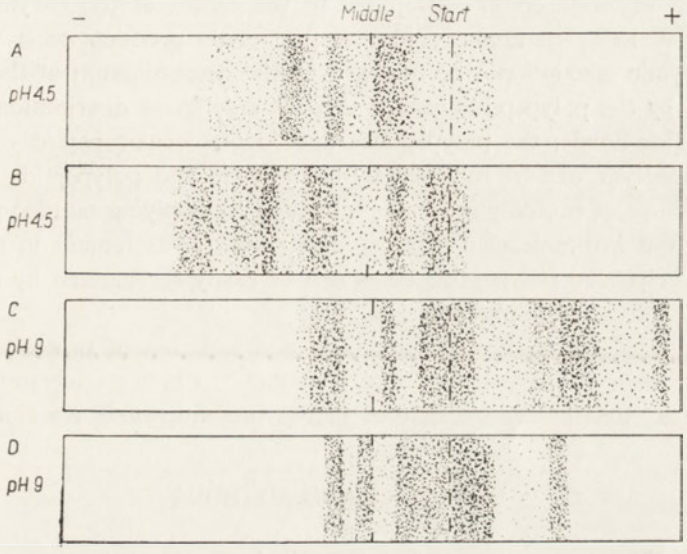

Fig. 5. Electrophoresis of end polypeptides of fibrin digestion by (*A* and *C*), trypsin, and (*B* and *D*), pepsin. After 20 hr. of incubation with the enzyme at room temperature, the polypeptides were concentrated by the tannin-caffeine procedure and submitted to electrophoresis as described in Methods. Samples (*A*) and (*C*) contained in 1 ml. of the incubation mixture 4 mg. of fibrin suspended in 0.1 M-phosphate buffer, pH 7.6, and 100 μg. of trypsin protein. Samples (*B*) and (*D*) contained in 1 ml. of the incubation mixture 4 mg. of fibrin in 0.06 N-HCl and 30 μg. of pepsin protein.

The polypeptides remaining after exhaustive proteolysis of casein or fibrin were concentrated by the tannin-caffeine procedure [5], and submitted to electrophoresis at pH 4.5 and 9. Figure 4 *A* and *C* presents the polypeptide fractions of casein obtained by trypsin digestion, and Fig. 4, *B* and *D*, by pepsin digestion. At pH 4.5, four cathodic fractions were obtained, both after pepsin and trypsin digestion, whereas at pH 9 a fast-moving anodic fraction appeared after pepsin digestion.

The electrophoretogram of the polypeptide fraction obtained from fibrin after 20 hr. of incubation at room temperature, is shown in Fig. 5. At pH 4.5, poorly resolved cathodic fractions were obtained both after trypsin and pepsin digestion (Fig. 5, *A*, *B*). At pH 9 the peptides released by trypsin (Fig. 5, *C*) separated into 6 fractions, 3 of which migrated towards the anode and 3 to the cathode. After

pepsin digestion at pH 9 (Fig. 5, *D*), one fraction migrated towards the anode, a group of fractions displayed low anodic mobility and another group migrated towards the cathode.

The application of tannin for studying the course of proteolysis permits to determine the activity of proteases toward any protein, and to evaluate the type of the products formed. By performing the three presented tests, not only the activity and, to a certain degree, the specificity of the enzyme can be determined, but also some information concerning the structure of protein could be obtained. When the substrate contains bonds susceptible to the action of the enzyme spaced at intervals of at least ten amino acids, all proteolysis products react with tannin, and the enzymic reaction can be followed by the determination of the undigested protein and by the polypeptide test. If there is a different distribution of the susceptible peptide bonds, the polypeptides and low-molecular peptides are formed, and the proteolysis can be followed by the peptide and polypeptide tests.

The products of proteolysis can be separated by applying tannin; polypeptides precipitate, and low-molecular peptides and amino acids remain in the supernatant. The precipitated polypeptide cores can be easily regenerated by caffeine and submitted to further examination.

Application of tannin for determination and isolation of proteolysis products can facilitate their further analysis and contribute to better understanding of the enzyme activity and of the mechanism of the protein-protein reactions.

MATERIALS AND METHODS

Enzymes. The trypsin preparation (Merck A. G., Darmstadt, Germany) contained 1.9% of protein and 20 000 Fuld-Gross units per gram; the pepsin preparation (Wytwórnia Fermentów, Warszawa, Poland) contained 0.3% of protein. The activity of the enzymes was tested by the Anson method [1].

Substrates. Soluble white casein was a product of B. D. H. (Poole, England); gelatin was a product of G. Grübler (Leipzig, Germany); fibrin was obtained from ox blood by recalcification of oxalized blood plasma. The clot was washed with water until the washings gave no reaction for protein. For determinations of trypsin activity, casein and gelatin were freshly dissolved and fibrin suspended in water, and mixed with an equal volume of 0.2 M-phosphate buffer, pH 7.6. For determinations of pepsin activity, solutions or suspension in 0.06 N-HCl were used.

Reagents. Tannic acid (Politechnika Śląska, Gliwice, Poland), no.contr. 1/3/61. Other chemicals were of analytical grade.

Determinations of protein and polypeptides were made by the tannin micromethod [4].

Concentration of polypeptides. The end products of exhaustive digestion of casein and fibrin by trypsin or pepsin were concentrated by the tannin-caffeine procedure [5]. The polypeptides were precipitated at pH 5 with a twofold amount of tannin, and regenerated at pH about 3 with a twofold amount of caffeine in

relation to protein. About 1% polypeptide solutions were obtained with a yield of 80%.

Paper electrophoresis of the concentrated proteolysis products was carried out on Schleicher-Schüll no. 2043a paper (3 × 36 cm.) in 0.1 M-acetate buffer, pH 4.5 and in the veronal buffer according to Dittmer [2] at pH 9; time of separation 6 hr., potential 200 v. On the paper strip, 0.5 mg. of polypeptides was placed 2 cm. from the middle in the direction of the anode. The electrophoretograms were stained with bromophenol blue dissolved in the tannin reagent [6].

Determination of trypsin and pepsin activity by the peptide test

On the basis of the obtained results, a rapid and simple micromethod for determination of trypsin and pepsin activity was elaborated; it is hoped that the procedure described below will be suitable for serial determinations in laboratory conditions.

Table 2

Determination of activity of trypsin and pepsin by the peptide test

One ml. of the incubation mixture contained 80 μg. of casein in 0.1 M-phosphate buffer, pH 7.6, and the amounts of trypsin protein indicated in the Table; or 80 μg. of casein in 0.06 N-HCl and the amounts of pepsin protein indicated in the Table. Incubation 5 min. at 30°. Mean values from 5 experiments are given.

Trypsin (μg./ml.)	Decrease of protein (μg./ml.)		(μg./μg. of trypsin)	Pepsin (μg./ml.)	Decrease of protein (μg./ml.)		(μg./μg. of pepsin)
	Mean	S. D.			Mean	S. D.	
0.25	5.5	1.29	22.0	0.5	6.35	0.88	12.6
0.50	10.3	1.71	20.6	1.0	10.8	0.52	10.8
0.75	14.5	1.29	19.4	1.5	18.0	1.38	12.0
1.00	18.8	1.92	18.8	2.0	22.8	1.54	11.4
1.25	21.8	0.96	17.5	2.5	26.5	0.45	10.7
1.50	27.5	1.04	18.3	3.0	30.7	1.00	10.2
1.75	31.0	0.82	17.7	3.5	34.3	1.82	9.8
2.00	33.0	1.82	16.5	4.0	37.2	0.77	9.0
2.50	37.0	0.82	14.8	4.5	40.3	0.67	9.0
3.00	42.0	1.41	14.0	5.0	44.2	1.86	9.0

The enzymic reaction is carried out at 30° at protein concentration not exceeding 100 μg./ml. of the incubation mixture. The reaction is stopped by adding the tannin reagent, and simultaneously the determination of protein is started.

For trypsin determination, 0.9 ml. of freshly prepared 0.01% solution of casein is placed in a water bath at 30° and after temperature equilibration 0.1 ml. of trypsin solution (10 μg. of protein/1 ml.), is added. After 5 min. the incubation is stopped by adding 1 ml. of the tannin reagent (10% tannin solution in 1 N-HCl, containing 2% of phenol) which was heated to 30°, and the whole is mixed. After 10 min., 2 ml. of 0.1% arabic gum solution is added. Then the sample is taken out from the

water bath, mixed, cooled to room temperature, and the extinction is read at 610 mμ (red filter, S 61), zero of the apparatus being adjusted to the reagent blank. Simultaneously a control is prepared, in which to the substrate solution first the tannin reagent and then the enzyme is added. The amount of protein in the proper and control samples is read from the standard curve, the difference corresponding to the activity of the enzyme. If the decrease of protein is greater than 30 μg., the determination should be repeated taking a smaller amount of the enzyme. If the difference is smaller than 10 μg., the determination should be repeated with a greater amount of the enzyme.

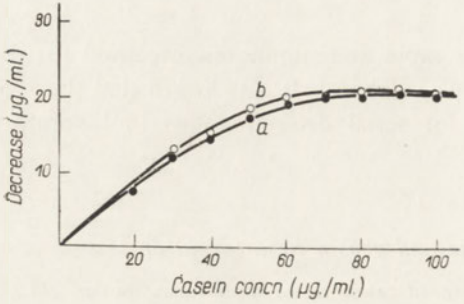

Fig. 6

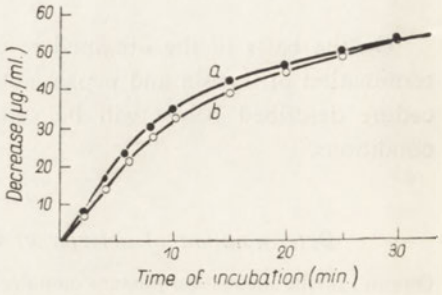

Fig. 7

Fig. 6. The effect of substrate concentration on the rate of proteolysis of casein, determined by the peptide test. (*a*), One ml. of the incubation mixture contained 20 - 100 μg. of casein in 0.1 M--phosphate buffer, pH 7.6, and 1 μg. of trypsin protein. (*b*), One ml. of the incubation mixture contained 20 - 100 μg. of casein in 0.06 N-HCl and 1.5 μg. of pepsin protein. Incubation 5 min. at 30°. The reaction was stopped by the addition of 1 ml. of the tannin reagent, and compounds reacting with tannin were determined. Mean values of 5 determinations are given.

Fig. 7. Time-course of digestion of casein by trypsin and pepsin, determined by the peptide test. (*a*), One ml. of the incubation mixture contained 80 μg. of casein in 0.1 M-phosphate buffer, pH 7.6, and 1 μg. of trypsin protein; (*b*), 1 ml. of the incubation mixture contained 80 μg. of casein in 0.06 N-HCl and 1.5 μg. of pepsin protein. Mean values of 5 determinations are given.

For pepsin determination the same procedure is applied except that casein dissolved in 0.06 N-HCl is used as substrate, and pepsin is dissolved in 0.06 N-HCl.

Using the peptide test, the effects of substrate and enzyme concentration on the reaction rate were studied. From the data presented in Table 2 it appears that at trypsin concentrations of 0.25 - 1.8 μg., and of pepsin below 3 μg. per 1 ml., the reaction rate was in linear relation to the concentration of the enzyme in the incubation mixture. The average decrease of casein per 1 μg. of trypsin was 18.7 ± ± 1.1 μg., and per 1 μg. of pepsin, 11 ± 0.8 μg.

The effect of casein concentration is shown in Fig. 6. In the range of 60 - 100 μg. of casein per 1 ml., the reaction for either enzyme was of zero-order. The time--course of digestion of casein with 1 μg. of trypsin or 1.5 μg. of pepsin is shown in Fig. 7.

REFERENCES

[1] Anson M. L. - *J. Gen. Physiol.* **22**, 79, 1938.
[2] Dittmer A., *Papierelektrophorese*, p. 58. 2nd ed., G. Fischer Verlag, Jena 1961.
[3] Kunitz M. - *J. Gen. Physiol.* **30**, 291, 1947.
[4] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **2**, 279, 1955.
[5] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **6**, 375 and 385, 1959.
[6] Mejbaum-Katzenellenbogen W. & Dobryszycka W. - *Clin. Chim. Acta* **4**, 515, 1959.
[7] Mejbaum-Katzenellenbogen W. & Lorenc-Kubis I. - *Acta Biochim. Polon.* **13**, 77, 1966.

OZNACZANIE I PREPARACJA PRODUKTÓW ENZYMATYCZNEJ DEGRADACJI BIAŁEK

Streszczenie

1. Przedstawiono możliwości zastosowania taniny do badania przebiegu proteolizy, zagęszczania polipeptydowych produktów enzymatycznego trawienia białek oraz oznaczania aktywności enzymów proteolitycznych.

2. Badano przebieg trawienia kazeiny, żelatyny i fibryny za pomocą trypsyny i pepsyny. Oznaczano ubytek substratu oraz przyrost peptydowych i polipeptydowych produktów reakcji. Polipeptydy zagęszczano taniną i regenerowano kofeiną.

3. Na podstawie uzyskanych wyników opracowano mikrometodę oznaczania aktywności trypsyny i pepsyny w warunkach prostego testu.

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K. TAYLOR

PHYSICAL AND CHEMICAL CHANGES OF Vi-POLYSACCHARIDE DUE TO Vi-PHAGE II ACTION

The Biochemical Laboratory, Institute of Marine Medicine, Gdańsk

1. Vi-phage II treated Vi-polysaccharide deprived of receptor activity, was compared with the initial preparation. The treatment did not cause depolymerization but resulted in a decrease of *N*-acetyl groups and loss of *O*-acetyl groups. 2. The loss of the ester bond was confirmed by infrared-absorption analysis. The acetic acid split off by the phage was identified by chromatography as acetohydroxamate. 3. The phage-treated Vi-polysaccharide regained the receptor activity after acetylation and lost it again after second phage treatment.

The reaction between Vi-phage II and Vi-polysaccharide leading to the loss of the receptor activity (phage-binding ability) of the polysaccharide was thought to be of an enzymic character [4, 13]. Previously, the general conditions of this reaction (ionic medium, pH, temperature, inhibitors) have been established [13], and a method for the isolation of considerable quantities of Vi-polysaccharide was elaborated [12]. The aim of the present work was to study physical and chemical changes of Vi-polysaccharide due to Vi-phage II action. The main results have been presented [14].

MATERIALS AND METHODS

The purified Vi-phage II preparation was obtained as described previously [13]. For control experiments, the phage unable to destroy the receptor activity of the polysaccharide was used. It was obtained by an overnight incubation at 37° in 0.15 M-NaCl - 0.01 M-phosphate buffer, pH 7.2, containing 5% formaldehyde, followed by 2 min. heating in a boiling-water bath and dialysis against an appropriate salt solution (Fig. 3c). The omission of the incubation with formaldehyde resulted in the ejection of the nucleic acid from the phage head (Fig. 3b) causing a drastic increment of viscosity. Fig. 3a represents the native phage preparation.

Vi-polysaccharide was isolated from *Escherichia coli* 5396/38 as described previously [12]. To obtain its sodium salt free from divalent cations, the Vi-polysaccharide was additionally dialysed against 0.01 M-Na-EDTA, pH 7.0, then against

bidistilled water saturated with chloroform, and the final product was freeze-dried.

Reagents used: EDTA (Light, Colnbrook, England); *p*-chloromercuribenzoic acid, crystalline bovine serum albumin, and acetylcholine (B. D. H., Poole, England); tubings for dialysis (Kalle, Wiesbaden, West Germany); Special Agar-Noble (Difco, Detroit, U.S.A.). Other reagents were products of Fabryka Odczynników Chemicznych (Gliwice, Poland).

Vi-phage II and Vi-receptor activity were estimated by the methods previously described [13, 17].

Acetyl groups (total) were determined according to Pregl & Roth [10], after 4 hr. hydrolysis in *p*-toluenesulphonic acid on a boiling-water bath. *O*-Acetyl groups were determined according to Hestrin [3], using acetylcholine as a standard. Protein was determined according to Lowry *et al.* [7], using crystalline bovine serum albumin as a standard. The turbidimetric test with albumin for acid polysaccharides was performed according to Webster *et al.* [19].

Agar electrophoresis of Vi-polysaccharide was performed in Wieme's apparatus, using light petroleum cooling [21]. The preparation was introduced to a well in agar, made as follows. A mould made of a brass sheet (10 mm. broad, 0.5 mm. thick) coated with paraffin was placed on a horizontal glass plate. Special Agar-Noble (0.8%) dissolved in the appropriate buffer was poured at 65° on the glass plate to form a layer 2 mm. thick, and left overnight in a moist box at room temperature. Then the mould was carefully removed and the well was washed with light petroleum. If the well was of a different shape or had been cut out of the agar, the trailing of Vi-polysaccharide during electrophoresis was observed. At concentrations higher than 0.5 mg./ml. additional false bands appeared and the outline of the polysaccharide zone became irregular. This disturbance was due to the electronegative character of Vi-polysaccharide which caused a local increase of the electro-osmotic flow in the agar to such an extent that, at concentrations exceeding 1 mg./ml., a depression in the agar moving towards the anode and corresponding to Vi-polysaccharide, could be observed. A voltage of 10 v/cm. was applied, usually for 40 or 80 min. Vi-polysaccharide was located by dipping the electrophoretogram in 0.2% bovine albumin solution in 0.1 M-acetate buffer, pH 4.0. The turbid bands corresponding to Vi-polysaccharide appeared already after 2 hr., but they were photographed the next day, in the dark-field illumination. The electrophoretograms immersed in 0.1 M-acetate buffer, pH 4.0, could be kept unaltered for several months.

Free electrophoresis was carried out in the Kern LK apparatus, sedimentation in the analytical ultracentrifuge Spinco model E. Specific viscosity ($\eta/\eta_0 - 1$) was estimated in a capillary viscometer, ultraviolet absorption in the Spektromom 201 (Hungary) spectrophotometer. Infrared-absorption spectrum of Vi-polysaccharide (in KBr discs) was estimated in the Perkin-Infracord SP-200 spectrophotometer.

RESULTS

Preliminary experiments

Koziński & Opara [4] could not find any serological difference between Vi-antigen and the same substance deprived of receptor activity by Vi-phage II action, hence it was at first assumed that the phage causes only a slight change in the structure of Vi-polysaccharide. Different Vi-polysaccharide preparations were found to possess the same, unidentified ninhydrin-positive components [12] which could be covalently bound with the polysaccharide. These components appeared in slight quantities and so, if one of them would be split-off by the phage this would not markedly change the serological properties of the polysaccharide. To verify this supposition, Vi-polysaccharide was incubated with purified Vi-phage II preparation in 0.1 M-ammonium acetate, pH 7.0, at 37°, under toluene until the receptor activity of the polysaccharide was lost. The phage was removed by centrifugation and the supernatant was separated by Sephadex G-25 filtration into two fractions: the high-molecular (polysaccharide) and the low-molecular one (ammonium acetate and presumably the digestion products). After acid hydrolysis of the high-molecular fraction and removal of ammonium acetate from the low-molecular fraction *in vacuo*, both fractions were examined by thin-layer chromatography. No ninhydrin-positive component appeared in the low-molecular fraction, and all the components of the high-molecular fraction remained unaltered.

Phage action could also consist in partial depolymerization of the polysaccharide to large fragments. In this case, the quantity of new terminal groups would be small and not easy to detect by serological methods. Therefore an attempt was made to verify this supposition by viscosimetry. The viscosity of the polysaccharide decreased during incubation with the phage (Fig. 1) in solutions containing considerable quantities (0.025 - 0.1 M) of magnesium or calcium ions. In the reaction of the polysaccharide with albumin, during incubation with the phage an increase of turbidity was observed. Both these phenomena might be regarded as manifestations of the splitting of the polysaccharide chain into large fragments. However, on agar electrophoresis no new fractions appeared. In the Mg^{2+} or Ca^{2+} containing medium, the phage-treated polysaccharide showed a diminished electrophoretic mobility (Fig. 4). The polysaccharide preparation consisting exclusively of the slower-moving fraction maintained its homogeneity (and showed a diminished electrophoretic mobility) following the incubation with the phage. The change of the properties of the polysaccharide which could be detected by the turbidimetric reaction and by agar electrophoresis was compared with the decrease of the receptor activity (Fig. 2).

The influence of the incubation with the phage on the polysaccharide viscosity diminished when lower concentrations of divalent cations were applied. On agar electrophoresis in the presence of EDTA no differences were found between the initial and the phage-treated preparation; under these conditions the mobility of both preparations was much greater than in the presence of divalent cations.

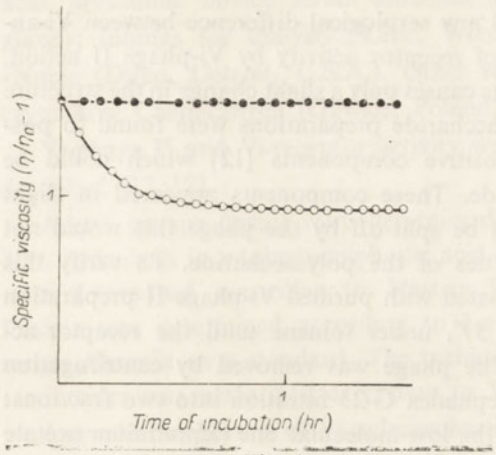

Fig. 1

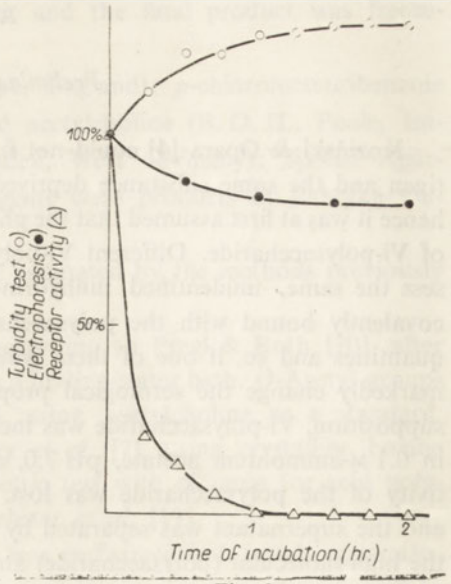

Fig. 2

Fig. 1. Specific viscosity of Vi-polysaccharide (2 mg./ml.): (○), incubated with Vi-phage II (2 × × 10^{12}/ml.) and (●), incubated with inactivated Vi-phage II (2 × 10^{12}/ml.) at 37°, in 0.05 M--HCl-veronal buffer, pH 7.6 (37°), containing 0.05 M-calcium acetate and 10^{-4} M-*p*-chloromercuribenzoate.

Fig. 2. Incubation of Vi-polysaccharide (2 mg./ml.) with Vi-phage II (2 × 10^{12}/ml.) at 37° in 0.05 M-HCl - veronal buffer, pH 7.6 (37°), containing 0.01 M-$MgSO_4$ and 10^{-4} M-*p*-chloromercuribenzoate. (○), Turbidity with albumin; (●), distance of the front of the polysaccharide zone from the start line in agar electrophoresis (10 v/cm., 80 min., HCl - veronal buffer, pH 8.4, I 0.05, containing 0.025 M-calcium acetate); and (△) receptor activity were estimated. The initial values amounted to 1400 ATU/ml. for the turbidimetric test, 12 mm. for the electrophoresis, 480 RU/ml. for the receptor activity. The formolized and heated phage preparation did not change the examined properties of the polysaccharide.

These observations seem to indicate that Vi-polysaccharide forms complexes with divalent cations, and that this ability is affected by the incubation with the phage. This supposition has been confirmed experimentally (to be published) and therefore in the further part of this paper the physico-chemical properties of the initial and the phage-treated polysaccharide are compared only in the media free from divalent cations.

Preparation of the phage-treated polysaccharide

Vi-polysaccharide (2 mg./ml.), a preparation homogeneous in agar electrophoresis, was incubated with Vi-phage II (2 × 10^{12}/ml.) in 0.01 M-$MgSO_4$ - 0.05 M-HCl--veronal buffer, pH 7.6 (37°), containing 10^{-4} M-*p*-chloromercuribenzoate, at 37°, for 4 hr. The phage was removed by centrifugation for 1 hr. at 16 000 *g*. The phage maintained its infectivity and the supernatant did not show increased absorption at 260 mμ. Hence it may be concluded that ejection of the phage nucleic acid did

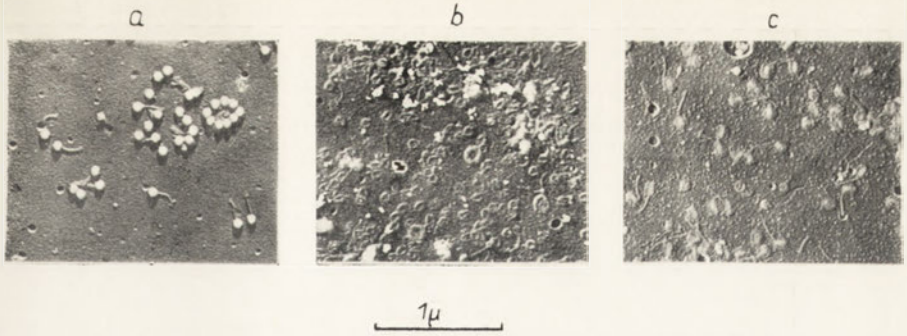

Fig. 3. Vi-phage II preparations: (*a*), native; (*b*), heated; (*c*), formolized and heated; explanations in the text. Agar-diffusion and chromium shadowing.

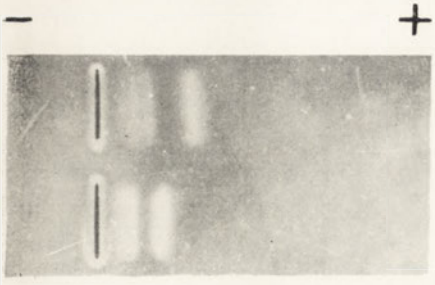

Fig. 4. Agar electrophoresis of Vi-polysaccharide (0.8 mg./ml.), a preparation separating into two fractions: above, before incubation; below, after incubation with Vi-phage II. HCl - veronal buffer, pH 8.4, *I* 0.05, containing 0.025 M-calcium acetate; 10 v/cm., 40 min. The electrophoretogram was developed in 0.2% bovine albumin solution in 0.1 M--acetate buffer, pH 4.0, and photographed in the dark-field illumination.

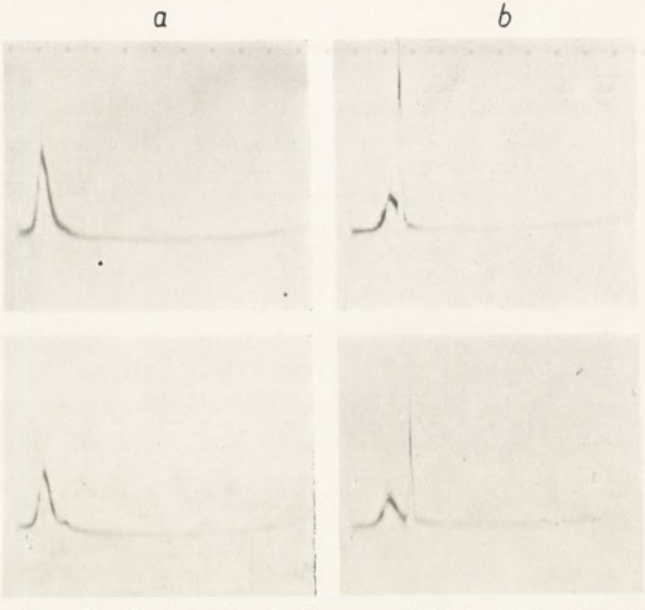

Fig. 5. Sedimentation of initial (above) and phage-treated (below) Vi-polysaccharide (2 mg./ml.) in (*a*) 0.05 M-HCl-veronal buffer, pH 7.8; and (*b*), 0.1 M-acetate buffer, pH 4.7, in analytical ultracentrifuge Spinco model E. Photographs were taken after 80 min. centrifugation at 56 100 rev./min. at 18°. Centrifugation from left to right.

Fig. 6. Free microelectrophoresis in Kern LK apparatus of initial (above) and phage treated (below) Vi-polysaccharide (5 mg./ /ml.) in acetate buffer, pH 4.7, *I* 0.05. Photographs were taken after 20 min. at 5 v/cm.

not occur during incubation and the polysaccharide has not been contaminated by nucleic acid. The supernatant was dialysed for 24 hr. against 10 volumes of bidistilled water (the diffusate being kept for further experiments), followed by dialysis against 0.01 M-Na-EDTA, pH 7.0, and against bidistilled water saturated with chloroform. The obtained preparation was freeze-dried yielding the phage--treated polysaccharide. The initial polysaccharide contained slight amounts of substances reacting with the Folin-Ciocalteu reagent which, calculated as a protein contamination, amounted to 0.6%. This value increased to 2.0% in the phage--treated polysaccharide.

Comparison of physical and chemical properties

The specific viscosity (Table 1) and the sedimentation in the analytical ultracentrifuge (Fig. 5) of the initial and phage-treated polysaccharides were assayed in 0.05 M-HCl-veronal buffer, pH 7.8, and in 0.1 M-acetate buffer, pH 4.7. The results of sedimentation analysis are difficult to interpret on account of the lack of basic studies on the sedimentation of acid polysaccharides [1]. In general, at pH 4.7 Vi-polysaccharide sedimented a little faster than at pH 7.8; this property has been observed earlier by Webster *et al.* [20] and was accounted for by aggregation. The presence of two peaks at pH 4.7 may be due to aggregation although it cannot yet be excluded that the Vi-polysaccharide examined consists of two fractions differing in molecular weight. This, however, is of no importance for the present work. On the other hand, it should be emphasized that both at pH 4.7 and 7.8 the sedimentation of the phage-treated polysaccharide was not slower than that of the initial polysaccharide, and that in the phage-treated preparation no new slower-sedimenting fractions could be found. Thus the sedimentation analysis together with the comparison of specific viscosity excluded the possibility of depolymerization of the polysaccharide by the phage.

Table 1

Specific viscosity $(\eta/\eta_0 - 1)$ *at 20° of the initial and phage-treated Vi-polysaccharide* (1 mg./ml.)

	Initial	Phage-treated
0.05 M-HCl - veronal buffer, pH 7.8	0.43	0.53
0.1 M-acetate buffer, pH 4.7	0.69	0.51

In free electrophoresis in HCl - veronal buffer, pH 8.4, I 0.05, both preparations had the same mobility but in acetate buffer, pH 4.7, I 0.05, the phage-treated polysaccharide seemed to move slightly slower than the initial one (Fig. 6). At pH values lower than 4.7 the phage-treated preparation, unlike the initial one, dissolved poorly.

It seems possible to explain this behaviour of the phage-treated polysaccharide by the decrease in the negative charge of the molecule. This effect could be due

to the liberation of free amino groups. The results of analyses (Table 2) confirm this supposition. The contents of *N*-acetyl groups, calculated by subtracting *O*-acetyl from the total contents of acetyl groups, amounted to 9.6% in the initial polysaccharide and to 2.1% in the phage-treated one. Moreover, it appeared that although the initial polysaccharide contained 8.4% of *O*-acetyl groups, the product of phage action was practically deprived of them (0.8%). The loss of the ester bond (*O*-acetyl group) was confirmed by the infrared-absorption analyses. To avoid interference

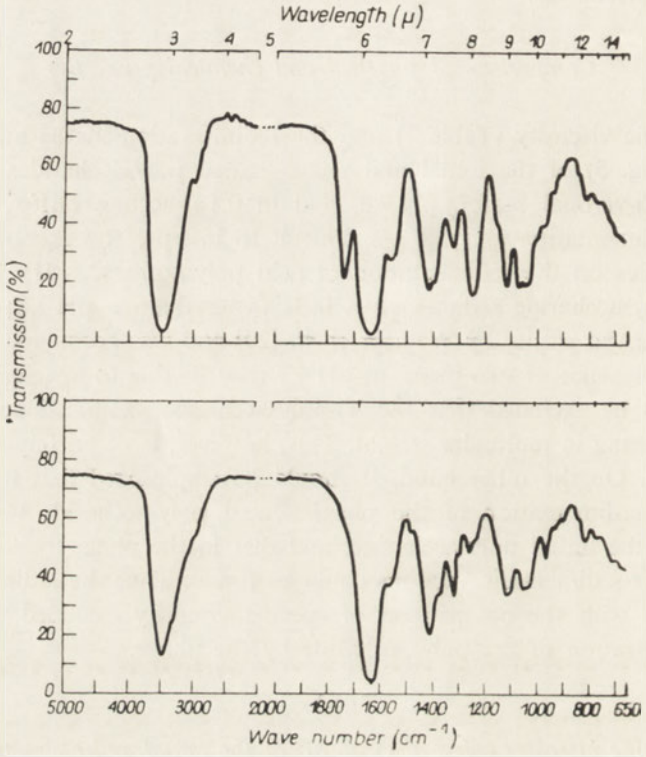

Fig. 7. Infrared absorption spectra of initial (above) and phage-treated (below) Vi-polysaccharide (sodium salt) in KBr. Perkin-Infracord SP-200 spectrophotometer.

caused by un-ionized carboxyl groups, sodium salts of the polysaccharides were used. The absorption peaks at 1740 cm.$^{-1}$ and 1240 cm.$^{-1}$ characteristic of the ester bond disappeared after incubation with the phage (Fig. 7). The nitrogen contents (Table 2) were in agreement with the presented results; the value calculated for sodium salt of deacetylated polyaminogalacturonic acid amounted to 6.76% and for sodium salt of polyaminogalacturonic acid containing two acetyl groups (one *O*- and one *N*-) per one sugar residue, 4.98%.

A formolized and heated phage preparation did not cause deacetylation of Vi-polysaccharide.

Table 2

Properties of initial and phage-treated Vi-polysaccharide

	Initial	Phage-treated
Receptor activity (units/mg.)	240	<1
Acetyl groups, total (%)	18.0	2.9
O-Acetyl groups (%)	8.4	0.8
N (%)	4.9	6.5

Identification of acetic acid

Acetic acid, which is split off and passes into the diffusate during the preparation of the phage-treated polysaccharide, was identified in the following way. The diffusate was concentrated to 5 ml. *in vacuo*, acidified with hydrochloric acid and left overnight in the refrigerator. The precipitated diethylbarbituric acid was removed by filtration, and 10 ml. of saturated sodium sulphate solution and 1 ml. of conc. sulphuric acid were added to the filtrate. After steam distillation, the distillate was neutralized with 1 N-NaOH to pH 12, concentrated and dried at 110°. The residue was used for the preparation of methyl ester and, subsequently, of hydroxamate, according to Block, Durrum & Zweig [2]. The product was chromatographed on Whatman no. 1 paper in the following solvent systems: (1), pentan-1-ol - formic acid - water (75:25:75, by vol.); (2), pentan-1-ol - acetic acid - water (4:1:5, by vol.); (3), butan-1-ol - acetic acid - water (4:1:5, by vol.); (4), phenol saturated with water. The chromatograms were sprayed with a solution of ferric chloride [2]. In all the solvent systems the substance examined moved as acetohydroxamate. In phenol saturated with water an additional unidentified spot, moving more slowly, was detected.

Acetylation

The phage-treated Vi-polysaccharide deprived of receptor activity and containing 2.9% of acetyl groups (including 0.8% of *O*-acetyl groups) was acetylated in the following way [8, 9]. The polysaccharide, dried *in vacuo* over P_2O_5 (40 mg.) was dissolved in 10 ml. of formamide by stirring for 2 hr. at 30°. Dry pyridine was slowly added to this solution with constant mixing; after addition of 8 ml. of pyridine the polysaccharide began to precipitate in the gel-form. Then 7 ml. of cool acetic anhydride was added to the cooled mixture with constant shaking. The mixture was kept for an hour at room temperature, then overnight at 30°. To the brown solution 12 ml. of pyridine and 11 ml. of acetic anhydride were added and the whole was again kept overnight at 30°. A part of the solvents was removed from the dark brown solution by distillation *in vacuo* carried out at a temperature lower than 35°. The residue was mixed with ice and dialysed against bidistilled water. The larger part of the colouring matter passed into the diffusate or underwent precipitation during dialysis. After filtration the solution was freeze-dried.

The acetylated preparation contained 18.3% of acetyl groups (including 8.3% of *O*-acetyl groups) and possessed the receptor activity amounting to about 80% of the activity of the initial polysaccharide (Table 3).

Table 3

Properties of phage-treated, acetylated Vi-polysaccharide

The phage-treated Vi-polysaccharide preparation was acetylated by acetic anhydride; then the same preparation was submitted again to phage-treatment.

	Phage-treated, acetylated	Phage-treated, acetylated, phage-treated
Receptor activity (units/mg.)	200	<2
Acetyl groups, total (%)	18.3	5.4
O-Acetyl groups (%)	8.3	1.7

The phage-treated and subsequently acetylated polysaccharide was submitted again to the phage action under the same conditions (Table 3). This treatment gave a preparation deprived of receptor activity and containing 5.4% of acetyl groups (including 1.7% of *O*-acetyl groups).

DISCUSSION

The reaction between Vi-phage II and Vi-polysaccharide leading to the loss of the receptor activity of the polysaccharide, was shown to consist in enzymic deacetylation of the polysaccharide by the phage. Thus it appears that the receptor activity of Vi-polysaccharide consists in its ability to bind phage enzyme. The electron micrographs [15] showing the adsorption of the phage on erythrocyte membranes coated with Vi-polysaccharide represent the binding of the substrate with the phage enzyme. This enzyme is situated on the tip of the phage tail, probably in the spikes found by Kwiatkowski [5], similarly to neuraminidase which according to Schäfer [11] seems to be localized in the spikes present on the surface of viruses of the influenza group.

The preparation of Vi-polysaccharide deprived of *O*-acetyl groups and partially depolymerized [6] obtained by Webster *et al.* [18] did not possess receptor activity [12]. It seems possible that, as suggested by Webster (private communication) the presence of *O*-acetyl groups is indispensable for the binding of phage enzyme with Vi-polysaccharide. *O*-Acetyl as well as *N*-acetyl groups are split-off hydrolytically by the enzyme of Vi-phage II; its mode of action is unknown but *O-N* transacetylation, with the possibility of the acetyl transfer on the water molecule, seems to be the most probable supposition.

From the quantity of Vi-II phages, 10^{11}, bound in the cold by 1 μg. of Vi-polysaccharide adsorbed on erythrocytes [17] it follows that one phage is bound by a quantity of polysaccharide corresponding to the molecular weight of 6×10^6.

As the molecular weight of Vi-polysaccharide is of the order of 10^6 [20] it appears that the phage is bound by at least one polysaccharide particle. In the cold the phage-polysaccharide linkage lasts several minutes [17] and after elution of the phage the receptor activity of the polysaccharide is completely lost. It seems therefore that, once bound with a polysaccharide particle, the phage can be detached only after having split off all the accessible acetyl groups. It is possible that during deacetylation Vi-phage II móves along the polysaccharide chain; this phenomenon could have some significance in the process of phage penetration through the bacterial cell wall [16].

The author wishes to express his gratitude to Prof. Dr. Z. Buczowski for his constant stimulating interest during the course of this work, and to thank Dr. Alina Taylor for the Vi-polysaccharide preparations, and Mr. B. Kwiatkowski, M. Sc., for the electron micrographs. Thanks are also due for making available the apparatus to Prof. Dr. D. Shugar and Dr. I. Niedźwiecka (the apparatus for free electrophoresis), Dr. T. B. Grela (the analytical centrifuge) and Doc. Dr. J. Sokołowski (the infrared spectrophotometer). The valuable technical assistance of Mrs. J. Starczewska, Mrs. J. Żabina and Miss A. Pióro is greatly appreciated.

REFERENCES

[1] Banks W. & Greenwood C. T. - *Advanc. Carbohydrate Chem.* **18**, 357, 1963.
[2] Block R. J., Durrum E. L. & Zweig G., *Paper chromatography*. Academic Press, New York 1955.
[3] Hestrin S. - *J. Biol. Chem.* **180**, 249, 1949.
[4] Koziński A. W. & Opara Z. - *Bull. Acad. Polon. Sci. Ser. Biol.* **2**, 39, 1954.
[5] Kwiatkowski B. - *Acta Microbiol. Polon.*, **15**, no. 1, 1966.
[6] Landy M., Trapani R. J., Webster M. E. & Jarvis F. G. - *Texas Rep. Biol. Med.* **21**, 214, 1963.
[7] Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. - *J. Biol. Chem.* **193**, 265, 1951.
[8] Masamune H., Sinohara H. & Okuyama T. - *Tohoku J. Exp. Med.* **68**, 165, 1958.
[9] Perkins H. R. - *Biochem. J.* **86**, 475, 1963.
[10] Pregl F. & Roth H., *Quantitative organische Mikroanalyse*. Springer Verlag, Wien 1958.
[11] Schäfer W. - *Bact. Rev.* **27**, 1, 1963.
[12] Taylor A. & Taylor K. - *Acta Biochim. Polon.* **12**, 123, 1965.
[13] Taylor K. - *Acta Biochim. Polon.* **12**, 157, 1965.
[14] Taylor K. - *Biochem. Biophys. Res. Commun.*, **20**, 752, 1965.
[15] Taylor K. & Kwiatkowski B. - *Acta Microbiol. Polon.* **12**, 107, 1963.
[16] Taylor K. & Kwiatkowski B. - *Acta Microbiol. Polon.*, **15**, no. 1, 1966.
[17] Taylor K. & Taylor A. - *Acta Microbiol. Polon.* **12**, 97, 1963.
[18] Webster M. E., Landy M. & Freeman M. E. - *J. Immunol.* **69**, 135, 1952.
[19] Webster M. E., Sagin J. F. & Freeman M. E. - *Proc. Soc. Biol. Med.* **81**, 263, 1952.
[20] Webster M. E., Sagin J. F., Anderson P. R., Breese S. S., Freeman M. E. & Landy M. - *J. Immunol.* **73**, 16, 1954.
[21] Wieme R. J. - *Clin. Chim. Acta* **4**, 317, 1959.

FIZYCZNE I CHEMICZNE ZMIANY WIELOCUKRU Vi SPOWODOWANE DZIAŁANIEM FAGA Vi II

Streszczenie

1. Wielocukier Vi pozbawiony aktywności receptorowej pod wpływem działania faga Vi II porównano z preparatem wyjściowym; wielocukier ten nie był zdepolimeryzowany, posiadał znacznie mniej grup *N*-acetylowych i był praktycznie pozbawiony grup *O*-acetylowych.

2. Utratę wiązania estrowego potwierdzono analizą absorpcji w podczerwieni. Odszczepiony przez faga kwas octowy zidentyfikowano chromatograficznie jako acetohydroksamian.

3. Wielocukier pozbawiony przez faga aktywności receptorowej odzyskiwał ją po zacetylowaniu i tracił ponownie po powtórnym działaniu faga.

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RECENZJE KSIĄŻEK

J. N. Attis and R. A. Khadif, MELANOTIC TUMORS. Biology, Pathology and Clinical Features. Charles C. Tomas, Springfield (Ill.) 1965, str. XI+346, cena 12.50 $.

W omawianej książce Autorzy zebrali zarówno z piśmiennictwa jak i z wieloletniej praktyki wszystko, co się odnosi do nowotworów melanotycznych. Książka przeznaczona jest głównie dla onkologów zainteresowanych tymi nowotworami, to też główną jej treść stanowi omówienie patologii i histogenezy, obrazu klinicznego oraz etiologii tego schorzenia, a przeszło trzecią część tekstu zajmuje terapia. Dla biochemika interesującego się procesem melanogenezy bezpośrednie znaczenie posiadają tylko dwa pierwsze rozdziały, poświęcone melanogenezie i biochemii melanin oraz histologii i pochodzeniu melanocytów.

Na wyjaśnienie procesu melanogenezy złożyły się przyczynki z bardzo różnych dziedzin naukowych. Stąd źródłowe informacje rozsiane są w piśmiennictwie biochemicznym, endokrynologicznym, chemicznym, histologicznym a nawet w ściśle zoologicznym. Wielką zaletą omawianej książki i zasługą Autorów jest skrupulatne zebranie tych różnorodnych informacji i cytowanie ich źródeł do każdego rozdziału z osobna. Dzięki temu książka ta, mimo że niewiele stosunkowo miejsca w tekście poświęca samej melanogenezie, stanowi także dla biochemika ważną pozycję w bibliografii tego zagadnienia. Jak wszystkie książki Thomasa i ta wydana jest nadzwyczaj starannie i estetycznie.

Józef Heller

M. Davies, SOME ELECTRICAL AND OPTICAL ASPECTS OF MOLECULAR BEHAVIOUR. The Commonwealth and International Library, Chemistry Division (General Editors, Sir Robert Robinson, H. M. N. H. Irving & L. A. K. Staveley), Pergamon Press, Oxford, 1965; X+190 pp., price 15 sh.

This small pocket-size volume of 7 chapters contains a remarkably lucid and concise account of those electrical and some optical properties of molecules which are of importance in studies of molecular structure and molecular interactions. Although the book is intended primarily for students of chemistry who are being introduced for the first time to the subject of dipole moments and molecular polarizability, the subject matter and clarity of presentation are such that it should prove of interest to a much broader audience. In particular, it is to be highly recommended to the increasing number of biochemists and biophysicists whose special interests embrace such fields as molecular structure, and conformation, properties of polypeptides and polynucleotides, energy transfer, etc.

It is perhaps not out of place to point out that one of the stumbling blocks to the adequate development of theoretical descriptions of the sources of hypochromicity and stability of polynucleotide helices (with a view to obtaining a better understanding of the structure of nucleic acids) is the lack of suitable experimental data on the dipole moments of the purine and pyrimidine bases. This booklet may be expected to stimulate further research in this field, as well as a wider application of dipole moment techniques to molecules of biological interest, the more so in that reasonably good measuring instruments for this purpose are now available commercially from a number of manufacturers.

The treatment throughout is extensively illustrated with a large number of experimental results and with brief descriptions of experimental principles and methods, as well as their scope and limitations. Particularly interesting is Chapter 6, which delves into some of the aspects of molecular behaviour associated with polarizability, e.g. Rayleigh scattering and its application to the investigation of molecular weights and shapes of large molecules; Raman scattering and molecular vibrations; the permittivity (or dielectric constants) of non-polar compounds, etc. Notwithstanding the short space necessarily assigned to each topic, the exposition is straightforward and simple.

The complete replacement of the term "dielectric constant" by the more precise term "permittivity" is perhaps open to question. The Author has done this on the grounds that "the 'dielectric constant' is ... constant in only a very limited way." (page 9). This is true enough. But the term "dielectric constant" has become so ingrained in the scientific literature that only some international body such as the International Union of Pure and Applied Chemistry (IUPAC) could authoritatively discountenance its use.

The book contains a number of representative problems at the end of each chapter, in some instances with hints as to how to solve them, and with numerical answers at the end of the volume, all of which undoubtedly enhance its value. Each chapter also includes a short list of references for additional reading for those interested in particular topics covered in the text. A 6-page subject index is a useful adjunct even to constant users of the text. Two remarkably good photographs of Peter J. Debye and C. P. Smythe vividly recall to the reader the major contributions made by these two authorities in this important field. Finally the publishers deserve to be commended for the excellent type and good paper, both of which contribute to provide a very readable text.

David Shugar

METABOLISM OF LIPIDS AS RELATED TO ATHEROSCLEROSIS (A Symposium compiled by Fred A. Kummerow) Charles C. Thomas Publ., Springfield, (Illi.) 1965; str. XXXIV+300, cena 14.50 $.

Recenzowana książka jest zbiorem (niekompletnym) referatów wygłoszonych na sympozjum poświęconym znaczeniu przemiany tłuszczów w patogenezie miażdżycy, które odbyło się w czerwcu 1963 r. w Uniwersytecie Illinois (Urbana, Ill., U.S.A.).

We wstępie i pierwszych rozdziałach Autorzy wprowadzają w aspekty kliniczne aterosklerozy; omówiono w nich występowanie tego schorzenia, jego następstwa oraz znane już od wielu lat zalecenia dietetyczne w miażdżycy: zredukowanie ilości dostarczanego tłuszczu do 25% zapotrzebowania kalorycznego, przy zwiększeniu w nim zawartości kwasów tłuszczowych nienasyconych. Z podawanych obliczeń statystycznych wynika, iż stosowanie tego zalecenia obniża śmiertelność z powodu następstw miażdżycy o 20 - 30%. W dwu rozdziałach omówione są badania histologiczne i chemiczne wczesnych zmian miażdżycowych w naczyniach. Podkreśla się, że wczesne zmiany, tj. nagromadzenia tłuszczu w komórkach mięśni gładkich mające charakter ogniskowy, występują dość powszechnie już u dzieci. Są to zasadniczo zmiany o charakterze odwracalnym. W populacjach o dużym procencie występowania następstw miażdżycy te nacieczenia tłuszczowe są już u dzieci otoczone odczynem zapalnym. W populacjach o niskim procencie występowania następstw miażdżycy tym nacieczeniom tłuszczowym nie towarzyszy odczyn zapalny. Autorzy zadają pytanie, czy obecność stanu zapalnego, która prowadzi następnie do rozwoju guzka miażdżycowego, nie jest uwarunkowana toksycznym charakterem nagromadzonego w komórce lipidu. W jednym z referatów porównano skład lipidowy fragmentów aort zmienionych miażdżycowo i zdrowych u różnych grup etniczych. Badania te jednakże nie dają odpowiedzi na poprzednie pytanie, gdyż dotyczyły zmian zbyt zaawansowanych, posługiwano się przy tym materiałem utrwalonym w formalinie.

W czterech rozdziałach omówiono własności fizyko-chemiczne i przemianę cholesterolu w organizmie w związku z rolą tego związku w patogenezie miażdżycy. Omówione są krytycznie farma-

kologiczne sposoby obniżania poziomu cholesterolu w surowicy. Mechanizm biochemiczny działania środków farmakologicznych hamujących ostatnie etapy biosyntezy cholesterolu oraz ich zestawienie podane są w referacie D. Steinberga (str. 207 - 224). Zwraca on uwagę na niebezpieczeństwa mogące wynikać przy stosowaniu tych związków. Dwa rozdziały poświęcono lipoproteidom osocza. W procesie tworzenia guzka miażdżycowego duże znaczenie odgrywa oksydatywna denaturacja tych ciał. Jest ona katalizowana przez związki hemowe i zachodzi przy współudziale mostów nadtlenkowych powstających w kwasach tłuszczowych cząsteczki lipoproteidu.

Cztery dalsze rozdziały poświęcono przemianie i roli kwasów tłuszczowych w organizmie. W referacie S. J. Wakila o biosyntezie kwasów tłuszczowych Czytelnik znajdzie dane o udziale termostabilnego koenzymu polipeptydowego w procesie powstawania kwasu tłuszczowego z malonylokoenzymu A. Peptyd ten przyłącza do swej grupy sulfhydrylowej resztę acylową z acylokoenzymu A i w czasie całego procesu wydłużania łańcucha kwasu tłuszczowego reszta acylowa jest z nim związana. Oznaczono skład aminokwasowy i ciężar cząsteczkowy tego peptydu oraz przeprowadzono syntezy chemiczne omawianych acylopeptydów. J. F. Mead w swym referacie omawia eksperymenty świadczące o występowaniu w mózgu procesu α-oksydacji kwasów tłuszczowych. R. T. Holman omawia badania nad ilościowym zapotrzebowaniem na niezbędne kwasy tłuszczowe u ludzi i zwierząt. A. L. Tappel omawia występowanie i lokalizację komórkową wielonienasyconych kwasów tłuszczowych, proces ich peroksydacji katalizowanej przez hemoproteidy oraz antyoksydatywne działanie aminokwasów i białek zawierających selen. Związki te wykazują 50 - 500 razy silniejsze działanie niż tokoferol. Tematami pozostałych rozdziałów są: fizjologiczne mechanizmy przeciwdziałające krzepnięciu krwi, budowa chemiczna i biochemia fosfolipidów oraz biochemia i funkcje koenzymu Q, witaminy E i witaminy K.

Poszczególne referaty zaopatrzone są w spis cytowanego piśmiennictwa; zamieszczono również wypowiedzi dyskutantów. Książka posiada indeks autorów i indeks rzeczowy. Można ją polecić klinicystom i biochemikom zajmującym się zagadnieniem lipidów.

Tadeusz Chojnacki

Maksymilian Pluta: MIKROSKOPIA FAZOWO-KONTRASTOWA I INTERFERENCYJNA. PWN, Warszawa 1965; str. 370, cena zł. 35.—

Oceniana książka jest bardzo wartościową pozycją, godną jak najszerszej popularyzacji wśród biochemików, tych zwłaszcza, którzy zajmują się ultrastrukturą, cytochemią, mikroanalizą czy badaniami izolowanych organelli komórkowych.

Autor jest wysoce kompetentny w omawianiu zawartego w książce materiału. Jest on twórcą polskiego mikroskopu interferencyjnego.

Mikroskopia fazowo-kontrastowa, jak i interferencyjna, spełniać mogą dwa, równie zresztą użyteczne, zadania. Przy pomocy obu tych systemów można oglądać tzw. obiekty fazowe, tzn. takie, jakimi najczęściej są niebarwione żywe komórki. Obiekty fazowe, których modelem mogłaby być płytka szklana zanurzona w wodzie, przesuwają fazę fali świetlnej w stosunku do fali świetlnej przechodzącej w środowisku je otaczającym. Ani oko ludzkie ani fotokomórka nie potrafią odczytać przesunięcia fazy światła. W układach kontrastujących fazy oraz w mikroskopie interferencyjnym przesunięcie fazy zostaje zamienione na zmianę w amplitudzie światła przechodzącego przez obiekt. Dlatego w układach tych obiekty „przeźroczyste" dają się oglądać.

Drugim, ważniejszym dla biochemika, zastosowaniem układów fazowo-kontrastowych oraz mikroskopu interferencyjnego jest możliwość dokonywania pomiarów suchej masy określonych struktur komórkowych, jak np. jąder komórkowych, włókien kolagenowych, izolowanych jąderek itp. Imponująca jest czułość tej metody ważenia, jednostką pomiaru jest bowiem 10^{-12} g.

Książka M. Pluty omawia w sposób systematyczny, jasny i dostępny dla niespecjalistów teorię oraz praktykę tych nowych i stale rozwijanych systemów optycznych. Dla nieznających analizy matematycznej rozważanych zagadnień, w każdym razie dostępna będzie prosta wektoralna interpretacja omawianych zjawisk.

Podkreślić trzeba doskonałą stronę ilustracyjną książki. Zarówno schematy układów optycznych, jak i fotografie istniejących instrumentów uprzystępniają wykład. Książka zawiera bardzo krótkie omówienie zastosowań mikroskopii interferencyjnej w poszczególnych gałęziach wiedzy, m. in. w dziedzinie biologiczno-medycznej. Mikrofotografie załączone do książki zbliżają wyobraźni tych, którzy nigdy tą metodą nie pracowali, efekty, jakie przy pomocy mikroskopu interferencyjnego można uzyskać. Jednym z błahych, ale wyzyskiwanych w cytologii efektów jest możliwość wykonywania barwnych fotografii obiektów żywych, niebarwionych, przez wykorzystanie powstawania barw interferencyjnych.

Książka M. Pluty jest podręcznikiem, którego użyteczność jest naprawdę duża, a grono zainteresowanych odbiorców, wbrew zawartemu w tytule terminowi „Mikroskopia", jest znacznie szersze niż grono cytologów i histologów.

Kazimierz Ostrowski