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Dawid Keilin

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Prof. Dawid Keilin, F. R. S.

Profesor Keilin zmarł nagle 27 lutego 1963 r. w Cambridge. Urodzony 21 marca 1887 r. w Moskwie, gdzie w tym czasie mieszkali jego rodzice, w okresie dzieciństwa powrócił do Polski i lata szkolne spędził w Warszawie. Ze świadectwem dojrzałości Liceum Górskiego wyjechał z Warszawy w celu dalszego kształcenia się, początkowo w Liège a następnie w Paryżu.

Właśnie w Paryżu, pod kierunkiem Maurice Caullery, do którego był głęboko przywiązany, Keilin zainteresował się żywo parazytologią i ogólną biologią komórki. Pierwotniaki pasożytnicze, nicienie, owady, ich morfologia, fizjologia oraz ich cykle rozwojowe były głównym przedmiotem jego badań do roku 1925 czyli do odkrycia cytochromu. Z okresu tego pochodzi około 60 publikacji, wiele z nich w języku francuskim, w tym jego praca doktorska, wykonana w Paryżu oraz słynne prace związane z wykryciem cyklu rozwojowego muchy *Pollenia rudis*, której larwy rozwijają się pasożytniczo w ciele dżdżownicy *Allolobophora chlorotica*.

Wybuch Pierwszej Wojny Światowej zastał Keilina we Francji, ale wkrótce w lutym 1915 r. na zaproszenie prof. G. H. F. Nuttalla z Uniwersytetu w Cambridge udał się do Anglii w charakterze asystenta naukowego. W Cambridge pozostał do końca życia i tylko na krótko powracał na kontynent, na dłuższe podróże nie pozwalała Mu bowiem przewlekła astma, przykra dolegliwość, od której rzadko udawało Mu się uwolnić. Nie wpływało to jednak na przebieg Jego pracy, zachował niezwykle pogodny i optymistyczny stosunek do życia i ludzi. W roku 1920 otrzymał tytuł Beit Memorial Research Fellow, w roku 1925 został wykładowcą parazytologii na Uniwersytecie, a w r. 1931 następcą Nuttalla jako Quick Professor of Biology oraz dyrektorem Molteno Institute of Biology and Parasitology.

W roku 1925 Keilin odkrył cytochrom. Jego praca „O cytochromie, barwiku oddechowym, wspólnym dla zwierząt, drożdży i roślin", opublikowana w Proceedings of The Royal Society, wprowadziła przełom w poglądach na biologię komórki i biochemię. Odkrycie to umożliwiło badanie wewnątrzkomórkowego oddychania nienaruszonych komórek czy to larwy *Gastrophilus*, czy mięśni skrzydeł owadów, czy też zawiesiny drożdży, przez bezpośredni pomiar w spektroskopie, metodą szczególnie prostą a jednak doskonałą. Wykazano, że „oksydaza indofenolowa" jest oksydazą cytochromową, a funkcja dawniej używanych

sztucznych układów modelowych „żelazo-węglowych" została właściwie oceniona.

O wpływie badań Keilina na dalszy rozwój biologii komórki najlepiej świadczy fakt, że po upływie lat czterdziestu z trudem znaleźć można numer jakiegoś czasopisma, poświęconego fizjologii komórki lub biochemii, w którym nie byłoby większych prac o istocie i funkcji barwików cytochromowych, co jest osiągnięciem wybitnym, nie ustępującym innym odkryciom naukowym naszych czasów.

Szczęśliwy zbieg okoliczności pozwolił autorowi niniejszego artykułu nawiązać współpracę z Keilinem w r. 1935. W tym czasie głównym kierunkiem Jego badań było oczyszczanie cytochromu c. Prowadził On również szeroko zakrojone badania porównawcze nad niektórymi hemoproteidami, w stałej, szczęśliwej współpracy z dr. E. F. Hartree. Molteno Institute w owych czasach był ośrodkiem wyjątkowo pobudzającym twórczo zarówno młodych jak i starszych pracowników nauki. Profesor, w białym laboratoryjnym płaszczu, biorący zawsze najczynniejszy udział w pracy doświadczalnej, otoczony był kolegami, przybywającymi do Molteno ze wszystkich zakątków świata, którzy poszukiwali u Niego porady, pokierowania i serdecznej przyjaźni. Dyskusje obejmowały zasięg od kinetyki tego czy innego świeżo oczyszczonego enzymu do zawiłych problemów parazytologii, nad którymi pracowali dr. P. Tate i dr. A. Bishop i kilku innych wybitnych parazytologów. Z badań, wykonanych w szczęśliwej współpracy z Keilinem najsilniej utkwiło w pamięci autora odkrycie, w r. 1936, kompleksu enzymu peroksydazy roślinnej z jej substratem, nadtlenkiem wodoru, a w latach następnych oczyszczenie oksydazy polifenolowej i lakazy z materiału roślinnego oraz hemokupreiny i anhydrazy węglanowej z krwinek czerwonych; dwa z tych białek były dla nas źródłem szczególnej radości; hemokupreina ze względu na piękne, niebiesko zabarwione kryształy; anhydraza węglanowa ze względu na to, że była ona pierwszym poznanym enzymem, zawierającym cynk.

Innym enzymem, którym Keilin był szczególnie zainteresowany, była katalaza; wraz z Hartree dokonali ważnego odkrycia tzw. sprzężonego utlenienia, które zależy od czynności peroksydatywnej tego enzymu. W tych i późniejszych badaniach nad katalazą, peroksydazą, cytochromem, oksydazą kwasu bursztynowego, oksydazą glukozy i hemoglobiną, które wypełniły około 200 publikacji, przejawia się niezwykle bystry zmysł obserwacji i bezcenna wprost, szeroka znajomość biologii zarówno świata roślin jak i zwierząt. W toku badań nad hemoglobiną brodawek korzeniowych roślin motylkowych wykazał Keilin, że sama roślina oraz sam, mogący żyć z nią w symbiozie, drobnoustrój *Rhizobium* nie posiadają zdolności wytwarzania hemoglobiny, zdolność ta natomiast pojawia się w symbiozie. Wykazał On także obecność hemoglobiny w drożdżach, pleśniach i u niektórych pierwotniaków. Wykonanie większości prac nad

wykrywaniem śladów hemoproteidów w różnych komórkach możliwe było dzięki umiejętnemu zastosowaniu metody spektroskopowej, którą Keilin znacznie udoskonalił w r. 1949, gdy zauważył, że wartości ekstynkcji widma absorpcyjnego hemoproteidów można znacznie zwiększyć obniżając temperaturę.

Dawid Keilin był nie tylko wybitnym naukowcem, lecz także człowiekiem o wysokiej kulturze i o wielkich zaletach charakteru. Niskiego wzrostu, energiczny, niezwykle serdeczny, był zawsze dostępny dla wszystkich, zarówno kolegów jak i studentów, gotów wysłuchać, poradzić, zaofiarować pomoc, poparcie i słowa zachęty. Dla kolegów z Polski był specjalnie gościnny i uprzejmy. Do końca życia zachował doskonałą znajomość języka polskiego i czytał wiele po polsku, zarówno czasopisma naukowe, jak też powieści i poezje. Warszawę specjalnie często wspominał jako swoje miasto ukochane, znane mu dobrze z lat dzieciństwa. Ogromną radość sprawiła Mu w roku 1959 wiadomość, że został wybrany członkiem Polskiej Akademii Nauk w Warszawie. Oprócz członkostwa Polskiej Akademii został odznaczony wielu innymi zaszczytami. W roku 1928 został członkiem Królewskiego Towarzystwa Nauk (Fellow of the Royal Society: F.R.S.), w roku 1939 otrzymał Medal Królewski (Royal Medal) za swoje osiągnięcia naukowe, a w roku 1951 tzw. Copley Medal, tj. najwyższe odznaczenie jakim bardzo rzadko obdarza naukowców Królewskie Towarzystwo. Był członkiem Francuskiej Akademii Nauk w Paryżu, Amerykańskiej Akademii Sztuki i Nauki w Bostonie, i doktorem *honoris causa* Uniwersytetów w Brukseli, Liège, Bordeaux i Utrechcie. O zaszczytach tych jednak wspominał rzadko. Gdy w toku rozmowy pytano Profesora, co mogłoby Mu sprawić specjalną przyjemność, odpowiadał niezmiennie: „Udane doświadczenie". Praca w laboratorium była istotnie dla Niego największą przyjemnością, a wykłady swoje dla studentów urozmaicał pokazem skomplikowanych ale zawsze udanych doświadczeń, które sam starannie przygotowywał. Najbardziej jednak znamienną cechą ludzką Keilina była niezwykła skromność, połączona z niezwykłą dobrocią, i ta cecha zaskarbiła Mu u Jego uczniów dozgonną miłość i szacunek. Profesor Keilin pozostawił wdowę, Dr. Annę Keilin, jak On pochodzenia polskiego, i córkę, biochemiczkę, Dr. Joan Keilin, która obecnie zajęta jest wydawnictwem książki, nad którą Ojciec Jej pracował w ciągu ostatnich lat Swego Życia.

Tadeusz Mann, Cambridge, England

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UNREACTIVE THIOL GROUPS IN HUMAN HAEMOGLOBIN

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Among the thiol groups of haemoglobin two types can be distinguished: reactive thiol groups, i.e. those which may be estimated in native protein using specific reagents, and unreactive groups which are estimated only after denaturation of the protein [6, 15, 2]. Most authors consider that the number of thiol groups in a haemoglobin molecule is from 6 [2, 8] to 8 [6, 13, 15]; four of them are unreactive, and the remaining 2 or 4 are reactive groups [15, 2]. The number of the reactive thiol groups is still discussed, and it has not been possible to elucidate this problem even by means of sensitive amperometric methods. These methods are based on titration of thiol groups with silver or mercury ions. However, the activity of these ions is so great that there is a possibility of their reacting also with other groups in the protein molecule. The titration is therefore carried out in the presence of compounds forming complexes with the metal ions used for titration. The silver ion complexed with tris (2-amino--2-hydroxymethylpropandi-1,3-ol) shows greatly diminished activity, and reacts with only a part of the thiol groups. The remaining thiol groups react very slowly, or not at all, and can be determined only after denaturation of the protein.

It is assumed that the unreactivity of thiol groups is due to their intramolecular linkages, the nature of which is not known. Some possibilities are: hydrogen bonds with amino groups, addition of thiol groups to the carbonyl of the peptide linkages, formation of esters with carboxyl groups, etc. [9].

In the haemoglobin molecule haem constitutes an additional grouping which can react with thiol groups. These groups are in close proximity [20], and the possibility of haem - thiol linkage might be assumed to be the cause of the diminished reactivity of thiol groups. This assumption is supported by the work of Riggs [19] who found that binding of thiol groups in haemoglobin abolished the phenomenon of haem - haem interaction during the oxygenation.

The close proximity of the thiol groups to the haem, and their influence on its function support the assumption of thiol - haem binding

in haemoglobin. The demonstration of such bonds might explain many problems concerning the structure and function of haemoglobin. In this paper we present some arguments supporting the above-mentioned assumption.

MATERIALS AND METHODS

Haemoglobin. Freshly drawn citrated human blood was centrifuged and the erythrocytes were washed with physiological salt solution. To lyse the cells five vol. of water was added. The stroma was centrifuged and the haemoglobin solution was dialysed at 1° for 20 hr. against distilled water and then against doubly distilled water. The dialysed solution was centrifuged again. In some experiments the erythrocytes after being washed with physiological saline were washed additionally with 0.15 M-sodium nitrate solution to remove chlorides.

In view of the nonhomogeneity of haemoglobin, for the successive series of experiments haemoglobin from the same individual was employed. The amount of haemoglobin used for determinations of thiol groups was from 0.09 to 0.23 μmoles.

Globin. This was prepared by the method of Rossi-Fanelli *et al.* [21].

Determination of thiol groups. This was carried out by amperometric titration, using a rotating platinum electrode, after Kolthoff & Harris [17] as modified for proteins by Benesch *et al.* [6]. The thiol groups were titrated with silver nitrate in the presence of tris as complexing agent. The reference electrode was connected by means of a KNO_3-agar bridge instead of KCl to allow titration also in chloride-free solution. The reactive thiol groups were estimated in native haemoglobin, and the remaining groups in the haemoglobin denatured with sodium dodecyl sulphate (SDS) [15, 2]. The difference between the number of thiol groups determined in native and in denatured haemoglobin permits calculation of the number of unreactive thiol groups.

Determination of protein. Concentration of HbO_2 was estimated by the method of Drabkin & Austin [12] and globin by dry weight or spectrophotometric method described by K. Murawski (unpublished) ($E^{1\%}_{280\,m\mu,\,1\,cm.} = 10.62$).

Spectrophotometry. A Hilger Uvispec spectrophotometer was used.

Reagents. Tris and sodium dodecyl sulphate were Light (England) products.

RESULTS

Determination of thiol groups in Hb and HbO_2

If at least some thiol groups of haemoglobin are bound with haem, in view of the electronic structure of haem, the energetic changes accompanying oxygenation of haemoglobin should produce a change in

the reactivity of thiol groups. Cecil & Snow [11] found that even HbO_2 and HbCO differ in this respect. Therefore an attempt was made to determine the reactive thiol groups in Hb and HbO_2. However, it is very difficult to titrate haemoglobin under anaerobic conditions with the rotating electrode, and the interpretation of the obtained results presents difficulties owing to the markedly different conditions in the medium as compared with those in which HbO_2 is titrated. To avoid these difficulties the determinations were made indirectly. Haemoglobin reduced in a Thunberg tube was treated with silver nitrate in an amount slightly in excess of that needed to bind all the thiol groups. After a determined interval of time, the remaining silver ions were precipitated with an excess of potassium iodide. Determination of the iodide ions remaining in solution allowed calculation of the amount of silver bound by the thiol groups. To prevent the possible reversal of the reaction between silver ions and unreactive thiol groups during conversion of Hb into HbO_2, Hb was removed by precipitation in the Thunberg tube with zinc hydroxide, and centrifugation.

Binding of silver ions by Hb was found to be greater than by HbO_2 by about 20 - 30% of unreactive thiol groups (0.8 - 1.2 SH groups/molecule). We are unable to decide whether this difference was actually due to the release of unreactive thiol groups, or only to partial change in their reactivity.

Determination of thiol groups in human globin

If the existence of a linkage between the thiol groups of globin and haem is assumed, an increase in reactive thiol groups following the splitting of haemoglobin into haem and globin should be expected. However, in the course of this estimation difficulties are apt to arise. Globin is a protein with a marked tendency to aggregation. Antonini *et al.* [3] showed that human globin, shortly after splitting off of haem, in the presence of salts and at low pH forms aggregation products of a molecular weight many times greater than that of haemoglobin. This aggregation can lead to a decrease in reactive thiol groups [1]. Moreover, in solutions of globin stored at low temperatures for several days, a diminution in the amount of thiol groups was observed; these groups were not restored even after protein denaturation with SDS. This was probably due to oxidation of closely adjacent thiol groups.

To avoid possible errors the titration was performed immediately after preparing the globin at -1°. The low temperature was necessary because globin is easily precipitated in the presence of salts at higher temperatures. The procedure was as follows. The globin sediment was washed several times with acetone, and dissolved in a volume of water 2 - 4 times as great as the volume of the haemoglobin solution, to diminish a possible effect of traces of acetone remaining in the sediment.

Centrifugation was then performed at temperature not exceeding 0°. The molar concentration of globin was determined and the titration results were compared with the corresponding results obtained with the same HbO_2 sample from which the globin was derived.

In all the experiments the amount of the reactive thiol groups was greater in globin than in HbO_2 by 20 - 35% of unreactive thiol groups (0.8 - 1.4 SH groups/molecule). This probably did not represent the full amount of the thiol groups released because in spite of very careful treatment a certain amount of globin was precipitated during titration.

Table 1

Thiol groups in human oxyhaemoglobin and globin

Tris-HNO_3 buffer, pH 7.47, final ionic strength 0.32; 1 mM-$AgNO_3$ used for titration.

Conditions ...	15°	-1°	-1° 4 M-urea	SDS denat.
Sample	Reactive thiol groups (μmole/sample)			
HbO_2, 0.2025 μmole	0.72	0.73	0.72	1.62
Globin, 0.2025 μmole	0.84	0.95	1.18	1.63
Increment	0.12	0.22	0.46	
Increment as % of unreactive groups	13.14	24.18	50.55	100
Increment of SH groups	0.53	0.97	2.02	4.00

To prevent the process of aggregation, urea was applied [1]. The titration of globin was carried out in 4 M-urea solution at -1°. Under these conditions no globin precipitated during titration and 50% of unreactive thiol groups was released (2.0 SH groups/molecule). When HbO_2 was titrated in the urea solution, the amount of thiol groups was not changed showing that this concentration of urea under conditions used did not alter the number of reactive thiol groups. The results of titration of free globin (Table 1) indicate that the splitting off haems release two unreactive thiol groups per molecule.

The effect of SDS concentration on the titratable amount of thiol groups

The denaturation of haemoglobin with sodium dodecyl sulphate (SDS) releases all the unreactive thiol groups [15]. The mechanism of this denaturation is not fully elucidated. SDS is an active compound and reacts steichiometrically with many compounds, especially with conjugated proteins [23]. Hence, besides the changes in haemoglobin due

to lowered surface tension, a reaction between SDS and protein, as well as with haem, must be considered. The susceptibility of the haem group to enter into reaction with SDS is shown by the fact that SDS even at low concentrations produces a change from the two-band spectrum of HbO_2 to the one-band spectrum of the SDS derivative of Hb, with maximum absorption at 535 mμ (Fig. 1). Apparently this is not yet the

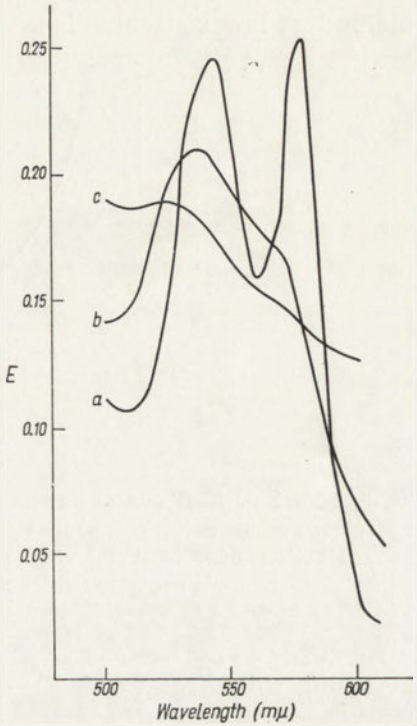

Fig. 1. Effect of SDS on absorption spectra of HbO_2. (*a*), HbO_2; (*b*), after the addition of SDS to final concentration 0.008%, and (*c*), after the addition of SDS to final concentration 0.4%.

spectrum of denatured haemoglobin since it changes further when the concentration of SDS is increased. Then the spectrum assumes a flatter and more diffuse form; at the same time, the dark red colour of the solution produced by low concentrations of SDS turns dark brown. A spectrum of this type can be produced by adding SDS to a neutralized solution of Hb denatured with alkali. It may be concluded that SDS reacts both with haem, giving rise to a new "SDS-Hb derivative", and, at higher concentrations, with protein causing its denaturation.

At wavelengths 530 and 560 mμ changes are observed in absorption consisting at first in increased extinction at low concentrations of SDS, and then in gradual decline of extinction as concentration increases and protein undergoes denaturation. This phenomenon confirms the above-mentioned assumption concerning the complexity of the reaction between SDS and Hb, and allows to analyse the course of both processes. If the change in extinction permits to distinguish between the reaction

of SDS with haem or some near situated group, and the denaturation of protein, it suffices to compare these changes with the simultaneous increment of thiol groups to ascertain which process is responsible for the release of thiol groups.

The effect of rising concentrations of SDS in HbO_2 solution on the extinction at 530 mμ and on the amount of reactive thiol groups is presented in Fig. 2. About 50% of unreactive thiol groups (2 SH) is released at low concentrations of SDS (Table 2), i.e. during the reaction

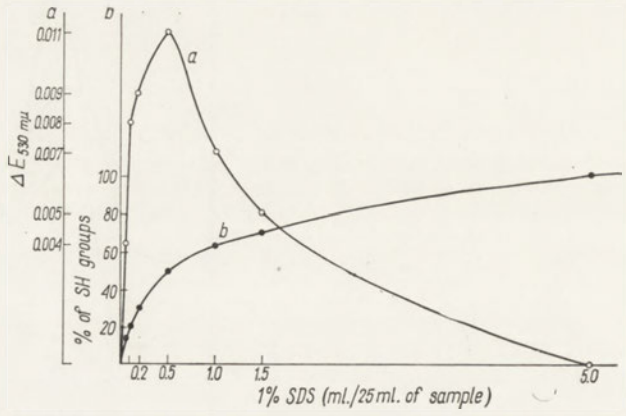

Fig. 2. Effect of SDS concentration on the absorption at 530 mμ of haemoglobin, and release of unreactive thiol groups. (a), Extinction of "Hb-SDS" after subtracting the extinction of HbO_2; (b), SH groups released expressed as percentages of unreactive thiol groups (details see Table 2).

between SDS and haem, as confirmed by changes in the spectrum. The remaining thiol groups are liberated gradually as denaturation proceeds. The release of the SH groups during the reaction between SDS and haem points to the existence of a linkage between thiol and haem groups of haemoglobin.

Table 2

Thiol groups in human haemoglobin at different concentrations of SDS

Tris buffer, pH 7.47, final ionic strength 0.32; temp. 15°. To 1 ml. of HbO_2 solution (0.135 μmole) in 8 ml. tris buffer, 1% (w/v) SDS was added as indicated in the Table, then water to 25 ml.

Addition of 1% SDS sol. (ml.) ...	0	0.05	0.1	0.2	0.5	1.0	1.5	5.0	10.0
Reactive thiol groups (μmoles/sample)	0.48	0.56	0.60	0.66	0.78	0.86	0.90	1.08	1.08
Increment of reactive thiol groups (μmoles/sample)		0.08	0.12	0.18	0.30	0.38	0.42	0.60	0.60
(% of unreactive thiol groups)		13.3	20	30	50	63.3	70	100	100

Behaviour of unreactive thiol groups bound with silver in the presence of tris

Thiol groups are thought to be combined with some undefined groups in the haemoglobin molecule, and this seems to be the cause of their unreactivity [9]. It may be that the binding of silver ions with unreactive thiol groups has a different character than binding with reactive groups, in which case the bond should be somewhat weaker. Free silver ions bind all the thiol groups although the reaction with unreactive groups is markedly slower. In the presence of tris the activity of silver ions

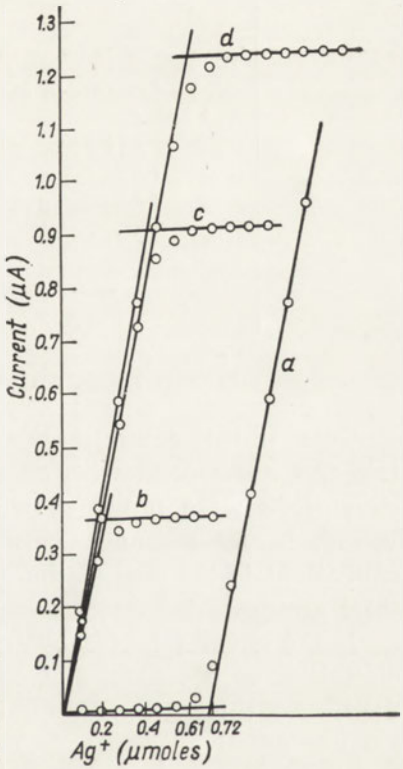

Fig. 3. Amperometric titration with iodide of silver ions released by tris from linkages with Hb thiol groups. (*a*), Titration with silver nitrate of Hb denatured by SDS; (b), Ag^+ released from Hb (the amount of $AgNO_3$ added was equimolar with thiol groups); (c) and (*d*), Ag^+ released from Hb after addition of larger amounts of $AgNO_3$. Details in Table 3 and in the text. Direction of electrical current in the galvanometer was reversed in iodide titrations.

is diminished about 10^5 times and at low concentrations of silver ions complexed with tris the binding of the unreactive thiol groups is negligible. Therefore, tris may be regarded as a factor competing with the unreactive thiol groups for silver ions. This competition proved to be sufficiently effective to enable removal of silver ions with tris from the already bound unreactive thiol groups.

Fig. 3 illustrates an experiment in which haemoglobin was added with silver nitrate in an amount sufficient to bind all the thiol groups, and then tris was added to release the unreactive thiol groups. The

amount of silver needed was calculated on the basis of titration of haemoglobin denatured with SDS. Silver released by tris was titrated with potassium iodide and was found to correspond to about 50% of the unreactive thiol groups (2-SH).

Table 3

Binding of thiol groups in human haemoglobin with silver ions and their release by tris

Tris buffer, pH 7.47, final ionic strength 0.32; temp. 15°. 1 mM-$AgNO_3$ and 1 mM-KI solutions used for titration. The amount of Hb per sample was 0.09 μmole.

Sample...	1	2	3
Thiol groups in haemoglobin denatured by SDS	(μmoles/sample) 0.72	0.72	0.72
Ag^+ added	0.72	0.93	1.14
Excess of Ag^+ added	0	0.21	0.42
Free Ag^+ ions found	0	0.02*	0.23*
Free Ag^+ ions found after addition of tris	0.20	0.40	0.61
Thiol groups released by SDS denaturation after removal of free Ag^+ ions	0.20	0.20	0.20

* In the absence of tris, a part of silver ions is bound in protein with other groups, not only with thiol.

Two control determinations were made, one without the addition of tris and the second with an excess of silver. In the first one no free silver ions were found showing that the whole amount of silver was bound. In the second experiment the amount of silver recovered was equivalent to the sum of the excess added and the amount released from thiol groups.

If the silver released by tris was removed from the solution by precipitation with iodide, and the haemoglobin was then denatured with SDS, free thiol groups appeared in a molar amount equivalent to the amount of silver released (Table 3). The results of these experiments are in agreement with the view that the unreactivity of thiol groups is due to their combination in the Hb molecule, and, moreover, support our assumption concerning the nonhomogeneity of unreactive thiol groups.

Determination of thiol groups in Hb after alkaline denaturation and in Hb oxidized by acidification

The thiol groups in haemoglobin are situated in close proximity [15]. Under the influence of denaturing agents they may easily undergo oxidation forming disulphide linkages [12, 20, 11]. This phenomenon is

seen very clearly after alkaline denaturation of haemoglobin which is followed by a reduction to one-half of the number of unreactive thiol groups. This may be observed by comparing the results of titration of HbO_2 and alkaline haemichrome in the absence of SDS and with SDS added to the concentration normally required for complete denaturation of Hb (Table 4). Oxidized thiol groups are not liberated even by high concentrations of SDS.

Table 4

The effect of alkali denaturation on human haemoglobin SH groups

Tris buffer, final ionic strength 0.32, final pH of the samples 8.6, temp. 15°. Each sample contained 0.223 μmoles haemoglobin. The samples were prepared in the following order: HbO_2: 8 ml. tris buffer, 0.025 ml. 2 N-NaOH, water to 24 ml., and 1 ml. HbO_2 solution; Hb denatured by alkali: 1 ml. HbO_2 solution, 0.025 ml. 2 N-NaOH, 8 ml. tris buffer and water to 25 ml.; Hb denatured by SDS: 1 ml. HbO_2 solution, 8 ml. tris buffer, 5 ml. of 1% (w/v) SDS solution, and water to 25 ml.

Sample (0.223 μmoles Hb)	SH groups (μmoles)	Molar ratio: SH/Hb	Origin of titratable SH groups
HbO_2	0.756	3.4	reactive: 3.4
Hb denatured by alkali	0.381	1.7	reactive: 1.7
Hb denatured by SDS	1.782	8.0	reactive: 3.4 unreactive: 4.6
Hb denatured by alkali, then by SDS	0.756	3.4	reactive: 1.7 unreactive: 1.7
Hb denatured by SDS, then by alkali	1.566	7.0	

A similar effect can be obtained in acid medium, beginning at the pH at which haem is oxidized. We observed that when iron is oxidized two thiol groups become undetectable by the amperometric method in spite of the denaturation of haemoglobin with SDS.

This confirms our observations on nonhomogeneity of the unreactive thiol groups and points to a relationship between the energetic state of haem and the behaviour of thiol groups.

DISCUSSION

In the present work only the behaviour of the unreactive thiol groups in haemoglobin, and not their number, was studied. The conditions under which each series of experiments was performed were uniform, and the obtained results were compared with those for normal HbO_2 under the same conditions of pH, temperature and ion concentration.

For determination of SH groups we applied the amperometric method using a rotating platinum electrode, and titration with $AgNO_3$ complexed

with tris. The method with dropping mercury electrode used by Allison & Cecil [2] was also considered; according to the authors, this method is well adapted to the determination of thiol groups. It seems, however, that the given conditions may affect thiol groups and their compounds. Borate buffer used as medium during mercury titration may facilitate oxidation of thiol groups in haemoglobin [20]. Sulphite or thiosulphate used as complex-forming agent with mercury might change the results by breaking disulphide bonds. Moreover, thiosulphate may react with protein (T. Szczepkowski, private communication). Presence in the solution of metallic mercury is a possible source of ions in the solution. In the opinion of some authors the mercury titration gives rise to difficulties in interpretation of results [14, 15, 7].

The electric charge of the complex containing the ion reacting with thiol groups and the spacial distribution of the charge may also exert an effect. Cecil & Snow [10, 11] employed mercury compounds complexed with the negatively charged sulphite ion. In our experiments, on the other hand, as complex-forming agent for silver ions we used tris which is positively charged and which, moreover, is entirely without effect on sulphur linkages.

Titration of standard solutions of cysteine under conditions of our experiments gave results in agreement with the theoretically expected ones. This fact as well as the comparative character of the study make the objection of some authors to the 1 : 1 proportion in the reaction of sulphur with silver [9] of little significance. There are, however, some discrepancies, probably due to different methods and conditions of determination, between our results and those of Cecil & Snow [10, 11] who found the same number of reactive thiol groups in human globin and in haemoglobin.

Our present results suggest a nonhomogeneity of the unreactive thiol groups pointing to the presence of two types of binding. We assume that one half of the unreactive thiol groups (2 SH) is bound with haem and influences its function. As haemoglobin contains 4 haems, this figure might seem surprising. However, studies of the processes of oxygenation [22] and splitting off haems [4] showed that not all of the haems are equally firmly bound with globin, and that the effect of haem - haem interaction is connected especially with one of the stages of oxygenation. There are several sites in haem which may bind with thiol groups; these are, especially, the vinyl groups [5], the propionic residue and the methine carbons [18]. Also the imidazoles of histidine combined with iron may form bonds with thiol groups affecting the function of iron. Further work is in progress and we are inclined to regard the vinyl groups as the most likely site of the thiol - haem linkage.

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SUMMARY

The behaviour of thiol groups in human haemoglobin, in its derivatives, and in globin was studied by amperometric methods using a rotating platinum electrode. The obtained results indicate that the unreactive thiol groups are not homogeneous, and that two of them may be combined with haem or in its close proximity. Such a linkage could explain, at least partly, some problems concerning the phenomenon of haem - haem interaction.

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BADANIA NAD NIEREAKTYWNYMI GRUPAMI TIOLOWYMI W HEMOGLOBINIE LUDZKIEJ

Streszczenie

Metodą amperometryczną z zastosowaniem obrotowej elektrody platynowej oznaczano grupy tiolowe w hemoglobinie i jej pochodnych oraz w globinie ludzkiej. Otrzymane wyniki wskazują, że niereaktywne grupy tiolowe są prawdopodobnie niejednorodne i że dwie z nich mogą być połączone z układem hemu lub jego najbliższym otoczeniem. Obecność takiego połączenia mogłaby przynajmniej częściowo wyjaśnić niezrozumiałe zagadnienia z zakresu zjawiska interakcji międzyhemowej oraz rzucić nowe światło na sposób wiązania hemów z globiną.

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THE GASTRIC SECRETION OF IODIDES, A FACTOR LIMITING THE USE OF ^{131}I-LABELLED FAT IN EXPERIMENTAL STUDIES ON INTESTINAL FAT ABSORPTION

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In continuation of our isotopic studies on fat absorption in rat [15] we made an attempt to study the kinetics of distribution of the lipid- and non-lipid radioactivity in various rat tissues following oral administration of triolein labelled with ^{131}I. The use of radioiodinated fat could be thought to provide an attractive method for studying the utilization of exogenous fat [17, 6], according to the assumption that the release of iodine follows the catabolism of oleic acid. In fact, however, there is a partial release of iodine already in the intestinal mucosa cells [8, 7], independent of the lipid metabolic process in the tissues. To examine in particular this intestinal deiodination we have estimated the lipid- and the non-lipid radioactivity in different parts of the digestive tract in the rat.

Special attention has been drawn to the stomach because of the known very active secretion of iodides by gastric mucosa [11, 13, 10]. It might therefore be expected that a part of iodides derived from the deiodination of the labelled fat would return to the gastric lumen *via* the blood circulation. This recycling of the radioactive label has not been yet demonstrated and was not taken into account either in critical evaluations of radioiodine as a label for fat [8, 7, 15] or in precise balance experiments on the absorption of compounds labelled with ^{131}I [12, 17].

MATERIALS AND METHODS

Adult male rats weighing 200 - 300 g. were used. A known amount (0.3 - 0.4 g.) of the olive oil containing 10 - 20 μc of ^{131}I-labelled triolein (The Radiochemical Centre, Amersham, England) was administered *per os*, the animals' mouths being opened with the aid of a widely perforated plastic plate.

To reduce the uptake of ^{131}I by the thyroid, 24 hr. before the experiment each animal received *per os* 1 mg. KI. Since this moment the animals were not given food but only water. We found the single dosis

of 1 mg. KI sufficient to block the thyroid which subsequently did not absorb more than 0.5% of the administered amount of ^{131}I. In some experiments, rats which had not been given KI, were also used.

In the studies on the distribution of iodides in rat tissues, 1 ml. of ^{131}I-labelled NaI (about 20 μc) in 0.9% NaCl solution was injected subcutaneously. To estimate the accumulation of iodides in the stomach, it was surgically separated from the other parts of the digestive tract. The animals were anaesthetized with ether and the ligatures were put on the oesophagus near the cardia and on the duodenum beyond the pyloric end of the stomach. After cutting off the oesophagus and the intestine, the abdominal wall was sutured, the stomach being left inside attached to its intact blood vessels. Soon after the operation the animals awakened, and were injected subcutaneously with [^{131}I]iodide.

After a determined time (maximum 6 hr.) since isotope administration the animals were anaesthetized with ether and samples of the blood were taken by heart puncture; samples of other tissues to be tested were also taken and weighed with the accuracy of ± 0.01 g. The small intestine was divided into four parts of equal length. To prevent any adsorption or oxidation of the [^{131}I]iodide present in the tissues, 1 - 2 drops of KI and Na_2SO_3 solution were added to the samples. The total radioactivity was determined in the tissue samples and the lipid-bound activity in extracts from homogenized tissues. Extraction of lipids was performed with a mixture of chloroform and methanol according to Bligh & Dyer [3]. The extraction procedure was checked in supplementary experiments; 96.4 - 99.2% (average 98%) of the ^{131}I-labelled triolein, added to the non-radioactive tissue extract, was recovered in the chloroform phase; the recovery of the added [^{131}I]iodide in the water phase of the extract was virtually complete (99.85 - 100%).

The radioactivity of the samples was measured with the scintillation counter as described previously [15]. The results are expressed as percentages of the administered dosis.

RESULTS

The distribution of the radioactivity in separate parts of the digestive tract following oral administration of 10 μc of [^{131}I]iodinated triolein is presented in Table 1. It can be seen that very high radioactivity, as much as 8% of the administered dosis, was found in the non-lipid extracts of the gastric contents. This seems to be in agreement with the secretion of iodides in the stomach. However, this accumulation, at least in part, could be also caused by iodides secreted into saliva, or by liberation of a part of ^{131}I-marker from the iodinated fat already in the stomach, and, finally, by the presence in the stomach of some polar substances, other than iodides.

Table 1

The total, lipid and non-lipid radioactivity in various parts of the digestive tract in rats after oral administration of ^{131}I-labelled triolein

The animals were killed 3 hr. after administration of 10 μc of ^{131}I-labelled triolein. The results are expressed as percentages of the administered dosis, found in the entire wall or contents of the respective segment.

Tissue		Expt. no. 1			Expt. no. 2		
		Radioactivity					
		Total	Lipid	Non-lipid	Total	Lipid	Non-lipid
Stomach	gastric contents	14.3	6.7	7.6	16.0	8.0	8.0
	gastric wall	3.2	1.1	2.1	4.1	1.3	2.8
Small intestine							
part I	intest. contents	0.9	0.4	0.5	1.0	0.4	0.6
	intest. wall	3.4	1.8	1.6	3.2	1.9	1.3
part II	intest. contents	1.4	0.6	0.8	1.6	0.8	0.8
	intest. wall	3.8	1.9	1.9	3.4	1.8	1.6
part III	intest. contents	1.0	0.2	0.8	1.3	0.2	1.1
	intest. wall	1.8	0.7	1.1	1.7	0.8	0.9
part IV	intest. contents	2.0	0.6	1.4	2.0	1.7	1.3
	intest. wall	0.6	0.2	0.4	0.6	0.2	0.4
Large intestine	intest. contents	1.6	1.0	0.6	1.3	0.6	0.7
	intest. wall	0.7	0.1	0.6	0.3	0.1	0.2

In order to estimate the possible role of these factors, experiments were performed concerning the distribution of iodides in the body following a subcutaneous injection of [^{131}I]iodide. Table 2 shows the results of the radioactivity determinations in some of the tissues. In all the other tissues tested (not mentioned in the Table), i.e. in the three lower parts of the small intestine, in the liver, heart, muscles, kidneys, epididymal fat, parotids, submaxillary glands, oesophagus and the area of injection, the radioactivity per gram of tissue was not greater than in blood plasma. A high concentration of iodides was found in the stomach both in rats with blocked and those with non-blocked thyroid gland; 1 - 2 hr. after the subcutaneous injection of [^{131}I]iodide the concentration of the label in the stomach was 17 - 78 times higher than the concentration of radioactivity in blood plasma. After 6 hr. the radioactivity in the stomach decreased considerably being only 8 times higher than in the plasma. The concentration of iodides in the saliva cannot account for their high concentration in the stomach because it was only 2 - 3 times as high as in blood plasma. The accumulation of iodides in the stomach was also confirmed by scintigraphic examination of rats made 1 or 1.5 hr. after the subcutaneous injection of 20 μc of [^{131}I]iodide. The areas corresponding to the position of the stomach exhibited several times higher radioactivity than those corresponding to other organs.

Table 2

The radioactivity in rat tissues after subcutaneous administration of [^{131}I]iodide

The administered dosis was 18 μc [^{131}I]natrium iodide. The thyroid was blocked by administration of 1 mg. of KI, 24 hr. before the experiment. Except for the thyroid, the results are expressed as percentages of the administered dosis per 1 g. of tissue.

Tissue	Thyroid blocked	Thyroid non-blocked				
	Time after injection (hr.)					
	1	1	2	1	1.5	6
Blood plasma	0.45	0.30	0.45	0.55	0.40	0.30
Stomach wall	2.0	1.6	1.95	3.0	4.7	1.0
Stomach contents	12.9	8.8	8.2	43.0	23.7	2.4
Ist part of the small intestine (wall and contents)	0.55	0.30	0.80	0.50	0.70	0.30
Urine	—	3.6	4.7	21.7	4.4	1.9
Saliva	—	1.0	0.7	0.6	0.6	0.3
Thyroid (per whole organ)	0.3	0.35	0.35	1.7	2.7	3.5

Table 3

The radioactivity in rat tissues 1 hr. after subcutaneous injection of [^{131}I]iodide to an animal with the stomach surgically separated from the digestive tract

The administered dosis was 17 μc [^{131}I]natrium iodide. The control animal was sham operated. For details see text. The results are expressed as percentages of the administered dosis per gram of tissue.

Tissue	Rat with the stomach separated	Control rat
Blood plasma	0.2	0.2
Stomach wall	1.2	1.0
Stomach contents	9.5	4.7
Ist part of the small intestine (wall and contents)	0.1	0.7
Urine	6.9	6.8
Saliva	0.5	0.4

Further experiments concerning the gastric accumulation of iodides were performed as follows. One animal was submitted to surgical separation of the stomach from the digestive tract as described above; another animal, on which only a sham operation was performed, served as a control. Both received subcutaneous injections of [^{131}I]iodide; after 1 hr.

they were killed and the radioactivity of the tissues was determined. From the data presented in Table 3 it can be concluded that the iodides accumulating in the stomach are transported *via* the blood vessels. Next, we examined the passage of iodides in the opposite direction. Two rats with non-blocked thyroid glands and with surgically separated stomachs were used. Through a very thin needle, 1 ml. of [^{131}I]natrium iodide solution was introduced into the stomach, and 80 min. later the radioactivity of some tissues was determined. The results presented in Table 4 show that virtually all the radioactivity remained in the stomach.

Table 4

The absorption of iodides from the surgically separated rat stomach

Into the stomach separated from the oesophagus and duodenum 12 μc of [^{131}I]natrium iodide was introduced, and the radioactivity was determined after 80 min.

Tissue	Expt. no. 1	Expt. no. 2
	Radioactivity (% of the dosis)	
Whole stomach	99.4	99.2
Blood	0.22	0.30
Thyroid (non-blocked)	0.05	0.06

Table 5

The deiodination of ^{131}I-labelled triolein in rat stomach

Into the stomach surgically separated from the oesophagus and duodenum 20 μc of [^{131}I]iodinated triolein was introduced. After 70 min. the radioactivity was determined.

Tissue	Expt. no. 1	Expt. no. 2
	Radioactivity (% of the dosis)	
Whole stomach, total activity	99.0	99.3
Gastric contents, lipid activity	> 98.2	> 98
Blood	0.007	0.008
Thyroid (non-blocked)	0.002	0.002

To check the possibility of deiodination of labelled fat in the stomach, [^{131}I]iodinated triolein was introduced into the surgically separated stomachs of two rats with non-blocked thyroid glands. The results presented in Table 5 show that 70 min. later, more than 98% of the radioactivity remained in the lipid-bound form. This indicates that, at least under the experimental conditions used, there was no deiodination of the iodinated fat in the stomach.

To examine the presence in the stomach of some other than iodide radioactive polar compound, the water phase of the gastric contents extract obtained from rats 1.5 hr. after feeding with [^{131}I]iodinated triolein, was analysed. The paper electrophorograms showed one homogeneous radioactive component of the same mobility at the $^{131}I^-$ ions. It was also shown that the non-lipid radioactive component of the gastric contents was quantitatively precipitated by silver salt in ammoniacal medium to form silver iodide. It can, therefore, be concluded that the non-lipid radioactive compounds found in rat stomach shortly after oral administration of [^{131}I]iodinated triolein are actually iodides derived from the extragastric process of deiodination of the iodinated fat.

DISCUSSION

The presented results demonstrate that a considerable part of $^{131}I^-$ ions derived from deiodination of fat accumulates in the rat stomach during the first few hours after oral administration of the ^{131}I-labelled triolein. The concomitant concentration of iodides in the stomach is several times higher than in the blood plasma. These findings are in agreement with the secretion of iodides into the stomach found by other authors [11, 5, 16]. The deiodination of [^{131}I]iodinated triolein does not take place in the stomach *in vivo*. The same was found to be true in experiments *in vitro* by Balint *et al.* [1]. The iodides present in the stomach result from the extragastric deiodination processes and are secreted from the blood by the mucosa into the lumen of the stomach. The gastric secretion of iodides was described by Lipschitz [11] and confirmed by Davenport [5]. Moreover, the present results indicate that iodides cannot be absorbed from the gastric contents into the blood, i.e. they can leave the stomach only through the pylorus.

The results obtained suggest that a part of the radioactive label of the introduced fat is recycled several times *via* the stomach. Such a circuit of the non-lipid iodine compounds is presented in the scheme of Riggs [14]. This recycling of iodides resulting from the metabolism of iodinated fat has not been yet taken into account in studies on lipid metabolism. Since the secretion of iodides from the blood into the lumen of the stomach is a regular and normal process, the deiodination products present in the digestive tract should not be regarded, as so far assumed, as derived only from the local process.

The accumulation of iodides in the stomach occurs not only in rats but in man as well [4, 9]. It seems that this phenomenon might provide the explanation of the incomplete recovery of non-lipid radioactivity in the experiments of Siurala *et al.* [17], a few hours after the administration of [^{131}I]iodinated triolein to human subjects; after 6 hr. they found 11 - 28% deficiency in the balance, and after 72 hr. 1 - 11% defi-

ciency in relation to the administered dose. In our opinion this incomplete recovery resulted from gastric accumulation of iodides whereby the iodide diffusion space was quantitatively insufficient.

The presented experiments seem to indicate that in the studies on the utilization of ^{131}I-labelled fat, both lipid and non-lipid radioactivity fractions should be examined.

SUMMARY

Following the administration *per os* of ^{131}I-labelled triolein, the radioactivity of various parts of the digestive tract in rat was studied. The lipid-bound and the water-soluble radioactivity was estimated. It was found that iodides derived from the deiodination of the administered fat were secreted from the blood into the stomach. The presented results indicate that in the studies on lipid digestion using iodinated fat, both lipid and non lipid radioactivity fractions should be examined.

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WYDZIELANIE JODKÓW W ŻOŁĄDKU JAKO OGRANICZENIE ZASTOSOWANIA TRÓJOLEINY ZNAKOWANEJ ^{131}J W DOŚWIADCZALNYCH BADANIACH JELITOWEGO WCHŁANIANIA TŁUSZCZU

Streszczenie

Badano rozmieszczenie radioaktywności lipidowej i nielipidowej w poszczególnych odcinkach przewodu pokarmowego szczura do 6 godzin po podaniu *per os* trójoleiny znakowanej ^{131}J. Stwierdzono, że jodki pochodzące z dejodacji podanego tłuszczu w znacznym stopniu są wydzielane ze krwi do żołądka. Podkreślono konieczność oznaczania frakcji lipidowej i nielipidowej w doświadczalnych badaniach dotyczących losów jodowanego tłuszczu w przewodzie pokarmowym.

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PROTEINS OF NORMAL URINE

III. ELECTROPHORETIC SEPARATION OF THE SULPHOSALICYLIC ACID-SOLUBLE PROTEINS *

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In the previous paper [1] a simple and fast method for concentration of proteins of normal urine was presented, the procedure being based on the precipitation by tannin and regeneration of proteins from the precipitate with caffeine according to Mejbaum-Katzenellenbogen [7]. In the present paper is described the isolation of 0.15 M-sulphosalicylic acid-soluble proteins (mucoproteins) by the tannin-caffeine procedure, and their paper electrophoretic patterns are compared with that of the total proteins of the urine.

MATERIAL AND METHODS

Fresh urine from healthy human subjects was used. Uromucoid was removed according to Tamm & Horsfall [13] as follows: to 1 vol. of urine, 1 vol. of distilled water was added and sodium chloride to 0.58 M-concentration. After 15 min. the solution was filtered and the precipitate of uromucoid discarded.

The urine proteins were concentrated according to Mejbaum-Katzenellenbogen [7] as previously described [1]. The pH of the uromucoid-free urine, about 800 ml., was adjusted to 4.7 with 0.1 N-HCl on bromocresol green, then a 4% water solution of tannin was added to obtain an amount of tannin 10- to 20-fold as great as that of the protein present, the final concentration of tannin being about 0.1% at protein concentration of about 0.01%. The protein-tannin precipitate was centrifuged, washed twice with 0.9% NaCl solution, then an amount of caffeine *in substantia* twice as great as that of the protein present in the precipitate, was added. The precipitate was thoroughly mixed with caffeine and centrifuged. The small quantity of the formed supernatant was found to contain protein in about 2% concentration. About 30% of the original amount of protein remained in the discarded tannin-caffeine precipitate.

* Parts I and II [1, 2].

The mucoproteins were separated from the concentrated urine proteins as follows: to 1 vol. of the solution (about 2 ml.), 1 vol. of 0.9% NaCl and 1 vol. of 0.45 M-sulphosalicylic acid, were added. After 15 min. the mixture was centrifuged and the precipitate discarded. In the supernatant, the mucoprotein was determined and an amount of tannin 10-fold as great as the content of mucoproteins, was added. After 30 min. at 5° the precipitate (sometimes gummy) was centrifuged and washed 3 - 4 times with distilled water to remove sulphosalicylic acid. Then an amount of caffeine twice as great as the amount of mucoproteins present in the precipitate, was added, stirred and about 0.1 - 0.2 ml. of 0.05 N-NaOH in 0.9% NaCl was poured in to obtain a suitable volume of, and concentration of mucoprotein in, the regenerated solution. After 30 min. at 5°, the mixture was centrifuged. The supernatant was found to contain mucoproteins in a concentration of 2 - 5%. About 20% of the original amount of mucoproteins remained in the discarded tannin-caffeine precipitate.

Determinations of protein were carried out by the tannin micromethod of Mejbaum-Katzenellenbogen [6].

Paper electrophoresis was carried out as follows. Whatman no. 1 paper, sodium veronal-acetate buffer of pH 9.0, ionic strength 0.08, or acetate buffer of pH 4.7, ionic strength 0.06 were used; a potential of about 360 V and a current of about 3 mA per paper strip were applied for 5 - 6 hr. After electrophoretic separation the proteins were fixed and stained by the tannin reagent containing 0.05% of bromophenol blue, and the protein content of individual fractions was determined in 0.1 N-NaOH eluates [8].

Acid mucopolysaccharides were stained with alcian blue according to Heremans & Vaerman [5].

RESULTS

Mucoprotein content in normal urine

When the amount of protein was determined by the tannin micromethod: (a) in normal untreated urine, (b) in the same urine diluted with distilled water, or (c) diluted with sulphosalicylic acid, a paradoxical phenomenon was observed. In diluted samples the amount of protein calculated per 1 ml. of urine was found to be higher than in the corresponding undiluted samples, and sulphosalicylic acid did not precipitate the protein. On the other hand, in uromucoid-free urine the protein content did not depend on the dilution and sulphosalicylic acid precipitated a part of protein (Table 1). This shows that the elimination of the uromucoid is important for the determination of the protein content by the tannin micromethod as well as for the precipitation of proteins

Table 1

Effect of uromucoid on the determination of protein by the tannin micromethod and on the precipitation of protein with sulphosalicylic acid

The uromucoid was eliminated according to Tamm & Horsfall [13]. The final concentration of sulphosalicylic acid (SSA) was 0.15 M. The protein was determined by the tannin micromethod [6]. The results represent the amount of determined protein in mg. calculated per 100 ml. of the original urine.

Sample no.	Urine with uromucoid			Urine without uromucoid		
	undiluted	diluted 3 times with		undiluted	diluted 3 times with	
		H_2O	SSA		H_2O	SSA
1	5.8	6.3	6.3	5.2	5.2	4.8
2	6.9	8.2	8.1	6.0	6.0	2.4
3	11.7	13.6	13.8	10.4	10.4	8.4
4	3.5	5.2	5.1	2.0	2.0	1.1

by sulphosalicylic acid. Therefore in further experiments the uromucoid was always removed from the examined urine.

The separation of mucoproteins from total protein by sulphosalicylic acid was examined in uromucoid-free urine directly and in the con-

Table 2

Determination of mucoproteins in normal urines and in the concentrated proteins of the same urines

Urine after removal of the uromucoid of Tamm & Horsfall was used. Determinations of protein were carried out by the tannin micromethod [6]. Proteins were concentrated by the tannin-caffeine procedure.

Initial	Sex	Urine			Concentrated protein of urine		
		Total protein	Mucoprotein		Total protein	Mucoprotein	
		(mg. %)	(mg. %)	(% of total protein)	(mg. %)	(mg. %)	(% of total protein)
W.D.	fem.	3.8	1.4	36.9	2400	850	35.5
B.B.	m.	3.4	2.5	73.5	2840	2050	72.2
B.M.	fem.	3.8	2.0	52.9	1420	760	53.5
J.K.	m.	2.0	0.5	25.0	1150	262	22.8
E.L.	fem.	4.5	2.2	49.0	2700	1400	51.9
E.M.	fem.	6.0	3.4	56.6	3540	2040	57.5
M.K.	m.	3.5	2.0	57.1	3400	2200	64.8
M.W.	m.	7.0	3.5	50.0	1840	980	53.2
H.I.	m.	5.0	2.0	40.0	2970	1250	42.1
M.M.	fem.	6.0	3.0	50.0	3420	1550	45.2
W.D.	fem.	5.2	4.8	92.3	1260	1170	92.8
P.D.	m.	6.0	2.4	40.0	1130	510	45.3
M.M.	fem.	10.4	8.4	80.7	2050	1600	78.0

centrated protein solution. As shown in Table 2, from the urine containing about 5 mg.% of protein the same proportion of protein was precipitated by 0.15 M-sulphosalicylic acid as from the solution concentrated to about 2% of protein. In samples of normal urine having similar protein concentrations the amount of mucoprotein was found to differ widely, amounting from 20 to 90% of the total protein (Table 2).

Paper electrophoresis of the total proteins and mucoproteins of normal urine

The concentrated mucoproteins were examined by paper electrophoresis at pH 9.0. Like the total urine proteins, the mucoproteins were separated into 5 fractions, poorly resolved in the region of α-globulins and slightly displaced towards the anode (Fig. 1, *A* and *B*). The concentrated total proteins and mucoproteins were also separated at pH 4.7 (Fig. 1, *C* and *D*).

Table 3

Relative distribution of electrophoretic fractions of total proteins and mucoproteins of normal urine

Mean values ± S.D for 10 urines at pH 9.0 and for 5 urines at pH 4.7 are given.

pH	Fractions with mobilities of	Total proteins (% distribution)	Mucoproteins (% distribution)
9.0	Albumin	25.6 ± 9.92	15.0 ± 4.03
	α_1-Globulin	12.7 ± 3.69	16.0 ± 7.81
	α_2-Globulin	17.5 ± 7.54	17.8 ± 7.76
	β-Globulin	28.2 ± 10.82	33.9 ± 12.24
	γ-Globulin	16.0 ± 5.32	17.3 ± 6.44
4.7	*I*	9.2 ± 4.69	5.3 ± 1.82
	II	7.4 ± 1.02	9.6 ± 2.65
	III	25.2 ± 2.51	33.9 ± 5.25
	IV	42.6 ± 0.59	24.0 ± 2.83
	V	10.7 ± 0.28	21.8 ± 2.81
	VI	4.9 ± 1.26	5.4 ± 1.76

The electrophoretic fractions of mucoproteins and proteins were eluted and determined. The results are presented in Table 3. The mean values for the distribution of mucoprotein fractions were similar to those for protein fractions of the same urine samples at either pH, the greatest differences being found at pH 9.0 in the fractions with the mobility of albumin and at pH 4.7 in the fractions *IV* and *V*. At pH 9.0 sulphosalicylic acid-soluble "albumin" formed 15% of the total mucoproteins whereas in the total protein, albumin accounted for 25.6%; this difference, however, was not statistically significant. The electrophoretic

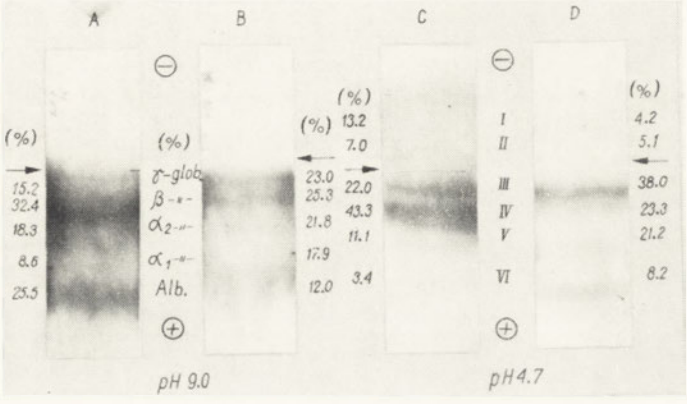

Fig. 1. Electrophoretic patterns at pH 9.0 (*A* and *B*) and at pH 4.7 (*C* and *D*) of proteins and mucoproteins of normal urine concentrated by the tannin-caffeine technique. (*A*), Urine proteins concentrated from the initial amount of 0.01 g./100 ml. to 1.7 g./100 ml.; 680 μg. of protein was placed on paper; (*B*), mucoproteins of the same urine concentrated from the initial amount of 0.006 g./100 ml. to 1.8 g./100 ml.; 720 μg. of protein was placed on paper; (*C*), urine proteins concentrated from the initial amount of 0.009 g./100 ml. to 1.75 g./100 ml.; 700 μg. of protein was placed on paper; (*D*), mucoproteins of the same urine concentrated from the initial amount of 0.005 g./100 ml. to 1.35 g./100 ml.; 540 μg. of protein was placed on paper.

pattern obtained at pH 4.7 displayed statistically significant differences in the distribution of the fractions *IV* and *V*.

The electrophorograms at pH 9.0 of concentrated proteins and mucoproteins were stained with alcian blue for acid mucopolysaccharides [5]. In both cases three fractions with a mobility greater than that of albumin were revealed.

DISCUSSION

Rigas & Heller [12] were the first to use electrophoresis for studying the proteins of normal urine. Although these proteins were found to consist of components having mobilities similar to those of serum proteins, they differ from serum proteins by their nature, origin and by the presence of some proteins derived from the urinary tract [4].

In normal urine the proteins are present in amounts too small for examination. The procedure of concentration of urine proteins as well as of mucoproteins by tannin and caffeine seems to fulfil three important conditions. The procedure is "mild" and no protein-denaturing reagent is used; the protein is released from the protein-tannin compounds without any detectable physico-chemical or biological changes [9, 10]; the procedure is fast enough to concentrate a 1 liter sample in about 3 hr. and so it is faster than other methods used. In the tannin-caffeine technique no desalting procedure is needed because the tannin-caffeine precipitate is virtually insoluble in distilled water and can be purified by washing out the salts and non-protein compounds unprecipitable by tannin (e.g. amino acids and peptides of low molecular weight).

From the examined urine the uromucoid of Tamm & Horsfall had to be removed because of its protective effect on the precipitation of protein by sulphosalicylic acid, and by tannin in the tannin micromethod for protein determination. Also in other studies the uromucoid had been removed before the electrophoretic separation of urine proteins was carried out. The uromucoid has electrophoretic mobility between that of α_1- and α_2-globulin. Even after it had been salted out, a small amount was still present in the urine and that was perhaps the cause of the diffuse pattern of α-globulins of urine, reported also by Rigas & Heller [12]. At pH 4.7 the separation was better probably because of the acidic character of mucoproteins.

In blood serum, the mucoproteins soluble in sulphosalicylic acid, called seromucoids, account for 1% only of the total protein whereas mucoproteins of normal urine form about 50% of the total urine protein. The results obtained by Morawiecka & Mejbaum-Katzenellenbogen [11] concerning the serum, and those presented in this paper indicate the existence of quantitative differences between the mucoproteins of serum and urine. For example, in the serum α_1-globulin accounts for about 4%

of the total protein and albumin for 65%, whereas in seromucoid the fraction with the mobility of α_1-globulin contributes to 50% and the fraction of albumin only to 5%. On the other hand, in the urine the distribution of the total protein fractions and of mucoprotein fractions is quite similar, particularly at alkaline pH.

Gabriel *et al.* [3] studied the chemical composition of urine proteins soluble in trichloroacetic acid but their results cannot be compared with those reported in the present paper as there is no indication that the proteins soluble in trichloroacetic acid are identical with those soluble in sulphosalicylic acid. Morawiecka & Mejbaum-Katzenellenbogen [11] studied the seromucoids soluble in sulphosalicylic acid and in perchloric acid, and pointed out that in spite of the same amount of total seromucoid being present in both filtrates, their electrophoretic patterns were different.

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SUMMARY

1. Total proteins and proteins soluble in 0.15 M-sulphosalicylic acid (mucoproteins) were determined in normal human urine free from the uromucoid of Tamm & Horsfall. The mucoproteins accounted for 20 - 90% of the total protein.

2. The tannin-caffeine technique was used for concentration of proteins and of the sulphosalicylic acid-soluble fraction.

3. At pH 9.0 the electrophoretic patterns of proteins and mucoproteins showed a similar distribution of fractions; at pH 4.7 differences in the distribution of two fractions were found.

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BIAŁKA MOCZU PRAWIDŁOWEGO

III. ELEKTROFOREZA BIBUŁOWA BIAŁEK ROZPUSZCZALNYCH W KWASIE SULFOSALICYLOWYM

Streszczenie

1. Oznaczono białka całkowite i białka rozpuszczalne w 0.15 M-kwasie sulfosalicylowym (mukoproteidy) w moczach prawidłowych pozbawionych uromukoidu Tamma i Horsfalla. Stwierdzono, że mukoproteidy stanowią od 20 do 90%, średnio około połowy, białka całkowitego moczu.

2. Do zagęszczenia białek moczu oraz frakcji mukoproteidowej zastosowano technikę taninowo-kofeinową.

3. Elektroforogramy w pH 9.0 białek całkowitych i mukoproteidów tych samych moczów wykazywały podobny rozkład frakcji, a przy pH 4.7 stwierdzono występowanie różnic w 2 frakcjach.

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THE REPRODUCIBILITY OF THE COMPOSITION OF DN-PROTEIN ISOLATED FROM PURIFIED THYMUS NUCLEI*

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A great variety of chemical bonds of different stability present in the deoxyribonucleoprotein complexes (DN-protein) makes a precise definition of their molecules impossible [3]. Every preparation procedure involves a partial disruption of these bonds under the influence of various factors such as ionic strength, kind of ions, pH, and enzymic degradation. Therefore, depending on the conditions used, different fragments of DN-protein may appear in solutions and the composition of the final preparations depends on the isolation procedure.

The aim of this work was to look for a method of preparation of DN-protein of a reproducible composition. DN-protein, characterized by an intermediate complexity between that of isolated chromosomes and that of purified DNA and protein molecules, is of major interest so far as its structure and fractions are concerned. Neither the original composition and structure of DN-protein are known, nor tests for the determination of its biological activity are available. Therefore during the isolation we tried to prevent the occurrence of serious changes in the structure by using procedures generally believed to be mild, and avoiding more drastic methods. It is known that most, if not all, of the activity of DNases and cathepsins is localized in the cytoplasm [e. g. 2, 5, 12]. We assumed therefore that a complete removal of cytoplasm at the very beginning of the procedure is essential in order to protect DN-protein against enzymic degradation. Consequently a more exhaustive procedure for purification of nuclei from cytoplasm was applied. Like other workers [e. g. 4], we adopted the N/P ratio as a basic analytical measure for comparison of successive preparations of DN-protein.

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MATERIALS AND METHODS

Isolation of nuclei from thymocytes

Cell nuclei were obtained from calf thymus in 0.24 M-sucrose - 2.5 mM-$CaCl_2$ medium [14] using a procedure similar to that of Allfrey [1]. Six to eight glands (about 0.5 kg.) removed from calves in a slaughter-house 30 - 60 sec. after the death of the animals, were placed immediately in a vacuum flask containing about 3 l. of 0.14 M-NaCl solution at 0°, transported to the laboratory and used for rapid preparation within about 30 min. following removal.

All steps of the procedure were performed in a cold room at 0 - 3°. Three samples of tissue, 150 g. each, roughly purified from the lymph nodes, fat and blood vessels, were minced in a meat grinder, suspended separately, each in 400 ml. of sucrose - $CaCl_2$ solution [14], and squeezed subsequently through 4 nylon mill gauze bags of diminishing diameter of meshes (twice 1 mm., once 0.4 mm., once 0.08 mm.). Connective tissue remaining in the bags was discarded and the filtrates, containing a suspension of thymocytes, were centrifuged for 15 min. at 600 *g*. The sediments were resuspended gently, each in 400 ml. of sucrose - $CaCl_2$, by means of a wooden stirrer operated by hand, and were centrifuged again. After each centrifugation the thin, coloured surface layers were removed to eliminate minute amounts of erythrocytes and fat. Then the three sediments, originating from 450 g. of tissue, were combined. The yield was about 80 g. of packed thymocytes. They were gently resuspended by means of the wooden stirrer in 50 ml. of sucrose - $CaCl_2$ until an uniform suspension was obtained. Two ml. of this suspension were left for examination. The rest was homogenized gently (3 min. at 60 V.) in a waring blendor (Niko, Yugoslavia), centrifuged for 10 min. at 600 *g*, and the surface layer of erythrocytes was discarded together with the supernatant. This procedure was repeated twice and gave finally purified nuclei free of cytoplasm, as judged by the following tests. (a) Examination in a phase-contrast microscope (Lumipan, C. Zeiss, Jena) using magnification × 1350. Unstained nuclei, nuclei stained with 0.2% methyl green or stained according to Kurnick & Ris [10] were used for examination. (b) DNase activity of suspensions of nuclei was estimated according to the spectrophotometric method of Rotherham *et al.* [13]. (c) Small amounts of supernatants after each homogenization were clarified by centrifugation at 28 000 *g* and their extinctions at 260 and 280 mμ were estimated.

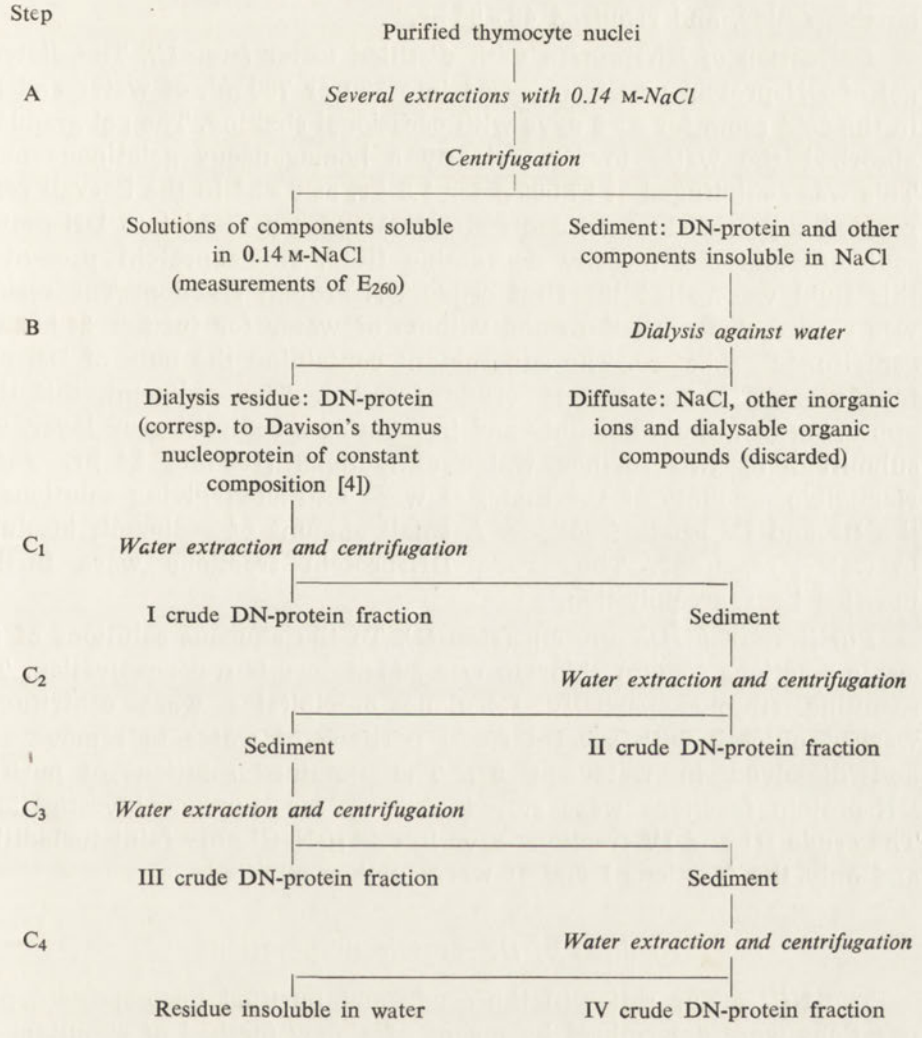
Preparation of DN-protein from isolated nuclei

The whole procedure for this preparation is shown in scheme 1.

Extraction with 0.14 M-NaCl (step A). The packed purified nuclei (40 - 80 g.) were suspended in 450 ml. of 0.14 M-NaCl, disintegrated in the

Scheme 1

Preparation of DN-protein from isolated nuclei



Niko blendor at high speed (15 sec., 180 V.) and extracted gently for 5 min. at low speed (65 V.). The homogenate was centrifuged for 10 min. at 3 000 *g*. Small amount of the supernatant was clarified by centrifugation at 10 000 *g* for 10 min. and its extinction at 260 mμ was measured.

Salt extraction procedure, as described above, was repeated with the sediment until constant extinction values (about 0.1 - 0.2) of two successive extracts have been reached.

Dialysis of sediments against water (step B). To remove traces of salts the gelatinous sediment of DN-protein was washed several times with water, transferred into cellophane bags (23/32 Visking tubing) and dialysed against successive 2 l. volumes of water with constant stirring of the outside fluid and gentle mixing of the DN-protein gel. The removal of ions was controlled by conductivity measurements of successive outside fluids, and required 48 - 96 hr.

Extraction of DN-protein with distilled water (step C). The dialysed gel of DN-protein was transferred into 350 or 700 ml. of water and left in the cold room for 2 - 3 days with occasional shaking. The gel gradually absorbed the water to form finally a homogeneous gelatinous mass. This was centrifuged at 27 000 *g* for 1.5 hr. at 0 - 2° in the Servall refrigerated superspeed centrifuge (SS rotor) and separated into DN-protein sediment and clear, easily decantable fluid. Nucleoprotein present in this fluid was called the first crude DN-protein fraction. The residue was reextracted with the same volume of water for further 24 hr. and centrifuged as before. The supernatant containing the bulk of DN-protein, was defined as the II crude fraction. The sediment, this time appearing in minute amounts and in form of a compact grey layer, was submitted to two further water extractions (100 ml., 24 hr. each). Negligible amounts of the material were extracted giving solutions of the III and IV crude fractions. A small amount of sediment, insoluble in water, remained. The crude DN-protein fractions were further purified by reprecipitation.

Purification of DN-protein (step D). To the aqueous solutions of the crude fractions sodium chloride was added to 0.14 M-concentration. The resulting fibrous, partially floculous precipitates were centrifuged, washed quickly with several small portions of water to remove salt, and dissolved in water at 3°. The obtained solutions of purified DN-protein fractions were stored at -20° for further investigations. The crude III and IV fractions gave in 0.14 M-NaCl only faint turbidities, and only the fractions I and II were further purified.

Analysis of DN-protein preparations

The N/P molar ratios in the crude and purified nucleoprotein preparations were determined by means of a new method of simultaneous mineralization [8] using selenium - $HgSO_4$ catalyst. Phosphorus was estimated according to Horecker *et al.* [9] and nitrogen by a modified Kjeldahl microprocedure according to Eisner & Wagner [7] in an improved automatic distillation apparatus (J. Skarżyński, in preparation).

RESULTS AND DISCUSSION

Isolation and purification of nuclei. Thymocytes and thymocyte nuclei, when blended in the sucrose - $CaCl_2$ medium at low speed (60 V.) form uniform suspensions and their samples taken by volume for quantitative measurements give quite reproducible results. At higher speeds of the blendor (70 V. and more) clumps of damaged cells or nuclei begin to form and quantitative volumetric sampling becomes impossible. Therefore, we did not use speeds higher than 65 V.

Microscopic examinations of the preparations, even after tenfold blending in sucrose - $CaCl_2$ solution at 60 V., have shown the presence of single undamaged cells and of slight amounts of cytoplasmic particles attached to the surface of some nuclei. The assays of DNase activity showed that even the exhaustively washed purified nuclei still possessed notable DNase activity. Fig. 1 presents DNase activity in the suspension of whole thymocytes, after the first blending, and in the suspension of

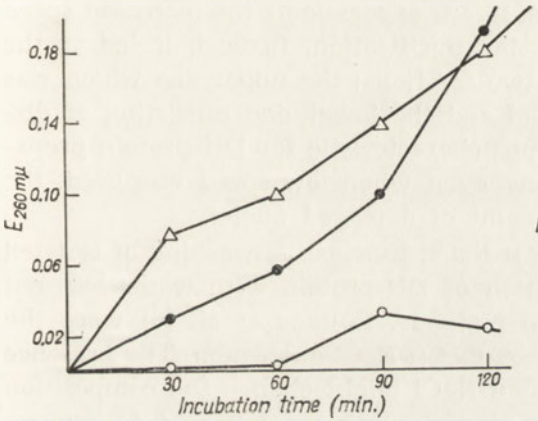


Fig. 1

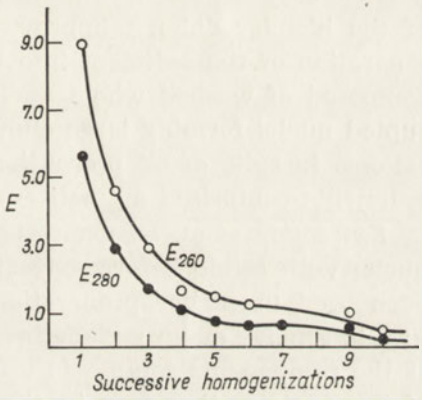


Fig. 2

Fig. 1. DNase activity of the suspensions of thymocytes and thymocyte nuclei. Composition of the samples: 4.0 ml. of 0.1% DNA from thymus, highly polymerized (obtained in this laboratory according to Skoczylas *et al.* [15]); 4.0 ml. of acetate buffer, pH 5.0, ionic strength 0.6; 4.0 ml. of the suspension containing 125 000 cells or nuclei in 1 µl. Incubation at 37° on a water bath with constant shaking. The reaction was stopped by addition of 4.0 ml. of 20% $HClO_4$ (final concn. 0.5).N After 20 min. the mixture was centrifuged for 10 min. at 3000 r.p.m. and in the supernatant the extinction was measured [13]. (●), Undamaged thymocytes; (△), thymocytes after 5 min. of blending; (○), purified nuclei after ninefold blending in sucrose - $CaCl_2$ medium.

Fig. 2. The extinction at 260 and 280 mµ of the supernatants after successive blendings of thymocytes in sucrose - $CaCl_2$. The supernatants after removal of thymocytes were clarified by centrifugation for 10 min. at 10 000 *g*, brought to suitable dilution with sucrose, and their extinction measured. The E values, calculated for undiluted extracts, are given.

purified nuclei after nine blendings. However, no quantitative conclusions can be drawn because of the complex enzymic composition of the suspensions. Both the digestion products of DNA and RNA and the products of their further enzymic decomposition are present in the examined acid-soluble fraction. Both from the enzymic and the microscopic examinations it is visible that a threefold blending is insufficient for the complete removal of the cytoplasm.

During the successive blendings of thymocytes in sucrose - $CaCl_2$ solution extinctions at 260 and 280 mμ of the supernatants were measured (Fig. 2). The observed decrease of extinction may be considered as an additional measure of the removal of the cytoplasmic components soluble in sucrose - $CaCl_2$. Their almost total removal was achieved after tenfold blending. It must be taken into account, however, that such a long and exhaustive washing causes a partial extraction of some nuclear material absorbing at these wavelengths. Neelin & Butler [11] isolated nuclei in diluted citric acid using a very similar blending procedure. They found that the isolation of thymocyte nuclei sufficiently free of cytoplasm was possible only after 8 - 9 blendings. In our experiments the increased speed of the blendor did not improve the purification. Instead, it led to the separation of the sediment into two fractions: the upper one which was composed of washed whole nuclei and the lower one consisting of disrupted nuclei forming large clumps characteristic for DN-protein preparations. In spite of all precautions, even when low speed was used, the sediments comprised a small amount of damaged nuclei.

Extraction of nuclei with 0.14 M-*NaCl.* Exhaustive washing of isolated nuclei with saline before extraction of DN-protein with water had not been used in earlier preparation methods. Dounce *et al.* [6] were the first to remove nuclear globulins by 0.14 M-NaCl extraction. The presence of the components soluble in 0.14 M-NaCl might change the composition of DN-protein, therefore in the present work we intended to remove them from nuclei after damaging the nuclear membrane but before water extraction of DN-protein. In this way we hoped to prevent an exchange between the proteins originally bound to DNA and the proteins soluble in 0.14 M-NaCl. Furthermore, saline extraction was expected to remove at least a part of DNase and cathepsin activities which, although very small, are still present in the purified nuclei.

The decrease of the amount of 0.14 M-NaCl-soluble substances absorbing at 260 mμ in the successive extracts of three different preparations is shown in Fig. 3. E_{260} values of the first extracts and the number of extractions necessary to reach the required final extinction value of 0.1 - 0.2, differed in particular preparations depending on the degree of purification of nuclei, on the ratio of NaCl solution volume per mass of nuclei, and possibly on the percentage of nuclei disintegrated by blending at higher speed. The E_{260} values of the first NaCl extracts (Fig. 3) show

that the amounts of substances absorbing at 260 mμ extracted from carefully purified nuclei were negligible. If the extinction of the last successive extracts had remained constant this could be considered to be a measure of the solubility of DN-protein in 0.14 M-NaCl. The extinction values depended, however, on the extraction time and the ratio of NaCl solution volume to the mass of nuclei, and these values could be still reduced by an increase in the number of extractions. This observation, quite important for the considerations on constant or reproducible composition of DN-protein complexes, will be a subject of further experiments and will be discussed separately.

To get some informations about the nature of substances extracted from nuclei by 0.14 M-NaCl, absorption spectra of the successive supernatants in the region from 230 to 300 mμ were measured. The results of determination of four successive extracts expressed as percentages of their extinction at 260 mμ, are presented in Fig. 4. The shapes of the

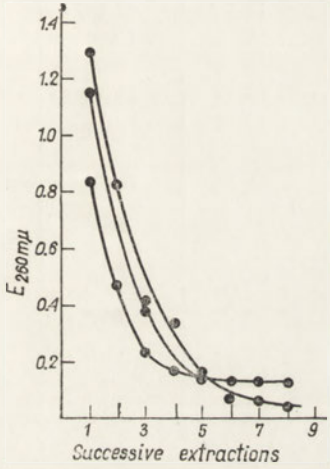


Fig. 3

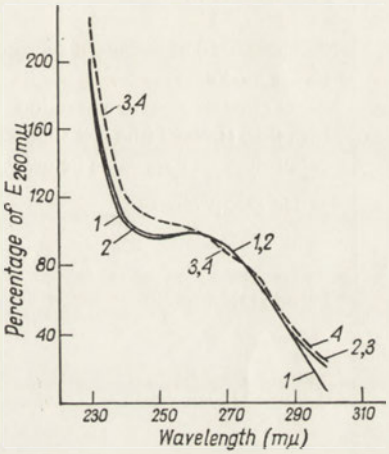


Fig. 4

Fig. 3. The extinction at 260 mμ of the supernatants obtained by successive extractions of thymocyte nuclei with 0.14 M-NaCl. The supernatants were clarified by centrifugation for 10 min. at 10 000 *g* and suitably diluted. The results for three separate preparations of nuclei are given.

Fig. 4. Absorption spectra of four successive 0.14 M-NaCl extracts of purified thymocyte nuclei. For qualitative comparison of the curves, the values are expressed as percentages of $E_{260\,m\mu}$. The actual E_{260} values for particular extracts were: 1, 0.838; 2, 0.464; 3, 0.240; 4, 0.172.

curves for the first and second extract indicate the presence of a mixture of larger amounts of protein and small amounts of nucleic acid derivatives. The third and fourth extract contained only protein.

Dialysis and water extraction of DN-protein. In these longlasting steps of preparation the material is exposed to the action of autolytic enzymes,

and both these procedures have always been the most controversial steps. Careful purification of nuclei, several extractions with 0.14 M-NaCl and low temperature diminish considerably the activities of autolytic enzymes but do not eliminate them completely. Changes occuring in the structure of DN-protein during these inevitable steps of procedure are so far unknown.

Table 1

N/P molar ratios during the preparation of DN-protein

The results are averages from four or more parallel experiments.

Fraction	Preparation no.	N/P molar ratio
Thymocytes	IV	12.45
	V	12.22
	VI	12.08
Nuclei after 3 blendings in sucrose-$CaCl_2$ medium	IV	12.15
	V	12.30
Nuclei after 10 blendings in sucrose-$CaCl_2$ medium	IX	11.63
Nuclei after exhaustive extraction with 0.14 M-NaCl (the last supernatant had an E_{260} value below 0.2)	IX	10.90
I crude DN-protein	VI	8.80
	VII	8.48
	IX	8.30
II crude DN-protein	VI	7.72
	VII	7.63
	IX	7.82
III crude DN-protein	VI	8.16
	VII	8.38
	IX	8.08
IV crude DN-protein	IX	12.05
Residue insoluble in water	IX	18.40
I purified DN-protein	VII	7.96
	IX	7.80
	X	7.88
	XI	7.95
II purified DN-protein	VII	7.50
	IX	7.61
	X	7.49
	XI	7.63

The N/P molar ratio in DN-protein preparations. Some values obtained are presented in Table 1, and regular changes of this ratio can be observed. In the first crude water extracts of DN-protein the ratio was high

(average 8.53); it was the lowest in the second extracts (av. 7.72) and rose again in extracts III (av. 8.21) and IV (av. 12.05). Crude water extracts had higher ratio values (up to 18.4) than the water-insoluble residues. During the purification of the crude fractions I and II the N/P ratios decreased (from 7.72 to 7.56 for the II fraction). This suggests that some losses of protein occur during salt precipitation of DN-protein from water solutions.

The reproducibility of the N/P ratio values was quite satisfactory; they seem to be the most reproducible in the purified DN-protein fraction but the average value of the N/P molar ratio is much lower than that of Davison & Butler [4] who found the N/P weight ratio to be 4.1, which corresponds to 9.07 molar ratio.

We would like to express our thanks to the Polish Women Association in Chicago, Ill., for the gift of the Servall Superspeed Refrigerated Centrifuge which made this work possible, and to the staff of the Municipal Slaughterhouse in Łódź for cooperation in the proper collection of material.

SUMMARY

A method for isolation of deoxyribonucleoprotein (DN-protein) from calf thymus nuclei, with reproducible N/P molar ratios in successive preparations, is described.

The nuclei were prepared in sucrose - $CaCl_2$ medium, washed thoroughly with 0.14 M-NaCl, DN-protein was extracted with water and purified by salt precipitation.

The results of microscopic examinations, estimation of DNase activity, spectrophotometric control of purification and N/P ratio estimations are discussed. The final average molar N/P ratio value of 7.56 was reasonably reproducible. It was lower than similar values reported in the literature.

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BADANIA NAD IZOLOWANIEM PREPARATÓW DN-PROTEIDÓW O POWTARZALNYM SKŁADZIE Z JĄDER KOMÓRKOWYCH GRASICY

Streszczenie

Opisano metodę otrzymywania dezoksyrybonukleoproteidów z grasicy cielęcej z nastawieniem na powtarzalność stosunku azotu do fosforu w kolejnych preparatach. Jądra tymocytów preparowano w środowisku sacharozy z dodatkiem $CaCl_2$ i przemywano starannie roztworem 0.14 M-NaCl. Dezoksyrybonukleoproteidy ekstrahowano wodą i oczyszczano przez wysolenie.

Przedyskutowano wyniki badań mikroskopowych, oznaczenia aktywności DNazy, kontroli spektrofotometrycznej procesu oczyszczania, oraz oznaczeń stosunku azotu do fosforu. Końcowa średnia wartość stosunku N/P wynosiła 7.56. Była ona dość powtarzalna lecz niższa od danych przytaczanych w piśmiennictwie.

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W. MAKAREWICZ

AMP-AMINOHYDROLASE AND GLUTAMINASE ACTIVITIES IN THE KIDNEYS AND GILLS OF SOME FRESHWATER VERTEBRATES

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It has been observed in this laboratory [8] that the kidneys and gills of carp could liberate about 7 - 10 times more ammonia from AMP than from glutamine. In other animals either glutaminase was several times more active than AMP-aminohydrolase (Mammalia) or both these enzymes had similar activities (Amphibia, Reptilia, Aves).

It is generally accepted that in mammals the urinary ammonia is formed in the kidneys from glutamine and from some other amino acids [14, 5]. Some experiments concerning the sources of urinary ammonia in amphibians were also presented [1]. However, very little is known about the sources of the ammonia excreted by freshwater fishes although this is their main nitrogenous waste product. Goldstein & Forster [7] established that in marine teleost *Myoxocephalus scorpius* 25% of ammonia may be accounted for by the disappearance of glutamine from the blood passing through the gills. Our previous results seem to suggest that in lower animals AMP might act as a precursor of ammonia excreted [8].

In this paper some data on the activities of glutaminase and AMP-aminohydrolase in the kidneys and gills of some freshwater vertebrates are presented.

EXPERIMENTAL

Animals and tissues. Lamprey (*Lamperta fluviatilis* L.) and five species of fishes were used for experiments. The animals were killed by a blow on the head or by decapitation. The kidneys and gills were rapidly removed and placed in an ice-cold beaker. The soft external layers of gill filaments were scraped out with a piece of glass and used for experiments. The tissues were weighed and homogenized with 9 vol. of 0.1 M-KCl containing 0.039 M-borate of pH 7.0, in a Potter-Elvehjem homogenizer chilled with ice. A part of the homogenate was diluted

with an equal volume of KCl-borate solution and used for glutaminase assay. The rest of the homogenate was centrifuged at 0° for 5 min. at 600 *g* and after discarding the sediment, the supernatant was centrifuged again at 0° for 15 min. at 16 000 *g*. The supernatant was collected, the pellet in the centrifuge tube was rinsed lightly and suspended in KCl-borate solution. The suspension called further the mitochondrial fraction, and the supernatant called the cytoplasmic fraction, were tested for AMP-aminohydrolase activity.

Glutaminase assay. The incubation was carried out at 25° for 15 min. in microdiffusion bottles. The incubation mixture contained in 1.0 ml.: 10 μmoles L-glutamine; 50 μmoles Na-phosphate buffer, pH 7.7; 25 μmoles tris, pH 7.7; 15 μmoles NaCl, and an amount of the whole homogenate corresponding to 0.2 - 0.4 mg. of nitrogen. The reaction was started by the addition of the homogenate and stopped by the addition of 0.5 ml. of saturated Na_2CO_3 solution. The ammonia formed was determined after microdiffusion according to Brown *et al.* [4]. Colour intensity was measured at 625 mμ in a Coleman-6 spectrophotometer or in a Leitz photometer fitted with a no. 620 filter. Two control assays were made simultaneously, one for phosphate-free hydrolysis, and the second for non-enzymic hydrolysis of glutamine. The results given represent the net phosphate-activated glutaminase activity and are expressed as μmoles of ammonia formed during 1 hr. of incubation per 1 mg. of nitrogen or 1 g. wet weight of tissue.

AMP-aminohydrolase assay. The incubation mixture containing in 2.0 ml.: 10 μmoles AMP; 200 μmoles KCl; 50 μmoles tris of pH 7.2; and amounts of the cytoplasmic fraction or mitochondrial fraction corresponding to 0.1 - 0.3 mg. of nitrogen, was incubated at 25° for 30 min. The reaction was started by the addition of the tissue preparation and stopped by heating for 5 min. on a boiling water bath. The samples were filtered and the remaining proteins removed by treatment with chloroform according to Sevag *et al.* [11]. The nucleotides and nucleosides present in the deproteinized incubation mixtures were separated by paper chromatography. The enzyme activity was calculated as described by Purzycka [9] and expressed in μmoles of IMP formed per 1 mg. of nitrogen or 1 g. wet weight of tissue per 1 hr.

Paper chromatography. The nucleotides and nucleosides were separated by the descending technique on Whatman no. 1 paper using saturated ammonium sulphate - isopropanol - water (72:2:19, by vol.) according to Deutsch & Nilsson [6]. The spots were localized by photography in ultraviolet light, cut out, and eluted with 5 ml. of 0.1 N-HCl for 12 hr. The extinctions of the eluates were read at 250 mμ for inosine, and at 260 mμ for adenosine derivatives in an Unicam SP 500 spectrophotometer against appropriate blank samples.

Total nitrogen determination. Nitrogen was estimated in the Parnas-Wagner apparatus, $CuSO_4$ being used as a catalyst for combustion, and boric acid for binding of ammonia.

Reagents. AMP and tris were L. Light (England) products. L-Glutamine was from Schuchardt (East Germany). Other reagents were obtained from the Gliwicka Fabryka Odczynników, Gliwice, Poland.

RESULTS

In all animals tested, AMP-aminohydrolase was localized in the cytoplasmic fraction, in which traces of 5′-nucleotidase activity were also present. The mitochondrial fraction possessed a distinct activity of 5′-nucleotidase. Fig. 1 presents a photograph in UV of a typical chromatogram

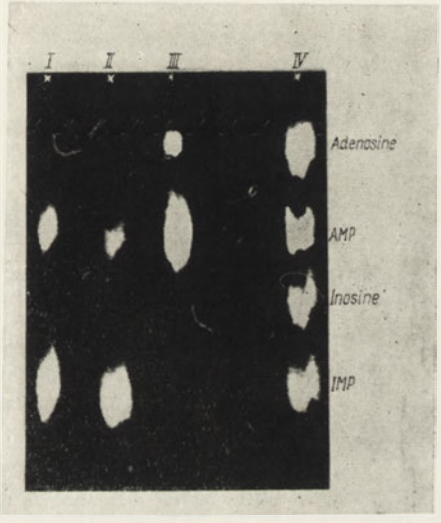

Fig. 1. Photograph in UV of the chromatographically separated products of AMP decomposition by gills and kidney of the eel. (*I*), Cytoplasm of the kidney; (*II*), cytoplasm of the gills; (*III*), mitochondria of the kidney; (*IV*), standards. For experimental conditions see text.

of the products arising on incubation with AMP. The cytoplasmic fraction of gills and kidneys deaminated AMP to IMP very actively, whereas in the mitochondrial fraction a small amount of adenosine was formed due to the 5′-nucleotidase activity. A faint spot of IMP was sometimes observed after incubation of the mitochondrial fraction with AMP. This was probably due to a contamination with the cytoplasmic fraction. It was possible to avoid the contamination by thorough washing of the mitochondrial fraction with KCl-borate solution but this procedure was not always applied.

In the cells of kidneys and gills of freshwater fishes, the localization of the enzymes deaminating and dephosphorylating AMP was found to be the same as that found by Żydowo [16] in rat kidney.

Fig. 2 shows the ability of the gills and kidneys of fish and lamprey to produce ammonia from AMP and from glutamine, expressed as μmoles ammonia per 1 g. of tissue. It may be seen that the activity of AMP-aminohydrolase exceeds several times the activity of glutaminase in the kidneys and gills of all species investigated. It means that during a given period 10 - 30 times more ammonia can be formed from AMP than from

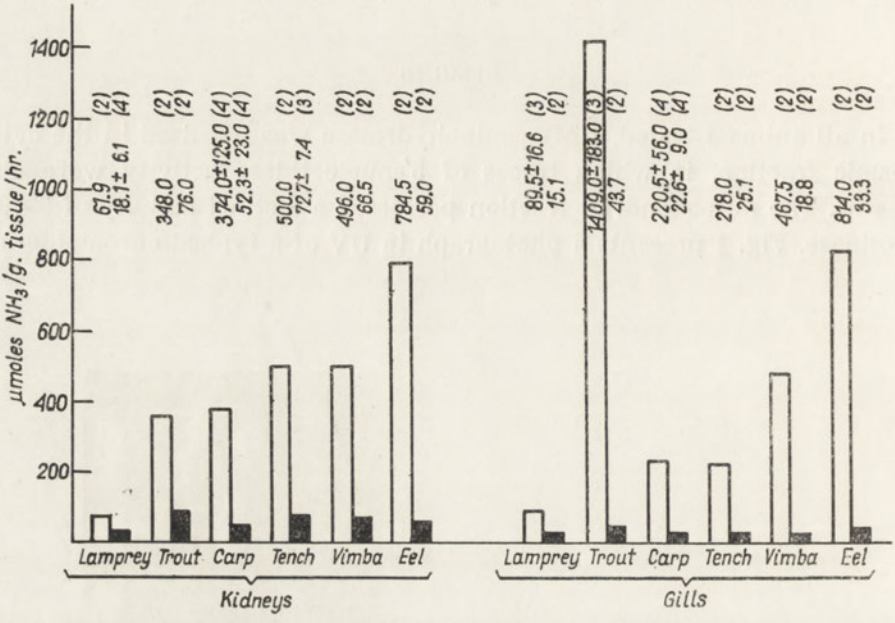

Fig. 2. The ability of kidneys and gills of some vertebrates to produce ammonia from glutamine and AMP. The values represent the mean, ± S.D. where applicable. In parentheses, the number of animals studied. For experimental conditions see text. AMP-aminohydrolase activity, white bars; glutaminase, black bars.

glutamine. The ratio of AMP-aminohydrolase activity to glutaminase activity is always higher in the gills than in the kidneys. The activity of glutaminase in the gills of fishes is lower than in their kidneys, being of the same order as in the kidneys of rabbit and guinea pig, the animals which are known to excrete only small amounts of ammonia.

The lamprey, which belongs to the lowest class of vertebrates, can also produce more ammonia from AMP than from glutamine. The activities of both enzymes studied are much lower in the tissues of this animal than in those of fishes; the activity of AMP-aminohydrolase is of the order observed in mammals [8].

Table 1 presents the activities of the two ammonia-producing enzymes calculated per mg. of total nitrogen. It may be seen from these data that in fishes as well as in the lamprey AMP-aminohydrolase constitutes a relatively large proportion of the proteins present in the excretory organs.

Table 1

The activities of AMP-aminohydrolase and glutaminase in the kidneys and gills of some freshwater vertebrates

The values represent the mean, ± S.D. where applicable. In parentheses, the number of animals studied. AMP-aminohydrolase activity was estimated in the cytoplasmic fraction and glutaminase in the whole homogenate as described in the text.

Species	Kidney		Gills	
	AMP-amino-hydrolase	Glutaminase	AMP-amino-hydrolase	Glutaminase
	μmoles IMP/ mg.N/hr.	μmoles NH_3/ mg.N/hr.	μmoles IMP/ mg.N/hr.	μmoles NH_3/ mg.N/hr.
Cyclostomata				
Lamprey (*Lamperta fluviatilis* L.)	(2) 7.1	(4) 1.1 ± 0.4	(3) 12.7 ± 1.0	(2) 1.0
Pisces				
Trout (*Salmo irideus* Gibbons)	(2) 39.0	(2) 3.9	(3) 172.0±25.4	(2) 2.7
Carp (*Cyprinus carpio* L.)	(4) 46.5±11.6	(4) 2.6±1.5	(4) 33.0±10.1	(4) 1.4±0.3
Tench (*Tinca tinca* L.)	(2) 49.0	(3) 3.8±0.7	(2) 25.8	(2) 1.5
Vimba (*Vimba vimba* L.)	(2) 60.3	(2) 3.5	(2) 65.0	(2) 1.2
Eel (*Anguilla anguilla* L.)	(2) 71.8	(2) 3.1	(2) 119.7	(2) 2.4

DISCUSSION

Since the experiments of Van Slyke *et al.* [14] it is generally assumed that in mammals the urinary ammonia is formed mainly from glutamine due to the action of a specific glutaminase [10]. In freshwater teleost fishes ammonia is a principal end-product of protein - nitrogen metabolism, and the gills are the main place of its excretion [12]. Many years ago Smith [12] suggested that ammonia excreted through the gills of fishes is not formed there but derives from the blood and is removed from the body by simple diffusion, however, no experimental evidence supporting this view was presented. Goldstein & Forster [7] demonstrated that most of the ammonia excreted by fishes is formed in the gills, and only about 12% derives from the blood ammonia. They found that in the gills of *Myoxocephalus scorpius* the deamidation of glutamine supplies about 25% of the ammonia excreted. As the deamination of glutamic acid may supply another 25%, they concluded that only the remaining 40% of the ammonia excreted by the gills is still not accounted for.

As ammonia is formed in the excretory organs of fishes and amphibians [15, 2] the high activity of AMP-aminohydrolase would suggest that AMP may serve as an important source of ammonia. However, the concentration of AMP and its turnover rate in the kidneys and gills

of fishes are not known. On the other hand, the concentration of glutamine is very similar to that observed in the kidneys of mammals (M. Żydowo and J. Purzycka, unpublished data).

Apart from fishes, only in the kidneys of amphibians such high activity of AMP-aminohydrolase was observed [8]. In amphibians the high activity of AMP-aminohydrolase is accompanied by a relatively high activity of glutaminase whereas in fishes the activity of the latter enzyme is much lower than that of the former. Amphibians are the transitory group of vertebrates between those living in water and the fully terrestrial animals. Their larval forms live in water and are ammoniotelic; then perhaps the ammonia is formed to a larger extent by deamination of AMP, like it is proposed for the teleost fishes and Cyclostomes. After metamorphosis the amphibians become terrestrial and then they are predominantly ureotelic and may use the glutamine - glutaminase system for ammonia production, as mammals do. *Xenopus laevis*, the adult form of which is living in water, has both very active AMP-aminohydrolase and glutaminase in the kidney. This animal preserved the ability of being either ammoniotelic or ureotelic depending on the external environment [13, 3].

Although the activities of both glutaminase and AMP-aminohydrolase in the kidneys and gills of lamprey are low as compared with fishes, also in this animal the latter enzyme is much more active than the former.

The present data as well as the previously described experiments [8] suggest that in lower vertebrates the deamination of AMP might supply a significant amount of the ammonia excreted.

The author wishes to thank Doc. Dr. M. Żydowo for his criticism and helpful discussion, and Mrs. Regina Kaczorowska for her technical assistance. The specimens of fish were kindly supplied by Mgr. R. Bartel from Instytut Rybactwa Śródlądowego in Gdańsk, Eng. A. Kosior from Ośrodek Doświadczalny M.I.R. in Tolkmicko, and Mr. M. Pietrusiński from Centrala Rybna in Gdynia.

SUMMARY

The activities of AMP-aminohydrolase and glutaminase were estimated in the kidneys and gills of five teleost fish species and of the lamprey. Kidneys and gills of fishes possessed very active AMP-aminohydrolase whereas the activity of glutaminase was low being of the order observed in higher animals. The activity of glutaminase in the gills was always lower than in the kidney. The possible role in various species of AMP and glutamine as precursors of the ammonia excreted, is also discussed.

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AKTYWNOŚĆ AMP-AMINOHYDROLAZY I GLUTAMINAZY W NERKACH I SKRZELACH KRĘGOWCÓW SŁODKOWODNYCH

Streszczenie

Oznaczano aktywność AMP-aminohydrolazy i glutaminazy w nerkach i skrzelach minoga oraz pięciu gatunków słodkowodnych ryb kostnoszkieletowych. W nerkach i skrzelach ryb znaleziono bardzo wysoką aktywność AMP-aminohydrolazy, natomiast aktywność glutaminazy jest niska; jest ona tego rzędu co w nerkach wyższych zwierząt. Aktywność glutaminazy w skrzelach jest u wszystkich badanych gatunków niższa niż w nerkach. Przedyskutowano rolę AMP i glutaminy jako prekursorów amoniaku wydalanego przez różne zwierzęta.

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TERESA STELMASZYŃSKA and J. M. ZGLICZYŃSKI

OXIDATION OF CYSTINE BY HYDROGEN PEROXIDE CATALYSED BY HORSE-RADISH PEROXIDASE

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Medes & Floyd [9] reported that cystine is oxidized by rat liver homogenate, and Chapeville & Fromageot [2] found that one of the products of cystine incubated with chick embryo homogenate is cystine disulphoxide.

Sulphoxide can be synthetized by oxidizing cystine with hydrogen peroxide in the presence of iodine as catalyst [22]. The oxidation of cystine by hydrogen peroxide suggests that peroxidase may be involved in the physiological oxidation of this compound. Therefore we undertook a study of the oxidation of cystine by hydrogen peroxide in the presence of a crude preparation of horse-radish peroxidase. The catalytic effect of iodine on non-enzymic oxidation of cystine [1, 22], as well as the fact that peroxidases are activated by KI_3 [15], induced us to employ potassium iodide as activator of the enzymic oxidation of cystine.

The properties of horse-radish peroxidase described by Theorell [18] and the observations of Knox & Mehler [5] on peroxidases indicate that an increase in hydrogen peroxide concentration decreases the activity of these enzymes. To avoid this effect, in the present experiments the hydrogen peroxide was generated in the incubation mixture by the oxidation of glucose by glucose oxidase. Sulphoxides, the expected product of the reaction, are unstable compounds which decompose readily, giving cystine. Hence, it was not possible to follow the reaction by determination of the decrease of cystine, or by chemical characterization of the reaction product. Manometric determination of oxygen consumption was therefore employed. Oxygen uptake was found to be proportional to the amount of hydrogen peroxide used in the presence of peroxidase for the oxidation of cystine. The products of the reaction were separated chromatographically and compared with the synthetically obtained sulphur amino acids. As some compounds containing the sulphoxide grouping were found to affect the activity of enzymes with active SH groups [21, 14, 13] and the presented experiments confirmed the inhibitory effect of cystine sulphoxide on the activity of papain, the effect of the products of enzymic oxidation of cystine on papain activity was also studied.

MATERIALS AND METHODS

A crude preparation of horse-radish peroxidase was obtained by the method presented by Maehly [7] and based on the procedures of Theorell and Keilin. The RZ value (the ratio of extinction at 403 mμ to extinction at 275 mμ) of the preparation was 0.2 - 0.3. Denaturation of peroxidase was carried out by heating on a boiling water bath for 15 - 25 min., which resulted in a loss of activity towards dihydroxyphenylalanine.

Cysteinesulphinic acid and cystine sulphoxide were obtained by the method described previously [22]. Cystine (Riedel, Hannover, Germany), 1 - 3 mM solution in Na_2HPO_4-HCl buffer of pH 7.2 was used. Cysteinic acid, papain and EDTA were L. Light (Colnbrook, England) products. The preparations of glucose oxidase (K. & K. Lab. Inc., U.S.A., and L. Light, England) contained catalase. Catalase was from K. & K. Lab. Inc., U.S.A.; glucose and potassium iodide from Fabryka Odczynników Chemicznych, Gliwice, Poland.

Human haemoglobin was dissolved in water and denatured by heating for 15 min. on a boiling water bath.

The incubations were carried out at 37°, the final volume of the samples being 4 ml. Substrates and enzymes were dissolved in 0.05 M-phosphate buffer of pH 7.2. The course of oxidation of cystine by peroxidase was studied manometrically, chromatographically, and by a method consisting in determination of the effect of the reaction products on the activity of papain. The experiments were performed with eight samples of different composition (Table 1). Substrate, peroxidase and glucose were placed in the Warburg flasks, 0.1 ml. of 20% NaOH in the central well, and glucose oxidase in the sidearm. The reaction was started by adding glucose oxidase, and incubation carried out until oxygen uptake ceased, i.e. until the whole amount of the glucose added was oxidized. The reaction was completed in about 60 min. Then each of the eight samples was divided into four parts. To the first part of each sample 50 μg. of catalase in 0.5 ml. of buffer was added and, after 5 min. incubation, 100 μg. of papain in 1 ml. of 1% EDTA in phosphate buffer; 10 min. incubation followed. Then the mixture was added with a suspension of 25 mg. of denatured haemoglobin in 1 ml. of phosphate buffer, and incubated at 37° for 15 min. After deproteinization with 5 ml. of 5% trichloroacetic acid, aromatic amino acids were determined in 2 ml. of the filtrate [4].

The remaining three parts of each of the 8 samples were analysed by paper chromatography. For this purpose they were desalted and deproteinized with a double volume of acetone. The acetone was removed by shaking with chloroform. Since sulphoxides decompose during chromatography, in two of the three portions the sulphoxides

were transformed into more stable compounds by reactions with CN^- and HS^- [16, 12]. To one portion 0.5 ml. of 0.01 N-KCN was added (reaction 1), and a current of H_2S was passed through the other portion (reaction 2).

$$RSO_2SR\,^* + CN^- \rightarrow RSO_2^- + RSCN \quad (1)$$

$$2\,RSO_2SR + HS^- \rightarrow RSO_2^- + RSO_2SH + RSSR \quad (2)$$

These reactions were carried out at 20° for 1 hr. After acidification with 0.1 ml. of 0.2 N-HCl, the solutions were concentrated in a current of air, then applied marginally on the circles of Whatman no. 1 paper, and developed using the solvent system of *tert.* butanol - formic acid - - water (70:15:15, by vol.). The chromatograms were dried at room temperature and the spots localized with isatin, ninhydrin [10], or reagents allowing differentiation of sulphur amino acids, i.e. KI in HCl, sodium azide, chloroplatinate, ferric chloride and silver nitrate [22]. In addition, standard sulphur amino acids were used for identification.

RESULTS

Reaction of cystine with the enzymically produced hydrogen peroxide catalysed by horse-radish peroxidase

Twenty experiments were performed; a typical one together with the manometric results, is illustrated in Table 1. The amount of oxygen consumed per hr. in control samples to which no cystine was added (sample *III*) and to which heat-denatured peroxidase was added (samples *II, IV, VI, VIII*) was 180 - 190 µl. The consumption of oxygen in the proper sample (*I*) was 200 - 220 µl., and after adding KI (sample *V*), 215 - 240 µl.

The consumption of oxygen by all 8 samples resulted from the oxidation of glucose by glucose oxidase. As the preparations of glucose oxidase contained catalase, the decomposition of hydrogen peroxide proceeded simultaneously, so that the manometric readings were the resultants of both reactions. In the proper samples in which cystine was also present together with the active peroxidase the consumption of oxygen was higher because a part of the hydrogen peroxide was used for the oxidation of cystine. The effect of possible changes in catalase activity was also examined by adding catalase in some experiments after the incubation. No manometrically detectable amounts of hydrogen peroxide were found in any of the samples.

The chromatographic analysis of the incubated samples containing cystine and active peroxidase revealed the presence of cysteinesulphinic

* In this paper the symbol R is used to denote the aminopropionic radical, $-CH_2-CHNH_2-COOH$.

Table 1

The oxidation of cystine by hydrogen peroxide catalysed by horse-radish peroxidase

Composition of the incubation mixture: cystine, 6 μmoles; glucose, 16 μmoles; glucose oxidase, 100 μg.; horse-radish peroxidase, 500 μg.; 0.06 M-phosphate buffer, pH 7.2; and, where indicated, 1 μmole of KI. Final volume 4 ml. Manometric readings were made as described in the text. Results are expressed as μl. of consumed O_2.

Composition of the sample	Incubation time (min.)		
	20	45	60
I. Whole sample	98	188	208
II. Whole sample, peroxidase denatured	85	166	187
III. Cystine omitted	85	162	185
IV. Cystine omitted, peroxidase denatured	89	171	190
V. Whole sample, KI added	101	198	226
VI. Whole sample, KI added, peroxidase denatured	86	170	190
VII. Cystine omitted, KI added	90	165	180
VIII. Cystine omitted, KI added, peroxidase denatured	89	169	190

acid and small amounts of cystine disulphoxide. After subsequent treatment with cyanide, chromatography showed the presence of cysteinesulphinic acid and cysteinic acid, and after treatment with hydrogen sulphide, traces of alaninethiosulphonic acid. In the samples without active peroxidase only cystine was found.

The effect of cystine oxidation products on the activity of papain

The experiments performed by Wills [21] on the effect of allicin ($C_3H_5SOSC_3H_5$) on the activity of enzymes depending on the presence of free SH groups, and the work of Pihl *et al.* [14, 13] on the effect of cystamine monosulphoxide on some of these enzymes, suggest that cystine sulphoxide may have a similar effect. The effect of this compound and of some other sulphur amino acids on the activity of papain was therefore studied. It was found (Fig. 1) that cystine sulphoxide is an inhibitor of papain, the effect being proportional to the concentration. Cysteinesulphinic acid inhibits papain activity only slightly, and cystine and cysteinic acid are without effect.

These results permitted to estimate the formation of cystine sulphoxide by determining the activity of papain. When the manometric experiment was terminated, 0.25 - 1 ml. samples were withdrawn from the Warburg flasks and incubated with papain solution and denatured haemoglobin. From the results (Fig. 2) it is apparent that during the incubation of cystine with hydrogen peroxide and peroxidase, some

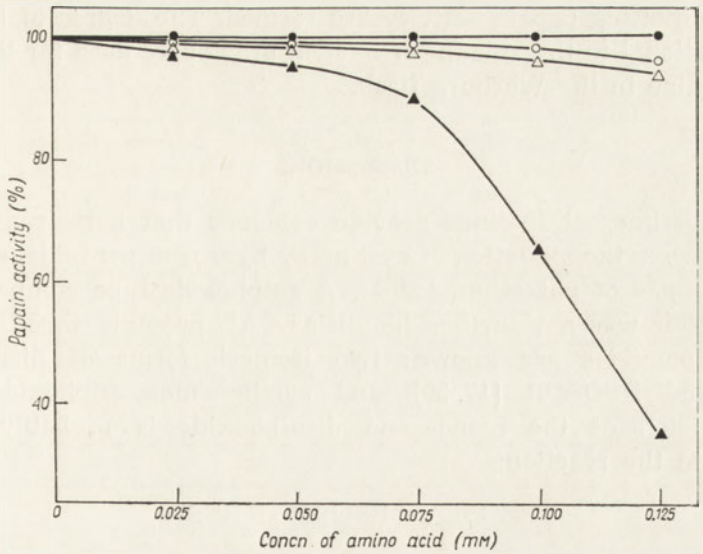

Fig. 1. The effect of sulphur amino acids on papain activity. (●), Cysteinic acid; (○), cystine; (△), cysteinesulphinic acid; (▲), cystine sulphoxide. Activity of papain with buffer added instead of sulphur amino acid, was assumed to represent 100% activity. Conditions: 1 ml. of suitably diluted sulphur amino acid in 0.06 M-phosphate buffer, 100 µg. papain, 10 mg. EDTA, 25 mg. denatured haemoglobin to total volume of 4 ml. at pH 7.2; incubation 37°, 15 min.

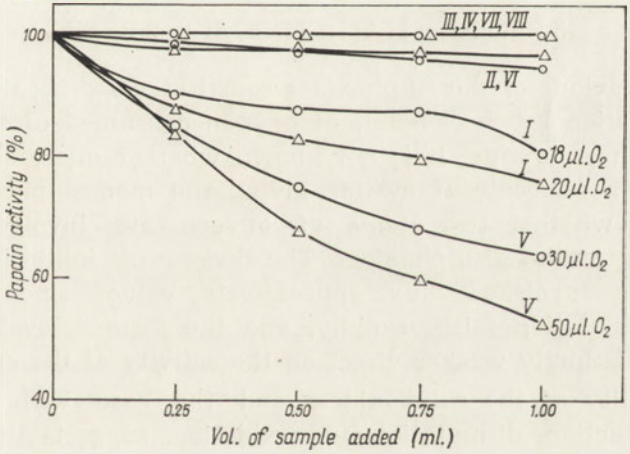

Fig. 2. The effect of cystine oxidation products on papain activity. The activity of papain was estimated in a mixture containing in 4 ml.: 100 µg. papain, 10 mg. EDTA, 25 mg. denatured haemoglobin and a sample of the incubation mixture after the manometric experiment. The original composition of the added samples as in Table 1; sample *I* contained cystine and active peroxidase; sample *V*, cystine, active peroxidase and KI. For samples *I* and *V* the amount of O_2 consumed in the manometric experiment is also given.

products inhibiting papain activity are formed. The degree of inhibition was proportional to the amount of oxygen previously used for the oxidation of cystine in the Warburg flask.

DISCUSSION

The experimental findings provide evidence that horse-radish peroxidase catalyses the oxidation of cystine by hydrogen peroxide, especially in the presence of potassium iodide. A sulphoxide-type compound was formed but it was not further identified. At present, three types of cystine sulphoxides are known: two isomeric forms of disulphoxide RSO_2SR and RSOSOR [17, 20], and cystine monosulphoxide [8, 19]. Interconversion of the mono- and disulphoxide is probably possible according to the reaction:

$$2\,RSOSR \rightleftharpoons RSO_2SR + RSSR \qquad (3)$$

This reaction may offer an explanation why workers using the same compound for oxidizing organic disulphides have obtained either sulphoxide [13] or disulphoxide [6, 8]. Presumably, all the methods of synthesis known at present lead to a mixture of the three above-mentioned forms of cystine sulphoxides. Furthermore, cystine disulphoxides and monosulphoxide undergo dismutation to cysteinesulphinic acid and cystine.

$$3\,RSO_2SR + 2\,H_2O \rightarrow 4\,RSO_2H + RSSR \qquad (4)$$

$$3\,RSOSR + H_2O \rightarrow 2\,RSO_2H + 2\,RSSR \qquad (5)$$

Such behaviour of the sulphoxides constitutes a difficulty in quantitative evaluation by the amount of oxygen consumed of the course of the oxidation of cystine. It is not known whether one atom of oxygen reacts with a molecule of cystine giving the monosulphoxide as first product, or whether two atoms of oxygen are involved, resulting in formation of the disulphoxide. The degree of inhibition of papain activity also furnished only approximate values since sulphoxides undergo dismutation fairly rapidly, and the formed cysteinesulphinic acid has a distinctly weaker effect on the activity of the enzyme.

The lability of the sulphoxides, and the ease with which they undergo reduction, dismutation and oxidation, suggests that they may play a role in tissue metabolism. The known reactions of disulphoxides, and especially of monosulphoxides, with thiol (reactions 6, 7) [12, 3] may explain the inhibitory effect of sulphoxides on papain activity and on other enzymes containing SH groups [21, 14, 13].

$$RSO_2SR + RSH \rightarrow RSO_2H + RSSR \qquad (6)$$

$$RSOSR + 2\,RSH \rightarrow 2\,RSSR + H_2O\,(?) \qquad (7)$$

The wide distribution in animal tissues of enzymes producing hydrogen peroxide, and of peroxidases [11] suggested that cystine sulphoxide may be a natural metabolite. The work of Chapeville & Fromageot [2] confirmed this supposition. Sulphoxides may be the first step in physiological oxidation of cystine, and their formation may be of importance for the activity of reactions catalysed by enzymes which require SH groups for their activity.

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SUMMARY

Oxidation of cystine by hydrogen peroxide catalysed by horse-radish peroxidase was demonstrated. Potassium iodide activates this reaction. Sulphoxide formed by oxidation of cystine inhibits the activity of papain.

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UTLENIANIE CYSTYNY NADTLENKIEM WODORU W OBECNOŚCI PEROKSYDAZY CHRZANOWEJ

Streszczenie

Stwierdzono utlenianie cystyny nadtlenkiem wodoru katalizowane przez peroksydazę chrzanową. Aktywatorem tej reakcji jest jodek potasu. Sulfotlenek wytworzony przez utlenienie cystyny hamuje działanie papainy.

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THE ACTION OF γ-RAYS ON EGGS, LARVAE AND PUPAE OF *BOMBYX MORI*

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The diapause in insects, at whatever stage of the life cycle it sets on, is always characterized by the arrest of development and the minimum in the metabolic activity. Thus, the diapausing insects when compared with the active ones are a suitable object for studies on the correlation between the metabolic activity and the radiosensitivity in animals. The results previously reported [4] indicated that the diapausing pupae of *Celerio euphorbiae* irradiated with γ-rays at the period of minimum metabolic rate are more sensitive than the adult moths.

This paper deals with the effect of γ-rays on *Bombyx mori*, an insect which diapauses at the egg stage. The aim of present experiments was to compare the effects of ionizing radiation when applied at the period of minimal and that of normal metabolic rate in the insect's life cycle.

MATERIAL AND METHODS

Bombyx mori of "warska" race, used in these experiments were purchased from an industrial culture. The eggs were kept from October to the first days of February at 15° and then to 20 May at 4°. Thereafter the temperature was raised gradually and amounted on 28 May, 4, 6 and 13 June to 10°, 15°, 20° and 25° respectively. At this last temperature the eggs were kept till the hatching of larvae. The larvae were grown at room temperature (20° ± 2°). For the spinning period the culture vessels were fitted with insets in order to facilitate the cocoon formation. The cocoons were stored at 25° till the eclosion of adults and these were kept at the same temperature till their natural death.

Each portion of living material was irradiated with a single dose of γ-rays delivered from ^{60}Co source (48 Curie), the average dose-rate being 200 rtg. per min. The samples to be irradiated as well as the control ones were taken out from the population at random. The eggs were irradiated with the doses of 2500, 5000 and 10 000 rtg. on 28 May and with the doses of 20 000 rtg. on 23 October, 13 April and 28 May. The purgating larvae (immobilized for the time of irradiation in thin-

-wall glass tubes of appropriate diameter) and the 3-days old cocoons were irradiated with 2500 and 10 000 rtg.

The growth of larvae was controlled by weighing. The average weight of larva for each group was calculated at early stages of development from the total weight of the whole group and later from the weight of 10 larvae chosen at random from the group.

RESULTS

Eggs. The dose of 20 000 rtg., at whatever period it was given, as well as the dose of 10 000 rtg. when given at the beginning of post-diapausal development of eggs inhibited completely the hatching of larvae. The doses of 2500 and 5000 rtg. delayed the hatching by 3 days, as compared to the control, and reduced the number of larvae to 50% and 10% respectively, as referred to the number of irradiated eggs.

The growth of larvae derived from irradiated eggs was impaired and the mortality in these groups was higher than in controls (Table 1).

Table 1

The effects of γ-irradiation of eggs on further development of Bombyx mori

As control non irradiated eggs were used.

Day of larval growth	Control	Dose applied	
		2500 rtg.	5000 rtg.
	Average weight of larva (mg.)		
11	27	22	15
14	38	34	20
17	71	62	30
20	128	88	53
23	169	148	110
26—27	290	264	181
29—30	550	401	549
32	741	602	672
35	1140	1196	1201
38	1809	1925	1911
41	2466	2080	1680
Observation	Number of animals		
Total number	30	30	30
Died within 40 days of growth	2*	9	18
Died at the spinning period	7	17	10
Formed cocoons	21	4	2
Formed pupae in cocoon	18	2	1
Eclosed adults	18	2	1

* One specimen injured mechanically.

The smaller weight increment in larvae from eggs irradiated with 2500 and 5000 rtg. could be observed beginning with the 20th and 10th day respectively. The difference in the average weight of larva between the irradiated and control groups vanished after 30 - 38 days of growth, owing to the earlier death of more injured animals. The mortality of larvae during their growth was greater in the group exposed to 5000 rtg. than in that exposed to 2500 rtg. At the spinning period the mortality in the group exposed to 2500 rtg. was higher than in that exposed to 5000 rtg. and finally only a few animals survived in either group. The irradiation did not affect the spinning nor the pupation and metamorphosis of survivors. No exterior abnormalities were observed in the eclosed adults.

Larvae and pupae. The irradiation of purgating larvae and of 3-days old cocoons with 2500 and 10 000 rtg. did not prevent the metamorphosis (Table 2). However, the exposure to 10 000 rtg. resulted in the injured metamorphosis process. The eclosed adults showed distinct exterior abnormalities (unexpanded wings, bare thorax and abdomen, malformations and paresis of legs) so that the percentage of normal adults in these groups was considerably lower than in controls. The effect was most pronounced when the γ-rays were given at the stage of purgating larvae.

Table 2

The effect of γ-irradiation at the larval or pupal stage on the metamorphosis in Bombyx mori

Observation	Purgating larvae			Pupae in 3-days old cocoons		
	Control	irradiated with 2500 rtg.	irradiated with 10000 rtg.	Control	irradiated with 2500 rtg.	irradiated with 10000 rtg.
	Number of animals					
Total number of observed animals	20	30	30	29	26	25
Pupated animals	15	21	18	29	26	25
Adults formed in the pupae	12	19	16	29	26	25
Eclosed adults (total)	11	17	13	24	24	18
Normal eclosed adults	9	12	6	19	20	11
Percent of normal adults referred to total number	45	40	20	65	76	44

The influence of irradiation of larvae or pupae on the reproduction in adults. The reproduction was markedly impaired in groups irradiated with 10 000 rtg. at larval or pupal stage (Table 3). In 7 out of the 12 observed pairs of adults no copulation occurred at all. A high percentage of eggs layed by the mated females were not fertilized. The hatching

Table 3

The effect of 10 000 rtg. of γ-rays given at the larval or pupal stage on the reproduction of eclosed adults of B. mori

Female	Male	Copulation	Number of eggs	
			total	fertilized
treated	treated	—	0	—
treated	treated	—	420	0
treated	treated	+	450	300
treated	non treated	—	170	0
treated	non treated	—	280	0
treated	non treated	—	0	—
treated	non treated	+	120	0
treated	non treated	+	240	180
non treated	treated	—	370	0
non treated	treated	+	550	400
non treated	treated	—	150	0
non treated	treated	+	170	90

of larvae from fertilized eggs did not exceed 25%; in one case, 300 out of 450 eggs layed, has been fertilized but only 5 larvae hatched and even these died within few days. In the group irradiated with 2500 rtg. at the larval or pupal stage, the copulation occurred as a rule. The number of layed eggs and the percentage of unfertilized ones did not differ from the control values (Table 4). In this group the hatching of larvae was normal.

Table 4

The effect of 2500 rtg. of γ-rays given at the larval or pupal stage on the reproduction of eclosed adults of B. mori

Irradiation applied at the stage of	Female	Male	Copulation	Number of eggs	
				total	fertilized
Purgating larvae	non treated	non treated	+	220	214
	non treated	non treated	+	240	235
	non treated	non treated	+	210	206
	treated	treated	+	220	100
	treated	treated	+	160	125
	treated	treated	+	380	340
	treated	non treated	+	200	185
	non treated	treated	+	110	101
Pupae in 3-days old cocoons	treated	treated	+	280	245
	treated	treated	+	190	183
	treated	treated	+	170	113
	treated	non treated	+	200	180
	non treated	treated	+	280	265

Table 5

The effect of γ-irradiation of parents at the prepupal period on the development of their offspring

The observed groups were chosen at random from each population.

Day of larval growth of the offspring	Parents					
	Control (non irrad.)	Irradiated, dosis (rtg.)				
		Female 2500	Male 2500	Female 10 000	Male 0	Female 0	Male 10 000
	Average weight of larva (mg.)					
10—11	9	12		5		6	
13—14	18	21		11		14	
16—17	28	42		16		22	
19—20	54	72		28		46	
22—23	87	102		68		73	
25—26	160	174		129		133	
29—30	310	365		265		215	
31—32	463	533		517		397	
34—35	623	723		1018		543	
37—38	1363	1140		1817		1150	
Observation							
Total number	30	30		30		30	
Died during the feeding period (%)	3	3		30		30	
Formed the cocoon (%)	54	26		26		28	

The development of the second generation. The irradiation at the larval or pupal stage affected the development of the offspring even when the parents were of normal exterior and behaviour (Table 5). The progeniture of individuals exposed to 2500 rtg. developed normally till the stage of purgation. The offspring of individuals irradiated with 10 000 rtg., however, showed early the radiation damage and about 1/3 of this population died within the feeding period. The mortality at the purgation and spinning period, however, made the difference statistically insignificant as finally only about 1/4 of each progeniture reached the stage of cocoon-spinning.

DISCUSSION

The adult insects are relatively resistant against ionizing radiation. The doses of 250 000 rtg. given on *Celerio euphorbiae* [4] and of 200 000 rads applied on the females of *Habrobracon* [1] did not cause their immediate death. High doses can result in temporary immobilization of adult insects [7, 1]. This effect was also observed in the diapausing pupae

of *Celerio euphorbiae*, when irradiated with 1 500 000 rtg. of γ-rays [4]. Clark [1] referred the low sensibility of adult insects to the lack of mitoses in their somatic tissue.

As a matter of fact the insects at the stages in their life cycle which are connected with the cell division are usually more susceptible to the ionizing radiation. We have previously reported [4] that the dose of 15 000 rtg. of γ-rays given on the diapausing pupae of *Celerio euphorbiae* resulted in exterior abnormalities in adults. In the present experiments the dose of 10 000 rtg. of γ-rays injured the metamorphosis in *Bombyx mori* too. This dose applied on larvae at the 5th instar damaged the tissues which had to develop only after the irradiation, like wings, and did not impair the already formed tissues and functions like spinning and pupal molting. Clark [1] observed that the irradiation of *Habrobracon* larvae with 3000 rads of X-rays did not inhibit markedly the eclosion of adults, the majority of whom, however, showed malformations of wings. Lucas [5] reported the high radiosensitivity of mouse-retina at the stage of cytological differentiation, but emphasized that the susceptibility is greater than one could expect taking into account the number of mitoses observed.

The functions involved in the reproduction seem to be more sensitive to ionizing radiation than the metamorphosis. The damage to reproduction was observed even in these adults in which the irradiation with 10 000 rtg. at the larval or pupal stage did not result in external malformations. This is in agreement with the experiments of other authors. Grosch [3] reported the sterility of adult females of *Habrobracon* after doses of a few thousand rads of ionizing radiation and Erdman [2] observed the same effect with doses of the same range applied on larvae or pupae of this insect. In our experiments the lowest dose of 2500 rtg. of radiation used, did not result in the damage to either metamorphosis or reproduction of eclosed adults. The effects were perceptible only in the second generation at the spinning period.

The larvae of *B. mori* at the 5th instar as well as the fresh pupae in the 3-days old cocoons are characterized by high level of respiration, i.e. by considerable metabolic activity. For the period of diapause in eggs the embryonal development is arrested at the stage "A" so that no mitoses occur [6]. The metabolic activity of diapausing eggs of *B. mori* drops dramatically at the third day after they were layed and remains on a level about 30 times lower than that of developing eggs. In our experiments all doses were applied at the period of minimal metabolic activity of eggs. The irradiation in October was given at the period of physiological diapause persisting even at the environmental temperature of 15°. In April the low storage temperature of 4° prolonged artificially the conditions as in diapause. The irradiation in May was applied on eggs stored at 10°, this temperature being below the threshold for

further development of embryo. The results indicate that the diapausing eggs of *B. mori* showed much greater sensitivity to ionizing radiation than the actively metabolizing larvae or pupae. This is particularly evident when the effects of 2500 rtg. dose are compared. This dose applied on diapausing eggs inhibited by 50% the hatching and impaired the further development so that only few percent of the population reached the imago-stage. When the same dose was given at the larval or pupal stage neither the metamorphosis nor the reproduction of the irradiated generation were affected.

Taking into account the results of our previous studies on the radiosensitivity of *Celerio euphorbiae* [4] and the present results on *Bombyx mori*, it can be stated that in the life cycle of the investigated Lepidoptera there are two periods of high sensitivity to ionizing radiation: the egg stage and the pupal one. This sensitivity persists as well if the insect diapauses at one of these stages. The possibility that the prolonged, physiological lowering of metabolic rate increases rather than decreases the radiosensitivity of insects will be the working hypothesis of our further studies.

SUMMARY

Bombyx mori were irradiated with single doses of γ-rays. The dose of 10 000 rtg. given on purgating larvae or on 3-days old cocoons injured the metamorphosis. The effect of 2500 rtg. applied at the same stages was revealed only in the second generation. The dose of 10 000 rtg. given on the diapausing eggs inhibited completely the hatching of larvae and the doses of 2500 and 5000 rtg. delayed the hatching of larvae and reduced their number respectively to 50% and 10% of the observed population. The high radiosensitivity of the stage of egg and that of pupae in Lepidoptera persists even if at these stages the metabolic activity is minimal owing to the diapause.

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WPŁYW PROMIENIOWANIA γ NA JAJA, GĄSIENICE I POCZWARKI *BOMBYX MORI*

Streszczenie

Naświetlano *Bombyx mori* jednorazowymi dawkami promieniowania γ. Dawka 10.000 rtg. podana w stadium oczyszczającej się gąsienicy lub 3-dniowego kokonu zaburza przebieg metamorfozy, a dawka 2500 rtg. ujawnia swe skutki dopiero w następnym pokoleniu. Natomiast dawka 10.000 rtg. podana w stadium diapauzującego jaja hamuje całkowicie wylęg gąsienic, a dawki 5000 i 2500 rtg. w tym okresie opóźniając wylęg obniżają go odpowiednio do 10 i 50% populacji. Zatem szczególna wrażliwość na promieniowanie stadiów: metamorfozy i jaj u Lepidoptera występuje również wówczas, gdy na dany okres przypada drastyczne obniżenie metabolizmu wywołane diapauzą.

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CHEMICAL SYNTHESIS OF ALIPHATIC ANALOGUES OF COBINAMIDE COENZYME AND THEIR EFFECT ON ENZYMIC ACTIVITY

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The discovery of the light-sensitive corrin derivatives and the demonstration of their role as coenzymes [1, 12, 2] posed the problem of their chemical structure, of the synthesis of these compounds as well as of their analogues, and of the effect of the analogues on enzymic reactions. The chemical structure of coenzyme B_{12} was elucidated by Lenhert & Hodgkin [9] who have shown that it contains the 5′-deoxyadenosine group directly attached to cobalt in place of the cyanide group in cyanocobalamin. The valency of cobalt in the coenzyme molecule was widely discussed (for review see [3]); several data indicated a bivalent cobalt, however, the recent data based on the rentgenographic (cit. after [4]) and electron paramagnetic resonance measurements [8] as well as some chemical features have shown that the trivalent cobalt is present in the coenzyme. The conclusions of the analytical studies on the structure of coenzyme B_{12} have largely been supported by the chemical synthesis of this compound [4, 18, 10] and of several analogues containing the Co—C bond. In all these syntheses advantage was taken of the nucleophilic character of the cobalt present in the reduced form of the corrin. The syntheses were based on the cryptoionic replacement of the proton in the corrin hydride for an alkyl, or 5′-deoxynucleoside residue derived from the alkyl halides or from 2′,3′-isopropylidene-5′-toluenesulphonylnucleosides, respectively. Smith *et al.* [18, 17] showed that the aliphatic and 5′-deoxynucleoside analogues were powerful inhibitors of the enzymic conversion of di-1,2-ol(s) into deoxyaldehyde(s) in the Abeles reaction [1]. It was shown in our laboratory that the alkyl analogues of cobinamide coenzyme are also inhibitors of the conversion of glycerol into β-hydroxypropionic aldehyde [14]. In the present study a number of new aliphatic analogues of cobinamide coenzyme (Co-alkylcobinamides) were prepared and their chemical structure is described.

EXPERIMENTAL

Materials. The coenzyme B_{12} and the cobinamide coenzyme were isolated from *P. shermanii* cells [20]. The coenzyme form of *abcdeg*--hexamidecobyrinic acid was isolated from *Nocardia rugosa* cells [9a]. The cyanocobalamin and cobinamide cyanide were obtained by the cleavage of the coenzymes in the presence of KCN. After desalting by phenol extraction they were purified by paper chromatography using the mixture of *n*-butanol - propan-2-ol - acetic acid - water (100:70:1:97, by vol.) as a solvent system in the presence of HCN in the chamber. The 3,5,6-trimethylbenzimidazolylcobamide cyanide was obtained by methylation of vitamin B_{12} dicyanide [6].

The enzyme extract was prepared from the cells of *Aerobacter aerogenes* no. 572; from the crude extract the corrinoids were removed by adsorption on active charcoal and nucleic acids by precipitation with streptomycin sulphate, then the extract was dialysed against distilled water for 12 hr. at 0 - 4° [21].

The methyl, ethyl, *n*-propyl, *n*-butyl iodides, ethyl chloroformate, and inorganic reagents were commercial products (Fabryka Odczynników Chemicznych, Gliwice, Poland). *n*-Amyl chloride and isobutyl chloride were prepared from appropriate alcohols by esterification with hydrogen chloride [19]. *n*-Octyl bromide and *n*-decyl bromide were obtained by the action of PBr_3 on the respective alcohols [19].

Methods. The corrin compounds were determined in their dicyanide forms by measuring the extinction at 580 mμ. The estimations were performed on the Unicam SP-700 or Hilger H-700 spectrophotometer in silica cells (length 10 mm.). β-Hydroxypropionic aldehyde was estimated by the method of Smiley & Sobolov [16] using the Spectronic 20, Bausch & Lomb, photocolorimeter. The electrophoretic separations were performed on Whatman no. 3 paper at 15 v/cm. in 0.5 M-acetic acid, pH 2.7, or at 4 - 6 v/cm. in 0.05 M-phosphate buffer, pH 6.5. For purification and identification of the compounds the descending chromatography on Whatman no. 3 paper was employed, the mixture of *n*-butanol - - propan-2-ol - water (100:70:100, by vol.) being used as a solvent system.

Abbreviation used. 5,6-DMBIA-cobamide, 5,6-dimethylbenzimidazolylcobamide.

Preparation of corrin derivatives

All steps of the procedure were performed in dispersed red light or in a completely dark room.

Cobinamide hydride. Cobinamide cyanide, 10 mg., was dissolved in 3 ml. of 10% aqueous solution of ammonium chloride, and 100 mg. of zinc fillings and 1 mg. of magnesium powder were added. Gaseous nitro-

gen was passed through the solution to remove the oxygen, then the reaction vessel was shaken for about 5 - 10 min. In the course of the reduction of cobinamide the red colour of the solution was gradually changing into cherry-red, brown and finally grey-green (as seen in the daylight). The grey-green hydride was used for subsequent syntheses without removing the excess of zinc. The same method can be employed for the reduction of cyanocobalamin; a grey-green cobamide hydride is thus obtained.

Co-*alkyl analogues of cobinamide coenzyme*. The solution of cobinamide hydride was treated in the absence of air oxygen with the appropriate alkyl halides or with their solutions in methanol (Table 1). On dropwise addition of the alkylating agent, the colour of the mixture rapidly changed into deep yellow. The sample was then shaken for 1 - 10 min. and after addition of 20 ml. of water the mixture was filtered to remove the unreacted zinc. The filtrate was desalted by phenol extraction. To the phenol phase 5 volumes of acetone and one of ether were added, and a Co-alkyl derivative was reextracted with water. The water solution was concentrated *in vacuo* over solid KOH. Crude preparations contained as impurities cobinamide cyanide and traces of a yellow unidentified compound. The impurities were removed by paper chromatography. The elution of the Co-alkyl derivative from the strip of paper was performed with water or methanol or with the propan--2-ol - water mixture according to the method of Pawełkiewicz & Walerych [13]. The volatile solvents were removed *in vacuo* and water in a vacuum desiccator over solid KOH.

Table 1

Preparation of Co-alkyl derivatives of cobinamide

For the synthesis, cobinamide hydride obtained from 10 mg. of cobinamide was used.

Reagent added (ml.)	Cobinamide derivatives obtained	Time of the reaction (min.)	Yield (%)
Methyl iodide or dimethyl sulphate, 0.05	Co-methyl-	1	90
Ethyl iodide, 0.05	Co-ethyl-	1	90
n-Propyl iodide, 0.05	Co-*n*-propyl-	1	90
n-Butyl iodide, 0.05	Co-*n*-butyl-	1	90
Isobutyl chloride, 0.05	Co-isobutyl-	5	90
10% methanolic solution of *n*-amyl chloride, 0.25	Co-*n*-amyl-	10	85
10% methanolic solution of *n*-octyl bromide, 0.25	Co-*n*-octyl-	10	80
10% methanolic solution of *n*-decyl bromide, 0.25	Co-*n*-decyl-	10	80

The same method was employed for obtaining Co-alkyl derivatives of 5,6-DMBIA-cobamide.

Co-*carbetoxycobinamide*. The cobinamide hydride was prepared from 10 mg. of cobinamide cyanide and then treated with 0.05 ml. of ethyl chloroformate in the absence of oxygen. The hydride reacted immediately and a yellow Co-carbetoxy derivative was formed. After shaking for 1 min., water was added, the mixture was filtered, and the product purified as described above, the yield being over 95%.

Co-*methyl-3,5,6-trimethylbenzimidazolylcobamide*. The 3,5,6-trimethylbenzimidazolylcobamide cyanide, 5 mg., was dissolved in 1.5 ml. of 10% aqueous ammonium chloride, and 100 mg. of zinc fillings and about 1 mg. of magnesium powder were added. The oxygen was removed by passing gaseous nitrogen, and the mixture was shaken for 5 - 10 min. A grey-green 3,5,6-trimethylbenzimidazolylcobamide hydride formed was treated with 0.05 ml. of dimethyl sulphate. After 1 min. shaking, about 10 ml. of water was added, the mixture was filtered and the solution was extracted with the chloroform - phenol (1:1, v/v) mixture. Then 10 vol. of trichloroethylen and 1 vol. of ether were added and the yellow derivative was reextracted from the phenol phase with water. The water solution was concentrated *in vacuo* over solid KOH. The product was freed from impurities by paper electrophoresis at pH 2.7. The derivative obtained moved slightly slower than the Co-methylcobinamide. The yield of the product was 80%.

The formation of Co-*methylcobinamide by chemical degradation of* Co-*methyl-5,6-DMBIA-cobamide*. (1) By using perchloric acid: 1 mg. of Co-methyl-5,6-DMBIA-cobamide was dissolved in 10 ml. of 65% perchloric acid and the mixture left at room temp. in complete darkness for 3 hr. The solution was then neutralized and desalted by phenol extraction. The water solution was concentrated *in vacuo* over solid KOH. The Co-methylcobinamide obtained did not contain the hydroxy(aquo) B_{12}. The product was purified by paper electrophoresis. It was found that perchloric acid caused an almost complete splitting off of the nucleotide from the Co-methyl-5,6-DMBIA-cobamide. (2) Degradation by cerous hydroxide: Co-methyl-5,6-DMBIA-cobamide, about 4 mg., dissolved in 4 ml. of water was treated with 3.2 ml. of 0.333 M-aqueous solution of $Ce(NO_3)_3.6H_2O$, followed by 2.7 ml. of 1 N-NaOH. Nitrogen has been passed through the suspension during 30 min. and the sample was subsequently heated at 95° on a water bath for 30 min. Then the sample was cooled, mixed with 50 ml. of water and centrifuged to remove the suspended cerous hydroxide. The supernatant was purified by phenol extraction. The water solution was concentrated *in vacuo* over solid KOH. For the separation of the obtained Co-methylcobinamide from the unreacted Co-methyl-5,6-DMBIA-cobamide, paper electrophoresis at pH 2.7 and paper chromatography were used. Under the conditions

employed only about 30% of Co-methyl-5,6-DMBIA-cobamide was transformed into Co-methylcobinamide.

Attempt at methylation of Co-methyl-5,6-DMBIA-cobamide. The compound, 1 mg., was dissolved in 5 ml. of water, 2 g. of sodium bicarbonate was added followed by the dropwise addition of 0.2 ml. of dimethyl sulphate during 2 hr. with continuous stirring. The temperature of the sample was kept within 19 - 21°. The solution was then acidified with acetic acid, desalted by phenol extraction, and the water solution was concentrated *in vacuo*. The reaction products were identified by paper electrophoresis at pH 2.7, and by chromatography. The main compound obtained was the unreacted Co-methyl-5,6-DMBIA-cobamide, and no Co-methyl-3,5,6-trimethylbenzimidazolylcobamide was found.

Physico-chemical and coenzymic properties of the synthetized derivatives

The obtained Co-alkylcobinamides and the Co-carbetoxycobinamide are light-sensitive; they are not affected by acids, alkali and cyanide on short exposure, except the Co-carbetoxycobinamide. In aqueous solutions they are yellow, and in solid state they are deep-yellow substances. They can be separated by paper chromatography with the mixture of *n*-butanol - propan-2-ol - water (100:70:100, by vol.). Their rates of movement relative to that of coenzyme B_{12} are shown in Table 2.

Table 2

Relative mobility values of Co-aliphatic derivatives of cobinamide

Conditions: Descending paper chromatography on Whatman no. 3 paper; solvent: *n*-butanol - propan-2-ol - water (100:70:100, by vol.). Mobility of the respective analogues is expressed in relation to that for coenzyme B_{12}.

Compound	$R_{coenzyme\ B_{12}}$
Coenzyme B_{12}	1.00
Co-methylcobinamide	1.70
Co-ethylcobinamide	1.78
Co-*n*-propylcobinamide	1.82
Co-*n*-butylcobinamide	1.92
Co-isobutylcobinamide	1.80
Co-*n*-amylcobinamide	1.95
Co-*n*-octylcobinamide	2.12
Co-*n*-decylcobinamide	2.15
Co-carbetoxycobinamide	1.91

On paper electrophoresis, the analogues of cobinamide coenzyme possess a positive charge at pH 2.7 and move similarly as cobinamide cyanide or coenzyme B_{12}. The Co-*n*-octyl and Co-*n*-decyl analogues move slightly slower than Co-methylcobinamide. At pH 6.5 the cobina-

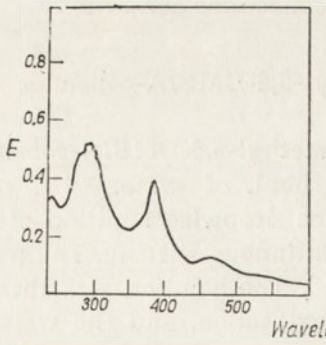


Fig. 1

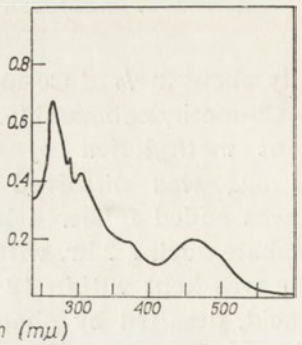


Fig. 2

Fig. 1. Absorption spectrum of cobinamide hydride in 5% NH_4Cl.

Fig. 2. Absorption spectrum of Co-methyl-3,5,6-trimethylcobamide in aqueous solution.

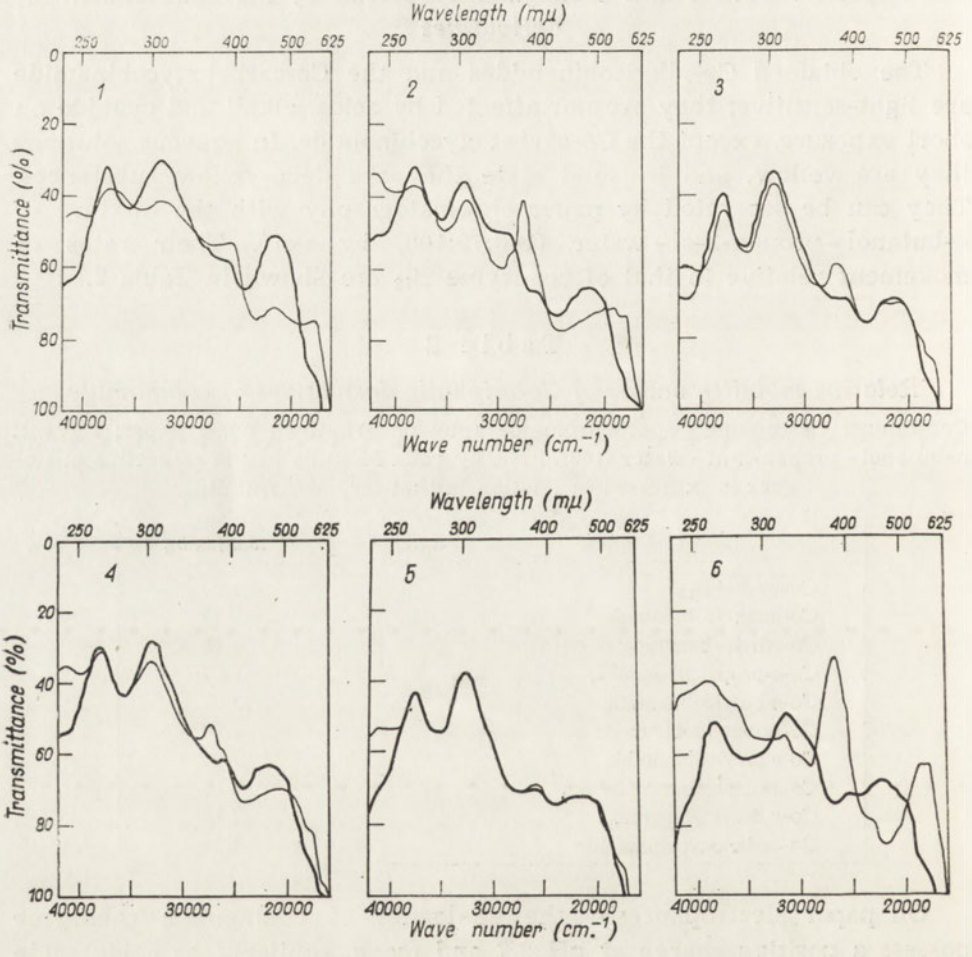


Fig. 3. Absorption spectra of Co-alkyl derivatives of cobinamide and Co-carbetoxycobinamide, (——), in 0.01 N-HCl solution; (– – –), in 0.01 M-KCN. (*1*), Co-methylcobinamide; (*2*), Co-ethylcobinamide; (*3*), Co-*n*-propylcobinamide; (*4*), Co-*n*-butylcobinamide; (*5*), Co-*n*-amylcobinamide; (*6*), Co-carbetoxycobinamide.

mide derivatives still possess a positive charge while cobamide derivatives are neutral. By means of paper electrophoresis at pH 2.7 the Co-alkyl-DMBIA-cobamides can be separated from Co-alkylcobinamides. Co-ethyl-5,6-DMBIA-cobamide moves slightly faster at pH 2.7 than the other Co-alkyl-5,6-DMBIA-cobamides, while Co-ethylcobinamide has the same electrophoretic mobility at pH 2.7 as the other Co-alkylcobinamides.

Table 3

Photolysis of Co-aliphatic derivatives of cobinamide and of other light-sensitive corrins

Aqueous solutions of the derivatives (38.2 μM-concn.) were irradiated in silica cell (length 10 mm.) with 60 watt tungsten lamp from a distance of 25 cm. The increase in extinction at 348 mμ was determined (max. for hydroxy(aquo)cobinamide). For complete degradation the samples were exposed to direct sunlight for 60 min.

Compound	Degradation (%)	
	after 2 min.	after 4 min.
Co-methylcobinamide	17.7	30.0
Co-ethylcobinamide	26.8	44.9
Co-*n*-propylcobinamide	22.6	33.0
Co-*n*-butylcobinamide	21.0	34.6
Co-isobutylcobinamide	22.3	37.0
Co-*n*-octylcobinamide	11.4	22.5
Co-*n*-decylcobinamide	11.2	22.0
Co-methyl-5,6-DMBIA-cobamide	18.4	37.7
Co-ethyl-5,6-DMBIA-cobamide	36.1	63.7
Co-*n*-butyl-5,6-DMBIA-cobamide	17.9	41.0
Co-isobutyl-5,6-DMBIA-cobamide	33.0	59.5
Co-carbetoxycobinamide	0.0	0.0
Cobinamide coenzyme	1.47	6.6
Coenzyme form of *abcdeg*-hexamide-cobyrinic acid	2.0	5.0

Absorption spectra. The spectrum of cobinamide hydride in ammonium chloride solution exhibits a wide absorption band in the region of 290 mμ, a maximum at 390 mμ and a less distinct band at 460 mμ (Fig. 1). The Co-alkyl derivatives of cobinamide show striking similarities in their spectra (Fig. 2, 3). The maxima are at 260, 310 and 460 mμ. The spectra do not undergo changes in acid, neutral and alkaline media, except a slight increase of absorption in the region of 240 - 255 mμ in alkali. The addition of cyanide causes a partial loss of absorption at 310 and 460 mμ and the appearance of absorption bands at 367 and 580 mμ. The spectra of the Co-alkyl analogues containing longer hydrocarbon chains (*n*-amyl, *n*-octyl, and *n*-decyl) were not considerably changed upon the addition of cyanide. Because of high light-sensitivity of the Co-alkylcobinamide cyanide, the assays of the spectrum were performed

in the Unicam SP-700 spectrophotometer equipped with an automatic recorder, the time of recording being about 15 min. The concentration of the compounds in the sample was 2.4×10^{-5} M.

The effect of cyanide. Co-alkyl analogues of cobinamide were kept in 0.01 M-aqueous solution of KCN in a completely dark room for several hours, then acidified and purified by phenol extraction. On chromatography they did not exhibit any changes following the KCN treatment, while the Co-carbetoxycobinamide was rapidly cleaved in aqueous solution of KCN, with the formation of cobinamide dicyanide.

The effect of light. The obtained compounds in aqueous solutions were cleaved in the presence of oxygen when exposed to light, with the formation of appropriate hydroxy(aquo)corrinoids. The light-sensitivity of different analogues is shown in Table 3. The cleavage of Co-carbetoxycobinamide was observed only after prolonged exposure to direct sunlight.

Table 4

Effect of the synthetized analogues of cobinamide coenzyme on enzymic activity

For the estimations 500 μg. of enzyme preparation, 0.05 mμmole of coenzyme B_{12}, a known amount of the compound tested, 45 μmoles of glycerol and 0.2 ml. of 0.2 M-potassium phosphate buffer, pH 8.0, were used. Final volume: 1.0 ml.; incubation time: 10 min. at 37°. The β-hydroxypropionic aldehyde formed was determined according to Smiley & Sobolov [16]. The sample was added with 0.5 ml. of tryptophane solution (300 mg. in 100 ml. of 0.1 N-HCl) followed by 3 ml. of conc. HCl. Then the sample was heated on a water bath at 60° for 5 min. and the extinction at 555 mμ was measured. The amount of aldehyde formed was calculated from a standard curve made for acrolein.

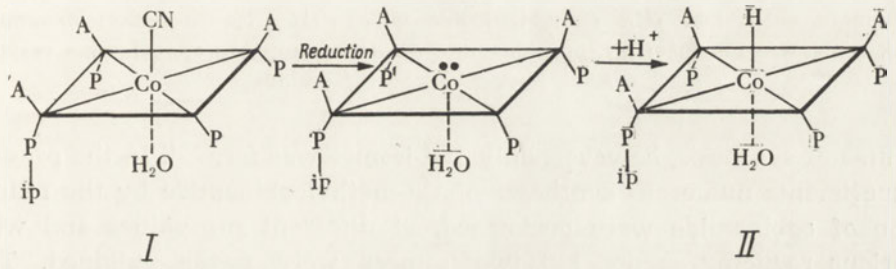
The inhibition index (I_{50}) denotes the concentration of the inhibitor relative to the concn. of coenzyme B_{12}, causing 50% inhibition of aldehyde formation.

Compound	I_{50}
Co-methylcobinamide	77
Co-ethylcobinamide	64
Co-*n*-propylcobinamide	150
Co-*n*-butylcobinamide	16
Co-isobutylcobinamide	70
Co-*n*-amylcobinamide	15
Co-*n*-octylcobinamide	18
Co-*n*-decylcobinamide	12
Co-carbetoxycobinamide	250
Co-methyl-5,6-DMBIA-cobamide	less than 1
Co-ethyl-5,6-DMBIA-cobamide	less than 1
Co-*n*-butyl-5,6-DMBIA-cobamide	less than 1
Coenzyme form of *abcdeg*-hexamidecobyrinic acid [9a]	without effect

Effect on enzymic activity. The enzymic reaction of conversion of glycerol into β-hydroxypropionic aldehyde dependent on the coenzyme B_{12}, was used as a test. None of the analogues tested exhibited the activity of the coenzyme B_{12}, on the contrary, they proved to be powerful inhibitors of this reaction (Table 4).

DISCUSSION

The results of the present experiments show that cobinamide readily forms organo-metallic derivatives possessing a characteristic Co—C bond, similarly as it was demonstrated for the 5,6-DMBIA-cobamide [4, 18, 17, 10]. The starting material for these syntheses was the cobinamide hydride obtained by the reduction of cobinamide cyanide. A slight addition of magnesium to the mixture containing zinc and ammonium chloride considerably enhanced the rate of reduction which was then



I *II*

Fig. 4. Formation of cobinamide hydride. (*I*), Cobinamide cyanide; (*II*), cobinamide hydride; (A), acetamide residues; (P), propionamide residues; (ip), isopropanolamine residue.

completed in a few minutes. No formation of by-products in the presence of magnesium was observed. The reduction by sodium borohydride in 0.1 M-versenate buffer at pH 9.5 as recommended by Smith *et al.* [18] gave rise to formation of some unidentified by-products.

For the formation of cobinamide hydride the reaction shown in Fig. 4 may be suggested. The spectrum of cobinamide hydride is very similar to that of the hydride of 5,6-DMBIA-cobamide reported by Schindler [15] and described also by Müller & Müller [10].

In the present experiments a number of aliphatic analogues of cobinamide coenzyme were obtained. In our syntheses advantage was taken of the observations of Bernhauer *et al.* [4] and Smith *et al.* [18] who pointed out the possibility of synthetizing Co-analogues of vitamin B_{12}. The German authors who had synthetized a number of Co-aliphatic analogues of 5,6-DMBIA-cobamide [4, 10] and the Co-methyl and Co-

-*n*-butyl cobinamides [10, 11] assumed that the alkyl residue is attached at the same position as 5′-deoxyadenosine in the natural coenzyme B_{12}. However, no experimental evidence was given to support this suggestion.

Theoretically a possibility exists of formation from 5,6-DMBIA--cobamide, and especially from cobinamide, of two stereoisomeric forms of each of these derivatives, as shown in Fig. 5 for cobinamide. In the

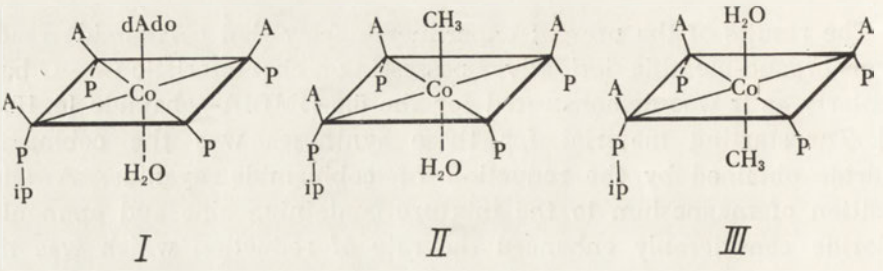


Fig. 5. The possible stereoisomers of Co-alkyl substituted cobinamide. (*I*), Co-binamide coenzyme; (*II*), Co-methylcobinamide; (*III*), iso-Co-methylcobinamide; (A), acetamide residues; (P), propionamide residues; (ip), isopropanolamine residue; (dAdo), 5′-deoxyadenosine residue.

course of synthesis, however, only one isomer was formed. In the present experiments numerous syntheses of Co-methylcobinamide by the reduction of cobinamide were performed, at different pH values and with various reducing agents, but two isomers were never obtained. The spacial arrangement of the methyl residue on cobalt in Co-methyl-cobinamide is unknown. The attempts to elucidate this problem were based on two previous observations of Bernhauer and co-workers: first, of the possibility of chemical degradation of 5,6-DMBIA-cobamide to cobinamide [6, 7] and of coenzyme B_{12} to cobinamide coenzyme [5], and secondly, on the specific methylation of $N_{(3)}$ of the benzimidazole moiety of 5,6-DMBIA-cobamide [6]. The methylation occurs only when the Co—$N_{(3)}$ co-ordinate bond is split as it is in the dicyanide form. On the basis of these facts it was assumed that if a substituent, e.g. an alkyl group, is on the same side as 5′-deoxyadenosine in the natural coenzyme B_{12}, no methylation should occur at the $N_{(3)}$ of benzimidazole by dimethyl sulphate at pH 8.4; and in fact it was found that at this pH Co-methyl-5,6-DMBIA-cobamide is not methylated by dimethyl sulphate. This suggested that a methyl group attached to cobalt occupies the position of 5′-deoxyadenosine residue in the coenzyme B_{12}. Knowing the structure of Co-methyl-5,6-DMBIA-cobamide it was possible by means of chemical degradation to establish the structures of Co-alkyl derivatives. The hydrolytic splitting off of the benzimidazole nucleotide by perchloric

acid or cerous hydroxide gave Co-methylcobinamide identical with that synthetized from cobinamide hydride. This indicates that the aliphatic substituents on cobalt in Co-alkylcobinamide and in the Co-alkyl-5,6--DMBIA-cobamide analogues are attached on the same side of the molecule.

It is interesting that while the natural coenzymes in the presence of KCN undergo degradation with the formation of dicyanides, the Co--alkyl derivatives of cobinamide form cyanides. The existence of cyanides of Co-alkyl derivatives of cobinamide is suggested by the spectral analyses. The absorption spectra of the aquo-Co-alkylcobinamide derivatives are almost identical in acid, neutral and alkaline solutions, the addition of cyanide to alkaline solution causes, however, distinct changes in the spectrum, especially for the analogues containing shorter alkyl chains. The formation of cyanide of Co-alkyl derivatives of cobinamide was demonstrated in 0.01 M-KCN solution and in 0.01 M-versenate - 0.01 M-KCN buffer, pH 9.5. On acidification of these solutions, the starting compounds could be recovered; this indicates the reversibility of the reaction. The addition of cyanide to the solutions of *n*-amyl, *n*-octyl and *n*-decyl derivatives did not cause changes in their spectra. This, however, is no proof that the long chain derivatives do not form cyanides.

The studies on the enzymic conversion of glycerol into β-hydroxypropionic aldehyde showed that all the analogues obtained are powerful competitive antagonists of coenzyme B_{12}. Further studies are in progress and will be reported separately. Recently, Smith & Mervyn [17] also demonstrated that methyl and ethyl analogues of coenzyme B_{12}, and even cyanocobalamin, are inhibitors in the Abeles reaction.

The author wishes to thank Professor Dr. J. Pawełkiewicz for valuable suggestions and advice during the course of this work.

SUMMARY

The chemical synthesis and some physico-chemical and enzymic properties of Co-alkyl derivatives of cobinamide and of Co-carbetoxycobinamide were described. The syntheses were based on the reduction of cobinamide in aqueous ammonium chloride solution with zinc and magnesium, to form cobinamide hydride, and on subsequent coupling with alkyl halides or with ethyl chloroformate. It was shown that the alkyl residue is attached to cobalt on the same side of the molecule as the 5′-deoxyadenosine residue of the natural coenzyme. The obtained Co-alkyl analogues are competitive antagonists of coenzyme B_{12}.

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CHEMICZNA SYNTEZA ALIFATYCZNYCH ANALOGÓW KOENZYMU KOBINAMIDOWEGO I ICH AKTYWNOŚĆ ENZYMATYCZNA

Streszczenie

Syntetyzowano i opisano niektóre własności fizyko-chemiczne i enzymatyczne Co-alkilo-pochodnych kobinamidu oraz Co-karbetoksy-kobinamidu. Ich synteza polegała na redukcji kobinamidu do wodorku kobinamidu przy pomocy cynku i magnezu w środowisku wodnego roztworu chlorku amonu, a następnie na sprzęganiu z halogenoalkilami lub chlorowęglanem etylu. Wykazano, że reszta alkilowa przyłącza się do atomu kobaltu z tej samej strony co reszta 5'-dezoksyadenozyny w naturalnym koenzymie kobinamidowym. Otrzymane analogi Co-alkilowe są kompetytywnymi antagonistami koenzymu B_{12}.

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INTRACELLULAR LOCALIZATION OF PHOSPHODIESTERASE BY A CYTOCHEMICAL METHOD

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What little is known about the intracellular localization of enzymes attacking nucleic acids has been obtained largely by cellular fractionation techniques. It is perhaps superfluous to insist on the importance of obtaining comparable data by means of standard histo- and cytochemical procedures. Several attempts in this direction have already been made [2, 1, 3, 20, 18] and the difficulties remaining to be resolved discussed in some detail in previous publications [14, 15]. It is important to note that these apply likewise to cell fractionation techniques.

One of these is the question of enzyme specificity. The use of natural nucleic acids as substrates for estimation of enzyme activities is warranted only if one recognizes that these are acted upon not only by RNases and DNases, but also by a variety of PDases [1]. This was what prompted us to employ pyrimidine nucleoside 2′:3′-cyclic phosphates as substrates for the cytochemical localization of RNase [14] and various oligo- and polynucleotide substrates for distinguishing roughly between different nuclease enzymes by means of the film-substrate technique [15] proposed by Daoust [2, 3].

More serious was the finding that RNase and DNase enzymes exhibited a pronounced tendency to diffuse from fixed tissue sections during incubation, thus seriously limiting the degree and validity of the localization attainable with normal cytochemical procedures [5, 14]. It is, of course, obvious that the existence of such diffusion of these

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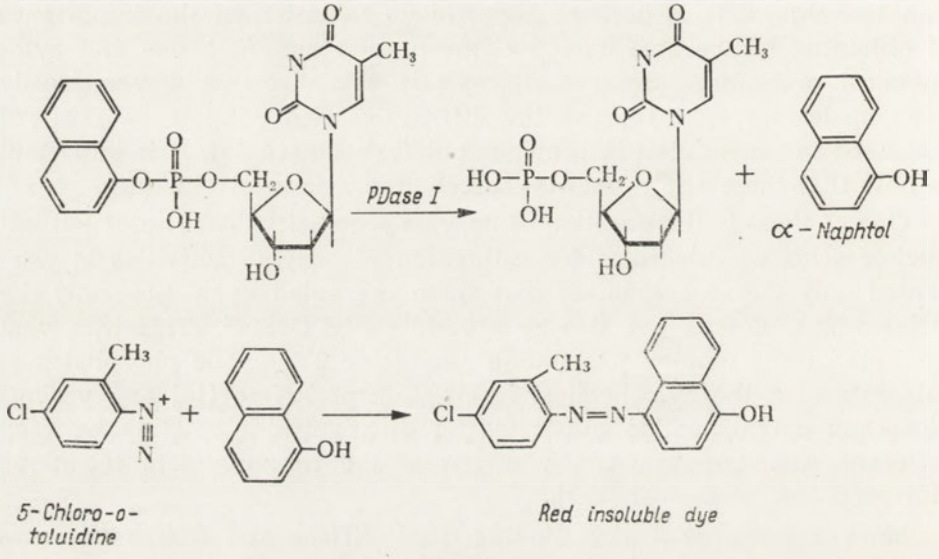
* This work is taken, in part, from a thesis submitted by H. Sierakowska to the Polish Academy of Sciences in partial fulfillment of the requirements for the Ph. D. degree.

[1] The following abbreviations will be used in this text: Tp, thymidine-3′-phosphate; pT, thymidine-5′-phosphate; Tp-naphthyl, α-naphthyl thymidine-3′-phosphate; naphthyl-pT, α-naphthyl thymidine-5′-phosphate; RNase, ribonuclease; DNase I and II, deoxyribonucleases I and II; PDase I and PDase II, phosphodiesterases active against naphthyl-pT and Tp-naphthyl, respectively, according to the designation of Razzell [10]; Fast Red TR, 5-chloro-o-toluidine; Fast Garnet GBC, 4-amino-3:1′-dimethyl azobenzene.

enzymes from tissues will equally affect the results obtained by cell fractionation procedures.

Both of the foregoing difficulties appear to be absent from a method which we have now developed for the cytochemical localization of PDase I. The starting point in the present investigation was the demonstration by Razzell [10] that *p*-nitrophenyl thymidine-5′-phosphate and *p*-nitrophenyl thymidine-3′-phosphate are hydrolysed specifically by kidney and spleen phosphodiesterases, PDase I and PDase II respectively. This suggested the possibility of utilizing similar substrates with substitution of the *p*-nitrophenol residues by α-naphthol; the enzymically liberated naphthol could then be coupled with suitable diazotates, according to standard cytochemical procedures [9], to form an insoluble dye at the sites of enzyme activity (scheme 1).



Scheme 1

Following synthesis of the necessary substrates, naphthyl-pT and Tp--naphthyl, it was found that while the former was, in fact, rapidly hydrolysed by PDase I, the latter was completely resistant to PDase II (see below). Like the substrates studied by Razzell, both were resistant to endonucleases [16]. Further investigation demonstrated that PDase I exhibited no detectable diffusion from fixed tissue sections. The necessary conditions therefore appeared to be fulfilled for the development of a cytochemical procedure for localization of PDase I activity in various tissues. This, in turn, should also prove useful as a blank to be taken into account in connection with localization of depolymerases by means of incubation of high molecular weight substrates which are slowly hydrolysed, amongst others, also by PDase I.

Notwithstanding that Tp-naphthyl proved inactive against PDase II, the synthesis of this compound is also described in the following, since it may be found subsequently to be susceptible to some other tissue PDase.

Attention should also be drawn to the fact that since the hydrolysis of naphthyl-pT in the presence of a diazonium salt results in immediate colour formation, this substrate should also prove extremely useful in investigations on the activity of various cellular fractions by ordinary colorimetry. Preliminary trials in this laboratory have shown that this is indeed so and the potentialities of this technique are being investigated.

MATERIALS

Thymidine was a commercial preparation (Fluke) which was chromatographically and spectrally homogeneous. Fast Red TR and Fast Garnet GBC salt were George Gurr, London, products. The dinucleoside monophosphate TpT was kindly made available by Dr. A. M. Michelson.

Snake venom (*Crotalus adamanteus*) was a gift of Dr. H. Fraenkel-Conrat. RNase, DNase I and DNase II were standard Worthington preparations. Prostatic phosphomonoesterase [8] was a gift of Dr. W. Ostrowski, Department of Physiological Chemistry, Medical School, Kraków. Alternatively the same enzyme, prepared in this laboratory according to Loring *et al.* [6] was employed. Both enzyme preparations were inactive towards Tp-naphthyl and naphthyl-pT.

Preparation of substrates

The following two solvent systems were employed for ascending paper chromatography with Whatman paper no. 1, unless otherwise indicated: *A*, water-saturated butanol; *B*, saturated $(NH_4)_2SO_4$ - isopropanol - 1 M-sodium acetate (80:2:18, by vol.).

5′-Tritylthymidine. Prepared according to the procedure of Weimann & Khorana [19] and isolated from the reaction mixture by the procedure of Michelson & Todd [7].

3′-Acetylthymidine. This was prepared by acetylation of 5′-tritylthymidine, followed by removal of the trityl group, according to Michelson & Todd [7].

α-Naphthylphosphoryl dichloride. Obtained essentially according to the procedure of Friedman & Seligman [4].

α-Naphthyl thymidine-3′-phosphate (*Tp-naphthyl*). This was synthesized by means of a modification of the procedure of Turner & Khorana [17] for *p*-nitrophenyl thymidine-3′-phosphate. 7.84 m-moles of dry pyridine in 5 ml. anhydrous dioxane was added dropwise to

a magnetically stirred solution of 1.98 m-moles 5′-tritylthymidine and 3.92 m-moles α-naphthylphosphoryl dichloride in 5 ml. anhydrous dioxane at room temperature. When the addition was complete (about 1 hr.), stirring was continued with maintenance of anhydrous conditions for additional 2 hr., following which 7.84 m-moles pyridine in 3 ml. water was added with stirring. Chromatography in solvent *A* exhibited a new product with $R_F = 0.26$, naphthylphosphate ($R_F = 0.46$) and pyridine hydrochloride ($R_F = 0.17$); and the complete absence of 5′-tritylthymidine ($R_F = 0.9$). The reaction mixture was evaporated under reduced pressure and the residue taken up in chloroform. Insoluble material was removed by decantation. The chloroform solution was then extracted with one-fifth its volume of water, again with one-fifth its volume of 1 M-pyridine hydrochloride solution of pH 5.5, and finally taken to dryness under reduced pressure. The resulting gum was dissolved in 25 ml. 80% acetic acid and heated under reflux on a water bath for 20 min. The solution was taken to dryness under reduced pressure, and the residue suspended in 15 ml. water and left for 18 hr. at 4°. The crystalline precipitate of triphenylcarbinol was filtered off, the aqueous solution brought to dryness under reduced pressure, and the residue dissolved in water and taken to dryness once more. The product was redissolved in water and passed through a column of Dowex 50(H^+). Chromatography in solvent *A* now exhibited the detritylated product ($R_F = 0.36$) and a trace of thymidine ($R_F = 0.52$). The product was, however, still contaminated with chromatographically undetectable naphthylphosphate which could not be removed on a Dowex 1 (Cl^-) column without serious losses of Tp-naphthyl. The contaminating naphthylphosphate was therefore removed by one of two procedures: (a) enzymically, and (b) by paper chromatography.[2]

(a) Enzymic removal of naphthylphosphate. The solution was concentrated to about 40 mg./ml., neutralized with 1 M-NaOH and acetate buffer, pH 5, added to a final concentration of 0.02 M. Acid phosphomonoesterase, free from diesterase activity, was added and the solution incubated at 37°. Fresh enzyme was added at intervals of about 4 hr. over a period of a day. During incubation, aliquots were removed and tested for naphthylphosphate content by addition of a small excess of Garnet GBC diazonium salt and centrifuging off the dye formed. When no more naphthol was liberated on further addition of enzyme, the total reaction mixture was extracted with about 2 ml. ether, passed through a column of Dowex 50(H^+), evaporated to dryness, and the residue taken up in 2 ml. anhydrous methanol. The insoluble enzymic

[2] We have now found that it is possible to separate thymidine naphthyl hydrogen phosphate from naphthylphosphate by elution with dilute hydrochloric acid from an ECTEOLA-cellulose column. (Added in proof).

protein was filtered off, the methanol removed under reduced pressure and the residue dissolved in water to a concentration of about 40 mg./ml. and brought to pH 7.5 with hot saturated barium hydroxide. The barium phosphate was centrifuged off, the supernatant concentrated to a small volume and the barium salt of Tp-naphthyl precipitated by addition of an excess of cold acetone. The flocculent precipitate was separated from the sticky, yellow, oil sedimenting to the bottom of the tube, centrifuged down and dissolved in water. Barium was removed with Dowex 50(H^+), the solution neutralized with 1 N-NaOH, concentrated to small volume and the sodium salt of Tp-naphthyl precipitated by addition of an excess of cold acetone. Yield of enzymically purified product, 193 mg. (23% theor.).

(b) Chromatographic removal of naphthylphosphate. This may be done on a semi-preparative scale by ascending chromatography on Whatman paper no. 3 with solvent *B*. The slower migrating band ($R_F = 0.1$) containing the diester was eluted with slightly acidified methanol, the eluate evaporated to dryness under reduced pressure, and the product dissolved in water, passed through a Dowex 50(H^+) column, and taken to dryness twice under reduced pressure at a temperature not exceeding 30°. The residue was dissolved in water at a concentration of 50 mg./ml. and sulphate ions removed by neutralization with hot saturated $Ba(OH)_2$. Barium sulphate was centrifuged off and the barium salt of Tp-naphthyl precipitated by addition of an excess of cold acetone. The barium salt was dissolved in water, barium removed with Dowex 50(H^+), neutralized with sodium hydroxide and the sodium salt of the product precipitated with cold acetone as above.

α-Naphthyl thymidine-5′-phosphate (naphthyl-pT). This was prepared as above according to Turner & Khorana [17] for *p*-nitrophenyl thymidine-3′-phosphate, with appropriate modifications. The crude product was isolated by a modification of the procedure of Michelson & Todd [7] for 3′-acetylthymidine-5′-dibenzylphosphate. 7.84 m-moles dry pyridine in 5 ml. anhydrous dioxane was added dropwise to a magnetically stirred solution of 1.98 m-moles of 3′-acetylthymidine and 3.92 m-moles α-naphthylphosphoryl dichloride in 5 ml. anhydrous dioxane at room temperature. When the addition was complete (1 hr.), the reaction mixture was stirred for additional 2 hr. under anhydrous conditions, following which 7.84 m-moles pyridine in 3 ml. water was added rapidly with stirring. Chromatography in solvent *A* demonstrated the presence of a new product ($R_F = 0.48$), pyridine hydrochloride ($R_F = 0.17$) and traces of acetylthymidine ($R_F = 0.72$). The reaction mixture was evaporated under reduced pressure, the resulting gum dissolved in chloroform, the chloroform solution extracted with water and then with 1 M-pyridine hydrochloride solution, pH 5.5, and taken to dryness under reduced pressure. The resulting gummy residue was evaporated twice from

ethanol, dissolved in a small quantity of ethanol, and 60 ml. cold ether added to precipitate an oil. The oil was dissolved in acetone, the solution filtered and evaporated under reduced pressure to a glass consisting mainly of 3′-acetylthymidine-5′-naphthylphosphate. Acetyl protecting groups were removed by heating the glass in 0.01 M-NaOH on a boiling water bath for 30 min., alkali being added to maintain a pH of about 12. The solution was then passed through a Dowex 50(H^+) column, taken to dryness twice, and dissolved in water. Paper chromatography in solvent *A* showed the presence of the deacetylated product ($R_F = 0.34$) and traces of thymidine. The product was still contaminated with naphthylphosphate, which may be removed as described above for Tp-naphthyl. Yield of enzymically purified product, 120 mg. (14% theor.).

Substrate specificity

Naphthyl-pT and Tp-naphthyl were each incubated under appropriate conditions [14, 15] at concentrations of 0.01 M in the presence of 100 μg./ml. of various enzymes. Hydrolysis products were sought for by means of paper chromatography with solvent *B*, and by analysis for enzymically liberated naphthol with the aid of GBC salt (final concn. 2 mg./ml.).

Tp-naphthyl was found to be completely resistant to RNase, DNase I, DNase II and crude snake venom (*Crotalus adamanteus*).

Naphthyl-pT was completely hydrolysed by snake venom to give pT, thymidine and free naphthol. It was resistant towards the other enzymes.

Tp-naphthyl was found to be resistant to PDase II. It was hydrolysed at a very slow rate by spleen homogenate, but the alkaline pH optimum indicated that this was due most likely to residual PDase I, rather than to PDase II, activity. The presence in spleen homogenate of PDase II, optimally active at slightly acid pH, was established by the rapid hydrolysis of TpT with an optimum at pH 5 - 6. TpT, while theoretically a substrate for both PDases, was found by Razzell [10] to be hydrolysed by PDase II at the same rate as TpTp and faster than Tp-nitrophenyl, but by PDase I at only 4% of the rate of hydrolysis of nitrophenyl-pT. If we assume PDase I activity in the spleen to be approximately twice that for PDase II [10], TpT should be attacked primarily by PDase II. The relatively rapid rate of hydrolysis of TpT by spleen homogenate at acid pH, as compared to the slow rate of hydrolysis of Tp-naphthyl with maximum rate at alkaline pH, was consequently regarded as reasonably good evidence for the resistance to PDase II of Tp-naphthyl.[3]

[3] The resistance of Tp-naphthyl towards PDase II has been confirmed by Dr. W. E. Razzell (Syntex Institute of Molecular Biology, Palo Alto, Cal.), using his purified enzyme, as well as in our laboratories with a commercial Worthington PDase II. (Added in proof).

The resistance of Tp-naphthyl to PDase II, contrasted with the rapid hydrolysis of naphthyl-pT by PDase I,[4] is not an altogether unexpected finding. Razzell [10] reported that PDase I hydrolysed nitrophenyl-pT 4.7 times as rapidly as pTpT, whereas PDase II attacked Tp-nitrophenyl at only 0.77 times the rate for TpTp. Since the substitution of *p*-nitrophenyl for the nucleoside reduces susceptibility towards PDase II, it is quite conceivable that substitution of naphthyl for *p*-nitrophenyl constitutes an important steric hindrance towards the action of the enzyme. The use of Tp-naphthyl for localization of PDase II activity was therefore abandoned. Nonetheless, since this substance may yet prove a substrate for some other phosphodiesterase, its synthesis has been described in detail in this paper.

Cytochemical procedure

Albino rats were sacrificed under ether anaesthesia and thin slices of tissue from various organs fixed overnight in formol-calcium at 4°. Sections were cut at a thickness of 15 μ on a freezing microtome, mounted on slides and allowed to dry in air for about an hour prior to incubation. Storage of mounted sections in the cold room for periods of 1 to 2 days was found to be without deleterious effect on enzyme activity.

With a view to economizing substrate, incubations were performed by flooding the horizontally placed sections with 0.1 ml. incubation medium, as previously described for quantitative cytochemical studies with isotopically labelled substrates and trapping agents [12, 13]. Alternatively freshly-sectioned free floating sections were transferred to a small test--tube containing 0.1 ml. incubation medium and were mounted following incubation. Results were identical with both systems of incubation, regardless of whether the volume of incubation medium was 0.05 ml. or 0.4 ml.

The incubation medium consisted of: naphthyl-pT, 2 mg./ml. and Fast Red TR, 4 mg./ml. in 0.1 M-tris-HCl buffer, pH 9. It is important to add the diazonium salt to the medium immediately prior to incubation since, when stored in buffer at room temperature for 1 hr., it forms a pale yellow precipitate. Precipitation of enzymically liberated naphthol is appreciably hampered in such a medium. The effectiveness of the medium may, in such instances, be restored by addition of diazonium salt. However, should incubation periods longer than 30 min. be necessary, it is more convenient to transfer the sections to fresh medium at 25 - 30 min. intervals.

The rate of the coupling reaction was tested over a range of substrate concentrations of 1 - 4 mg./ml., and of Fast Red TR concentrations of

[4] A direct comparison of the susceptibility to purified PDase I of naphthyl-pT and *p*-nitrophenyl-pT, kindly performed by Dr. Razzell, showed that both substrates are hydrolysed at about the same rate. (Added in proof).

1 - 4 mg./ml. The substrate concentration of 2 mg./ml., giving the maximum rate of precipitate formation, necessitates the use of the rather high concentration of diazonium salt. Lower concentrations, e.g. 1 mg./ml., resulted in a poorer capture reaction for naphthol, with consequent formation of diffuse precipitates.

The optimal pH value for the enzymic reaction within the pH range 5.2 - 9.5 was determined with 0.1 M-acetate, phosphate and tris-HCl buffers and with two diazonium salts, Fast Garnet GBC and Fast Red TR. Optimal activity for kidney sections was found at pH 9, although some diffuse activity persisted even at pH 5.2 upon incubation for 30 min. at room temperature. Activity was completely absent in pancreatic sections incubated for 30 min. at pH 5.2.

Routine controls included: (a) heat inactivated sections (100° for 10 min.) incubated in full medium, and (b) sections incubated in substrate-free medium. Incubations were normally at room temperature for from 1 to 30 min., but control sections were always incubated for periods at least three times longer.

Effect of fixation. The effects of cold formalin fixation, cold absolute acetone fixation with subsequent dehydration and paraffin-embedding, and cold alcohol fixation with subsequent dehydration and paraffin-embedding, on rat kidney PDase activity were investigated.

Morphologically similar slices of fresh kidney were weighed and treated as described above. Homogenates of fresh tissue served as controls. Formalin-fixed tissue was rinsed in cold water and homogenized. Paraffin-embedded tissues were sectioned, the paraffin removed in benzene and the tissue air-dried and homogenized. Then 0.9% homogenates of experimental and control tissues were incubated with naphthyl-pT, 2 mg./ml. in 0.1 M-tris buffer, pH 9, at 37°, and aliquots of 35 µl. removed at intervals of 15 min., 30 min. and 1, 2 and 4 hr. Hydrolysis of naphthyl-pT was estimated chromatographically in solvent *B*. Comparison of the rates of hydrolysis of naphthyl-pT by the homogenates indicated that about 20% PDase I activity survived formalin fixation, but only about 5% activity remained following acetone and alcohol fixations, due almost entirely to the effect of paraffin embedding.

Fresh frozen kidney and pancreatic sections were employed in several experiments designed to test the validity of the localization patterns obtained with formalin, alcohol and acetone fixed materials. The results were in all instances identical. Variations in incubation times at room temperature, viz. 15 sec. for fresh frozen sections, as contrasted with 1 - 2 min. for formalin fixed material and 3 min. for alcohol and acetone fixed sections, are roughly in accord with the semi-quantitative results obtained, above, with homogenates of tissues subjected to the same treatment.

Enzyme diffusion

In view of our previous experience with RNase and DNase enzymes [14], where enzyme diffusion was found to be a serious obstacle to the development of a suitable cytochemical procedure, special attention was devoted in the initial stages of this investigation to the question of possible PDase I diffusion.

Formalin-fixed sections were preincubated in 0.06 ml. of 0.1 M-tris--HCl buffer, pH 9, for 30 min. at room temperature. The buffer was removed, substrate and diazonium salt added to make up a normal incubation medium, and this was then transferred onto heat-inactivated control sections and incubated for 30 min. The section did not exhibit any precipitate formation, nor did the incubation medium itself demonstrate the formation of a coloured precipitate, indicative of the release of naphthol. When the preincubated section and an identical untreated section were incubated with the normal complete incubation medium for 1 - 2 min., a similar intense reaction was observed for both sections.

The foregoing experiment was repeated, using paper chromatography to control possible substrate hydrolysis. Preincubation was carried out as above, and the preincubation buffer then combined with substrate and incubated for 45 min. The preincubated section and an untreated section were also incubated for 45 min. in a buffered substrate solution. The diazonium salts were omitted since they were not necessary, while they interfered with the development of the chromatograms. Paper chromatography with solvent *B* demonstrated the absence of any hydrolysis of naphthyl-pT by the buffer used for preincubation. On the other hand the normal and preincubated sections partially hydrolysed the substrate, to pT in the case of pancreas, and to a mixture of pT and thymidine with the kidney.

The foregoing experiments were performed only with formalin-fixed pancreatic and kidney sections and were regarded as indicating the absence of any significant diffusion of enzyme from these materials.

Effect of inhibitors

Attempts were made to confirm cytochemically the reported inhibitory effects of EDTA and AMP on PDase I [10]. No inhibition was observed with formalin-fixed kidney sections incubated for up to 5 min. at room temperature in incubation media containing EDTA over a concentration range of 0.8 to 3 mM. Pancreatic sections incubated for up to 5 min. at room temperature in normal incubation medium with ATP added to 1 mM-concentration likewise gave no indication of inhibition of enzyme activity. On the other hand, the introduction of NaF at a concentration of 20 mM in the incubation medium resulted in a decrease of precipitate formation of about 30% with pancreatic sections.

Distribution of PDase I

The localization described in each case applies to sections incubated for the time interval specified. Upon prolonged incubations sites containing less enzymic activity might have become evident. No staining was observed in any of the control sections.

Kidney. Figs. 1, 2, incubation 3 min. The enzyme was found in the epithelial cells of the proximal tubules of the cortex, although the straight portions of the tubules exhibited more pronounced activity than the convoluted ones. The nuclei were unstained, and cytoplasmic activity was concentrated in the brush border zone.

Pancreas. Fig. 3, incubation 3 min. The enzyme was found centred in the supranuclear portion of the acinar cells, adjacent to the lumen, with lesser activity in the intima of arteries, adventitial coat of the blood vessels and the connective tissue coat surrounding the excretory ducts. The islets of Langerhans and the epithelium of the excretory ducts were negative.

Duodenum. Fig. 4, incubation 5 min. The brush border of the epithelium of the villi was highly active, with slight activity in the epithelium of the crypts of Lieberkuhn. Capillaries, intima and adventitia of blood vessels, and other elements of the lamina propria and submucosa were also active.

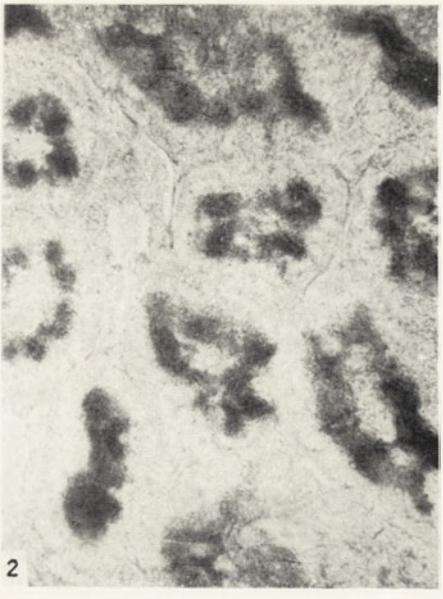
Thyroid. Fig. 5, incubation 15 min. The cytoplasm of the follicular cells stained intensely, especially in the vicinity of the basement membrane. Capillaries, intima and adventitia of blood vessels reactive.

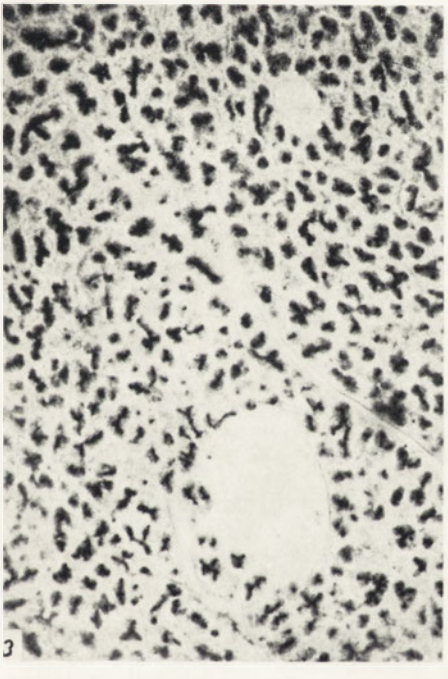
Liver. Figs. 6, 7, incubation 30 min. Intense activity in proximity to bile canaliculi. Cytoplasm of hepatic cells weakly active with somewhat greater activity at the lumen of sinusoids. The nuclei were negative. Capillaries, intima and adventitia of blood vessels also reactive. Kupfer cells and epithelium lining the bile ducts were negative.

Tongue. Fig. 8, incubation 15 min. Activity in capillaries and the intima of small arteries and veins. The perineurium was also positive. A strong reaction was evident in the cytoplasm of mast cells.

Spleen. Fig. 9, incubation 20 min. Staining in the cytoplasm of certain reticular cells and free macrophages in the red pulp. Slight reaction in the free macrophages and the endothelial cells, lining the capillaries in the germinal centers. Lymphocytes in the nodules were unstained. Blood vessels reacted as usual.

Submaxillary gland. Fig. 10, incubation 10 min. The enzyme was localized in the cytoplasm of the serous acini with greatest activity at what seemed to be the edges of the intercellular secretory canaliculi. The nuclei were negative and the different acini showed varying intensity of reaction. Epithelium of salivary and excretory ducts reacted slightly





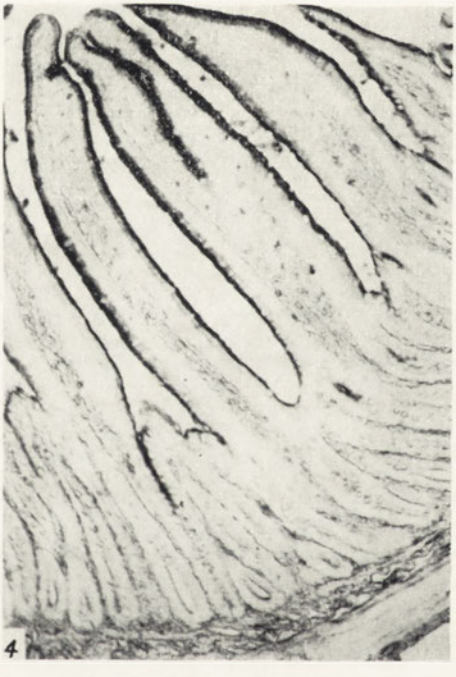


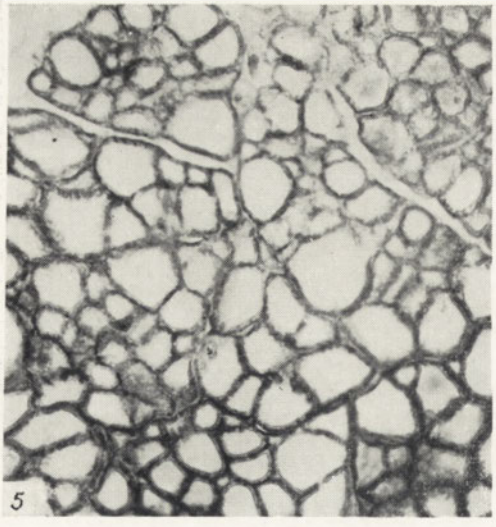
Fig. 1. Kidney cortex. Most intense activity in the straight portions of proximal tubules (× 70). For further details with reference to this, and subsequent figures, see text.

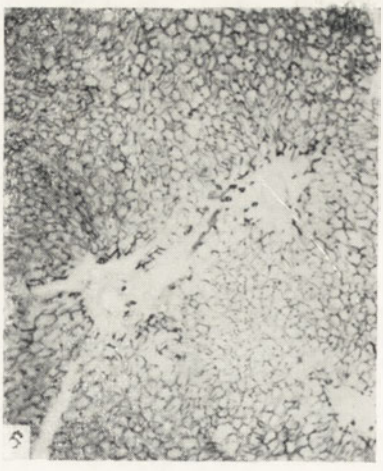
Fig. 2. Inner kidney cortex. Activity in the brush border zone with nuclei negative (× 400).

Fig. 3. Pancreas. Activity in acinar cells. The islets are negative (× 150).

Fig. 4. Duodenum. Intense brush border activity (× 90).

H. Sierakowska and others, Acta Biochim. Polon. vol. X, 1963 (Plate 1).





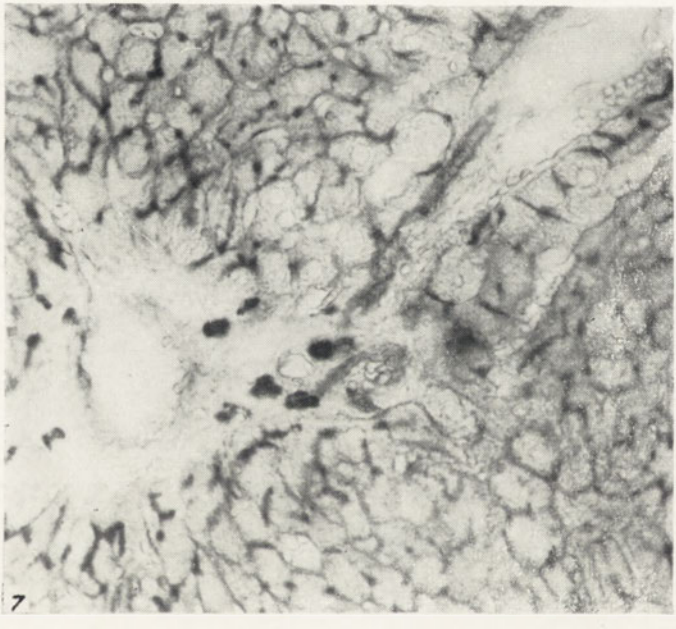


Fig. 5. Thyroid. Cytoplasm of follicular cells reactive (× 75).
Fig. 6. Liver. Intense activity in proximity to bile canaliculi (× 80).
Fig. 7. Liver. Note the negative nuclei of hepatic cells (× 300).

H. Sierakowska and others, Acta Biochim. Polon. vol. X, 1963 (Plate 2).

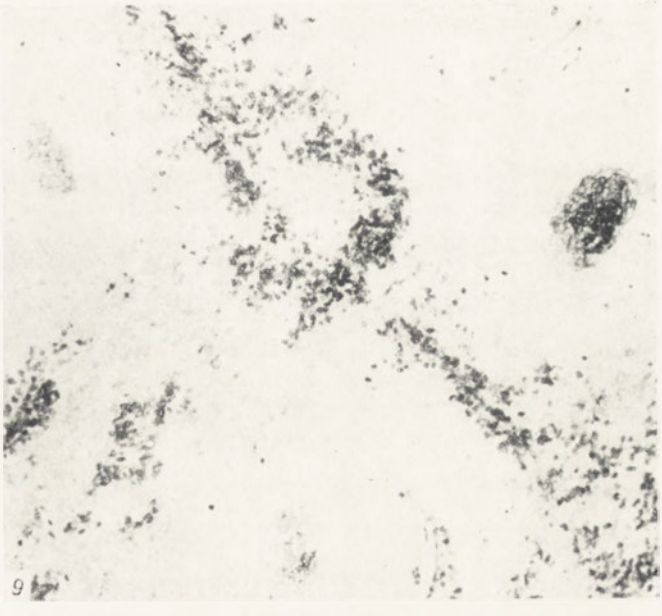
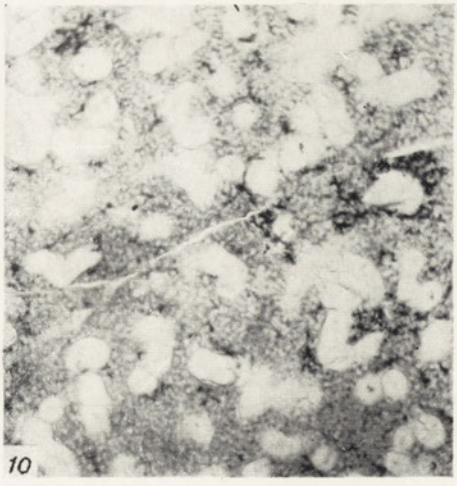
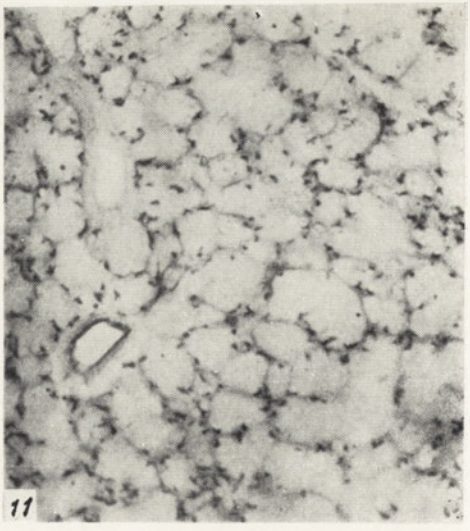
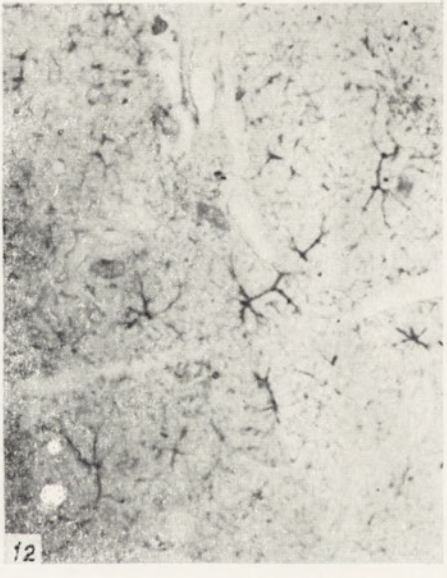


Fig. 8. Tongue. Activity in blood vessels, perineurium and mast cells (× 100).
Fig. 9. Spleen. Activity in certain red pulp cells and the endothelium of blood vessels (× 75).

H. Sierakowska and others, Acta Biochim. Polon. vol. X, 1963 (Plate 3).







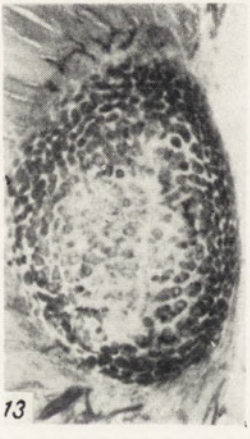


Fig. 10. Submaxillary gland. Activity in serous cells (× 75).

Fig. 11. Sublingual gland. Activity in semilunar cells (× 80).

Fig. 12. Parotid gland. Intense activity in proximity to intercellular canaliculi (× 100).

Fig. 13. Tracheal hyaline cartilage. Intense activity in subperichondrium cells and walls of lacunae (× 80).

H. Sierakowska and others. Acta Biochim. Polon. vol. X, 1963 (Plate 4).

in the supranuclear portion of cells adjacent to the lumen. The mucous acini were negative.

Sublingual gland. Fig. 11, incubation 10 min. The enzyme was localized in the cytoplasm of semilunar cells with greatest activity at the edges of the intercellular secretory canaliculi. The epithelium of excretory ducts reacted at the lumen.

Parotid gland. Fig. 12, incubation 10 min. Enzyme in cytoplasm of serous cells with most intense activity at the edges of the intercellular canaliculi. The epithelium of salivary and excretory ducts reacted in proximity to the lumen. In all the salivary glands the endothelium of capillaries and the intima and adventitia of blood vessels exhibited enzymic activity.

Tracheal hyaline cartilage. Fig. 13, incubation 20 min. The cells of subperichondrium reacted intensely, whereas the cytoplasm of chondrocytes showed moderate activity. The walls of the lacunae, particularly the outer surface, stained intensely.

DISCUSSION

A general conclusion which may be drawn from the foregoing is that PDase I is particularly active at adsorptive surfaces, as exemplified by the brush border of the kidney and duodenum, and in cells involved in secretory processes, as illustrated by the serous acini of the pancreas and the salivary glands. This relative abundance of the enzyme in secretory cells, actively engaged in protein biosynthesis, is of some significance. In the cell fractionation investigations of Razzell [10], the enzyme was found to be localized largely in the microsomes, it being inferred from this that PDase I may play some specific role in the sequential hydrolysis of ribonucleic acid chains which participate in protein synthesis. It is nonetheless of interest to note the intracellular distribution of the enzyme at the secretory surfaces of these cells.

The present cytochemical findings demonstrate the complete absence of PDase I from the nuclei of the various tissues investigated. This is to be contrasted with the results of Razzell [10] who, although finding no positive evidence for nuclear localization, nonetheless entertained the opinion that PDase I may be present in, or bound to, rat liver and kidney nuclei. It should consequently be emphasized that even prolonged incubation, by the cytochemical procedure, of highly active kidney tubular epithelium cells, exhibited no evidence of nuclear PDase I activity.

The cytochemical results do not indicate as high a PDase I activity for the testis and thymus as reported in the cell fractionation studies of Razzell [10]. In the testis PDase I occurred almost exclusively in the blood vessels of the stroma with no activity in the seminiferous tubules

and could hardly account for an activity of the order of 25% that for the kidney, as proposed by Razzell.

A rather puzzling feature of the present results is the failure to observe any marked inhibition of enzyme activity by either EDTA (versene) or AMP as found by Razzell [10] with homogenates as well as with the individual cellular fractions. No obvious interpretation of this discrepancy suggests itself, and it certainly merits further study.

While the present cytochemical method appears to fulfil adequately the requirements for intracellular localization of at least one of the important enzymes involved in nucleic acid metabolism, it is somewhat difficult at the moment to determine to what extent its range of application may be broadened to include other such enzymes. Theoretically the naphthyl esters of the ribonucleoside-2′(3′)-phosphates should be suitable substrates for the localization of ribonucleases. It is, on the other hand, conceivable that a naphthyl ester may be as resistant to ribonucleases as Tp-naphthyl is to PDase II. Even more important is the fact that the ribonucleoside-2′(3′)-phosphate esters are hydrolysed relatively slowly by ribonucleases. Taken in conjunction with the relatively rapid rate of diffusion of these enzymes from tissue sections [5, 14], accurate localization might be difficult of achievement.

We should like to thank Dr. W. Szer for his invaluable assistance in the preparation of the substrates. We are indebted to Dr. P. Czerski for identification of the spleen cells.

SUMMARY

A cytochemical method for the localization of phosphodiesterase I based on hydrolysis of α-naphthyl thymidine-5′-phosphate and coupling the enzymically liberated naphthol with diazotate has been elaborated. The syntheses of α-naphthyl thymidine-5′-phosphate and α-naphthyl thymidine-3′-phosphate have been described. The resistance of α-naphthyl thymidine-3′-phosphate to PDase II has been shown and discussed.

The optimal conditions for the localization of PDase I have been determined and the intracellular localization of the enzyme in several rat tissues presented. The effects of fixation and various inhibitors on PDase I have been investigated. The problems of substrate specificity and PDase I diffusion have been discussed.

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CYTOCHEMICZNA METODA WEWNĄTRZKOMÓRKOWEJ LOKALIZACJI FOSFODWUESTERAZY

Streszczenie

Opracowano cytochemiczną metodę lokalizacji fosfodwuesterazy I polegającą na zastosowaniu jako substratu 5′-α-naftylofosforanu tymidyny oraz sprzęganiu enzymatycznie uwolnionego naftolu z solami dwuazoniowymi. Opisano metodę syntezy 5′-α-naftylofosforanu tymidyny oraz 3′-α-naftylofosforanu tymidyny. Wykazano oraz omówiono odporność 3′-α-naftylofosforanu tymidyny na działanie fosfodwuesterazy II.

Opracowano optymalne warunki lokalizacji fosfodwuesterazy I oraz przedstawiono wewnątrzkomórkową lokalizację enzymu w szeregu tkanek szczura. Ustalono wpływ utrwalaczy oraz szeregu inhibitorów na aktywność enzymu. Zbadano zagadnienia specyficzności substratu oraz dyfuzji fosfodwuesterazy I.

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AN ARGINASE INHIBITOR FROM SUNFLOWER SEEDS (*HELIANTHUS ANNUUS*)

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In an earlier paper concerning the occurrence and activity of arginase in higher plants [1] we have reported that in some of the investigated plants no or practically no arginase activity could be detected. This applied particularly to sunflower seeds in the dormant and in the germinating stage, as well as to young 14-day old seedlings of the sunflower plant.

The interpretation of this rather unexpected observation was at that time impossible. However, a tentative suggestion was made, namely that the inability of demonstrating arginase activity need not at all be the result of absence of this enzyme, but that certain plants may contain substances acting as specific enzyme inhibitors. In this paper experimental evidence is presented which indicates that the seeds of the sunflower contain a very active arginase inhibitor.

MATERIAL AND METHODS

Extracts from sunflower seeds. Ten grams of kernels from sunflower seeds obtained from commercial sources were finely ground in a porcellain mortar and suspended in 100 ml. of distilled water. After addition of 1 ml. of glacial acetic acid, the suspension was thoroughly mixed, centrifuged for 5 min. at 5000 r.p.m. and the supernatant filtered. The solution was then evaporated to dryness on a steam bath, dissolved in 10 ml. of distilled water, filtered off, washed with 5 ml. of distilled water and precipitated with 40 ml. of a 2% solution of barium hydroxide. The precipitate was centrifuged, washed with 50 ml. of distilled water, centrifuged again, suspended in 10 ml. of distilled water and treated with 20 ml. of 0.1 N-H_2SO_4 bringing up the pH to 4.0 - 4.5. The precipitate of $BaSO_4$ was centrifuged off and discarded. The supernatant was again precipitated with 20 ml. of barium hydroxide, centrifuged, washed as above and treated with 12 ml. of 0.1 N-H_2SO_4. Barium sulphate was again centrifuged off and the supernatant solution made up with distilled water to 100 ml. The dry weight of the acetic acid extract amounted to about 15% and the dry weight of the extract from

the second barium hydroxide precipitate was equal to about 1.5% of the original weight of the kernels used for the preparation.

Acetone powder from bitter blue lupins (Lupinus angustifolius, *variety Bielak*). Acetone powders proved to be a very good and reliable source of highly active arginase preparations, which could be kept for many months at -18° without any appreciable loss of activity. The preparation of the acetone powder as well as the determination of arginase activity was carried out as previously described [1].

Determination of arginase activity. Urea obtained on hydrolysis of arginine was hydrolysed with added urease and the resulting ammonia distilled by the usual Conway technique.

RESULTS AND DISCUSSION

In order to determine the effect of the sunflower seed extract upon arginase activity, aliquots containing from 35 μg. to 700 μg. of the preparation dissolved in 0.5 ml. of water were used for each assay of arginase activity. The total volume of the incubated mixture was equal to 4.5 ml. and all determinations were carried out in quadruplicates.

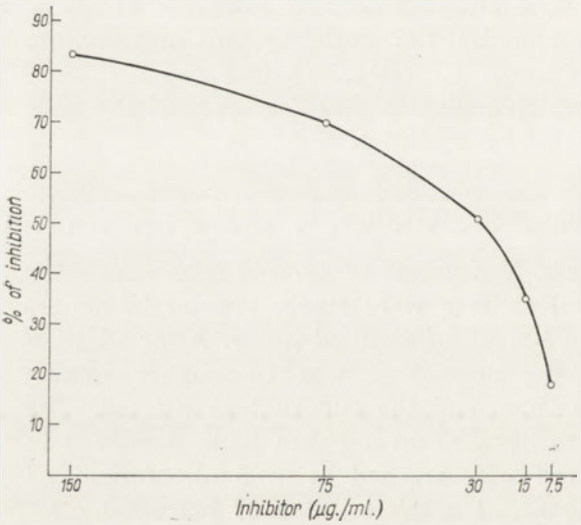

Fig. 1. Influence of inhibitor from sunflower seeds on arginase activity.

Fig. 1 demonstrates the effect of the sunflower seed extract on arginase activity, expressed in per cent inhibition as compared with control samples without addition of the extract from sunflower seeds. As can be seen, the inhibition of arginase activity amounts to 83% at the concentration of the inhibitor equal to 150 μg./ml. and to 18% at the concentration equal to 7.5 μg./ml. No further increase of inhibition could be obtained even when the concentration of the inhibitor was raised to 600 μg./ml. Preincubation at 0° and at room temperature for a period

of 30 min. remained without any noticeable effect. In the course of work a very interesting observation was made, namely that L-cysteine reactivated arginase which had been previously inhibited with the sunflower seed extract. Provided that enough cysteine was added, the original arginase activity could be quantitatively restored. As can be seen from Table 1, about 10 μmoles of L-cysteine restored completely the activity of arginase which had been inactivated with 75 μg./ml. of the inhibitor; 1 μmole remained without effect probably because it was oxidized to cystine by some reducible substances present in the acetone powder as well as in the inhibitor preparation. Cystine applied in the above concentrations had no effect on the reaction and did not restore arginase activity which had been inhibited with the extract from sunflower seeds.

Table 1

Influence of cysteine on reactivation of arginase inhibited by the extract from sunflower seeds

In 4.5 ml. of the incubation mixture 4 mg. of acetone powder from seedlings of the bitter blue lupin, and the given amounts of the inhibitor from sunflower seeds with or without cysteine, were added.

Addition	0.01 N-HCl used (μl.)	Activity restored (%)
None	528	—
70 μg. inhibitor	217	—
70 μg. inhibitor, 1 μmole CySH	215	0
70 μg. inhibitor, 5 μmoles CySH	518	98
70 μg. inhibitor, 10 μmoles CySH	527	100

An interesting question arose whether plants which contain the inhibitor also possess arginase, the activity of which would merely be masked (partially or wholly) by the presence of the mentioned inhibitor. If this be the case, then the addition of cysteine to arginase preparations containing the inhibitor should appreciably increase the amount of ammonia arising from the two-step hydrolysis of added arginine with the native arginase, followed by the addition of urease. Arginase preparations which do not contain the inhibitor, i.e. acetone powders from which the inhibitor has been washed out, should show no such increases. Extracts were therefore prepared from plant material in the following way: 1 g. samples of plant material were thoroughly macerated with 10 ml. of distilled water in the presence of 20 mg. of cysteine per 1 g. of dry weight, centrifuged at 3000 r.p.m. for 5 min. and the supernatant suspension filtered through a thin layer of cotton wool. A parallel sample was prepared in the same manner as above but without the addition of cysteine. Control determinations were carried out in the

same way, except that the arginase present was inactivated by immersion of the sample for 5 min. in a boiling water bath.

It is evident from the results presented in Table 2 that sunflower seeds as well as young lupin plants not only contain a very active inhibitor of arginase activity, but also that the apparent detected activity can be considerably enhanced by the addition of cysteine, which causes the unmasking of the blocked enzyme. Arginase activity was increased about four times in the samples containing the inhibitor, but no increases at all were observed in acetone powders which had the inhibitor removed in the course of preparation.

Table 2

Influence of cysteine on activation of arginase inhibited with the native inhibitor

Suspensions (10:1) of plant material or acetone powder were assayed for arginase activity.

Material used	0.01 N-HCl used (µl.)		Difference (%)
	No CySH added	CySH added	
Sunflower seeds, dormant 0.5 ml.	37	144	+ 390
1.0 ml.	63	244	+ 388
Sunflower seeds, germinating 0.5 ml.	59	232	+ 394
1.0 ml.	98	404	+ 412
Lupin plants, 22 day old 0.5 ml.	182	771	+ 423
Bitter blue lupin seedlings, acetone powder 1.0 ml.	645	587	— 9

The difference in arginase activity obtained with and without the addition of cysteine would suggest that it is essential to distinguish between "true" and "apparent" enzyme activity. It would therefore appear that much additional information could be gained if arginase activity, particularly in comparative and physiological studies, would always be determined in samples prepared without and with the addition of cysteine, in the amount of about 20 mg. per 1 g. of dry weight of the sample. Evidently the apparent detected activity may depend not only on the amount of the enzyme present, but also on the quantity of the inhibitor, the amount of which varies considerably during the various stages of plant growth and development.

Certain physical and chemical properties of the inhibitor are given below. The inhibitor was tested for its specificity on another enzyme of the ornithine cycle, namely on ornithine carbamoyl transferase, and on urease, and it was shown to have no effect on their activity. The preparation is easily soluble in water, in mineral and organic acids and

is gives copious precipitates with barium hydroxide and copper sulphate in the presence of an excess of calcium hydroxide (Van Slykes' reagent). The inhibitor is thermostable and can be evaporated at 100° on a water bath without any loss of activity. It passes through semipermeable membranes indicating a substance of moderate molecular weight.

Finally it must be pointed out that the preparation of the inhibitor despite its remarkably high activity is still greatly contaminated with inactive material as the dry weight of the preparation amounts to as much as 1.5% of the original mass of the sunflower seed kernels. Work on the purification, identification and on the kinetic properties of the substance is in progress.

SUMMARY

1. Sunflower seeds contain a very active inhibitor of arginase.

2. With the described preparation of the inhibitor at a concn. of 150 μg./ml., an 83% inhibition of arginase activity can be obtained. No further inactivation can be obtained by increasing the concentration of the inhibitor.

3. L-Cysteine restores quantitatively the inhibited arginase activity.

4. There is a considerable difference between "true" and "apparent" arginase activity in the sunflower seeds. It is therefore suggested that the determination of arginase activity should be carried out with and without the addition of cysteine.

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INHIBITOR ARGINAZY Z NASION SŁONECZNIKA

Streszczenie

1. Nasiona słonecznika zawierają bardzo czynny inhibitor arginazy.

2. Opisany preparat inhibitora w stężeniu 150 μg./ml. hamuje aktywność arginazy w 83%. Zwiększenie koncentracji inhibitora nie zwiększa stopnia inhibicji.

3. L-Cysteina ilościowo przywraca aktywność inhibowanej arginazy.

4. W nasionach słonecznika występuje znaczna różnica między faktyczną i oznaczalną aktywnością arginazy i dlatego oznaczenie aktywności tego enzymu należy przeprowadzać z reguły z dodatkiem i bez dodatku cysteiny.

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J. ROGULSKI

THE SULFHYDRYL GROUPS OF CELLULAR FRACTIONS IN THE KIDNEY OF MALEATE-TREATED RATS

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It has previously been shown [12, 13] that in the kidney of maleate-treated rats the content of both protein and non-protein SH groups was considerably diminished. Simultaneously, the content of SH groups in the blood and liver was unaffected. These results suggested that the diminution of SH content in the kidney tubules may be one of the causes of renal disfunction. Other inhibitors of SH groups, such as *N*-ethylmaleimide or mercury compounds, did not produce the same kind of disorder, but caused the death of the animals even when administered in doses 10 - 100 times smaller than those of maleate. Therefore we assumed [3] that, contrary to the other inhibitors, maleate affects the SH groups involved in special metabolic pathways associated with the excretory function of the kidney.

However, an effect of maleate on some metabolic reactions in mitochondria was also considered. The inhibition of the SH groups which are involved in such reactions may cause a shift of the hydrogen pathway, leading to energetic disturbances in the cells of renal tubules. In the last series of experiments [4, 15] we have shown that the oxidation of the Krebs cycle intermediates by kidney homogenates of maleate-treated rats is considerably diminished. The inhibition of α-ketoglutarate oxidation was the greatest resulting in the accumulation of α-ketoacids in the kidney, and affecting the biosynthesis of amino acids.

The examination of the effect of maleate on the content of SH groups in particular cell fractions of rat kidney was the main purpose of this work.

METHODS

Wistar rats fed the milk diet [1] were used. Maleic acid was injected subcutaneously, in doses of 300 mg. per kg. of body weight. The animals were killed by decapitation; the tissues to be tested were removed immediately, cooled, weighed and homogenized in a glass Potter homogenizer with 9 volumes of cold 0.25 M-sucrose solution. The homogenate was layered over the same volume of 0.34 M-sucrose solution and centrifuged at 600 - 700 *g* for 10 min. [11]. The sediment was suspended in

0.25 M-sucrose and centrifuged as above. The washed sediment was rehomogenized with 0.25 M-sucrose, and is referred to as the nuclear fraction. The first supernatant and the washings were pooled and centrifuged at 18 000 *g* for 15 min., the sediment was washed, centrifuged and rehomogenized in 0.25 M-sucrose; this fraction is referred to as the mitochondrial fraction. The pooled two supernatant fluids are referred to as the cytoplasmic fraction. The whole procedure was carried out at 0 - 5°. The sulfhydryl groups were determined in duplicate samples of the whole homogenate and of each fraction.

The total SH groups were determined at room temperature by amperometric titration with an apparatus constructed as described by Benesch *et al.* [6] with the modification of Cole *et al.* [9]. The rotating platinum electrode was devised according to Bhattacharya [8]. The titration was carried out in an aqueous solution of tris buffer at pH 7.4 [6] by means of 0.0025 M-$AgNO_3$ added from a microburette in 50 µl. doses at 1 min. intervals. The galvanometer deflections were read 55 seconds after the addition of $AgNO_3$. The electrode was standardized with a glutathione solution and the results obtained were 95% ± 3 of the theoretical. The non-protein SH groups were determined after deproteinization with sulfosalicylic acid [7]. The determinations by amperometric titration were in agreement with the values obtained with the nitroprusside method of Grunnert & Phillips [10] with the modification of Beck *et al.* [5]. Therefore the latter method, the quicker and more convenient one, was used. The content of protein SH groups was calculated by subtracting the content of non-protein SH groups from the total SH. The homogenates and all fractions were kept at 0 - 5° until the determinations were finished (about 5 hr. from the death of the animal).

Significance of difference (*P*) was obtained according to Student's *t* test [18].

RESULTS

The contents of SH groups in cellular fractions of rat kidney and liver are given in Table 1. In both tissues about 70% of total SH content was found in the cytoplasmic fraction, and about 15% in each granular fraction. This is in agreement with the data reported by Surthin & Yagi [17] and recently by Shore & Shore [16]. The percentage distribution of SH content in particular fractions was similar in the kidney and the liver but the amounts per gram of tissue were markedly higher in the liver. It should be pointed out that the ratio of protein SH groups to non-protein SH groups was distinctly higher in both granular fractions than in the cytoplasmic one.

Table 1

The content of SH groups in cellular fractions of rat kidney and liver

The total and non-protein SH groups were determined, and protein SH groups were calculated. Sum denotes the amount of SH groups calculated by adding the values for all three cellular fractions. Mean values from 10 experiments ± S.D. are given.

SH groups	Sulfhydryl groups (μmoles per g. tissue)				
	Kidney				
	Homogenate	Nuclei	Mitochondria	Cytoplasm	Sum
Total	13.98 ±1.01	2.18 ±0.30	1.71 ±0.12	10.33 ±0.98	14.22
Non-protein	3.83 ±0.46	0.32 ±0.07	0.31 ±0.07	3.30 ±0.40	3.93
Protein	10.15 ±0.91	1.86 ±0.18	1.40 ±0.21	7.03 ±0.79	10.30
	Liver				
Total	20.59 ±1.80	3.19 ±0.45	3.06 ±0.40	13.65 ±1.51	19.80
Non-protein	5.10 ±0.52	0.35 ±0.07	0.55 ±0.16	4.41 ±0.27	5.31
Protein	15.49 ±1.51	2.84 ±0.31	2.51 ±0.18	9.24 ±1.12	14.49

The effect of maleate intoxication on the content of total SH groups in the kidney and liver cellular fractions is presented in Table 2. In agreement with our previous data [12, 13], a marked decrease of kidney SH groups occurred during the first 15 min. after the injection of maleate amounting in the homogenate to 1/3 of the normal value. Although the greatest change in SH content occurred in the cytoplasm, the percentage decrease was highest in the mitochondrial fraction. It reached in mitochondria 45% ($P < 0.001$) whereas in the nuclear fraction 30% ($P < 0.001$) and in the cytoplasmic fraction 23% ($P < 0.01$). Three hours after the administration of maleate the content of SH groups in the kidney showed an increase confined, however, to the cytoplasmic and nuclear fractions. The mitochondrial SH content was still by 40% smaller than the normal value ($P < 0.001$). Within 48 hr. after the administration of maleate, the content of SH groups reached the normal values in all fractions.

The decrease of the SH groups in the kidney of maleate-treated rats concerned both protein and non-protein groups (Table 3). The decrease of protein SH groups was found in the mitochondrial and nuclear fractions; in the cytoplasmic fraction no changes were observed. As it has

Table 2

The content of total SH groups in cellular fractions of the kidney and liver of maleate-treated rats

Maleic acid, 300 mg. per kg. body weight, was injected subcutaneously. Non-treated rats were used as controls. The mean values from 6 - 10 experiments ± S.D. are given, and *P* values indicating the level of significance of the differences between the results obtained with treated and with normal rats.

Time after maleate injection	Sulfhydryl groups (μmoles per g. tissue)				
	Kidney				
	Homogenate	Nuclei	Mitochondria	Cytoplasm	Sum
Control	13.98 ±1.01	2.18 ±0.30	1.71 ±0.12	10.33 ±0.98	14.22
15 min.	9.93 ±1.12	1.51 ±0.28	0.95 ±0.12	8.01 ±0.90	10.47
P	<0.001	<0.001	<0.001	<0.01	
3 hr.	11.81 ±0.96	1.71 ±0.31	1.05 ±0.18	9.13 ±0.75	11.93
P	⩽0.01	⩽0.05	<0.001	⩽0.05	
48 hr.	14.60 ±1.37	2.15 ±0.28	1.70 ±0.16	11.08 ±1.04	14.93
P	>0.1	>0.1	>0.1	>0.1	
	Liver				
Control	20.59 ±1.80	3.19 ±0.45	3.06 ±0.40	13.65 ±1.51	19.80
15 min.	20.00 ±1.75	3.23 ±0.38	3.10 ±0.35	13.50 ±1.87	19.83
P	>0.1	>0.1	>0.1	>0.1	
3 hr.	19.42 ±1.92	3.15 ±0.41	3.17 ±0.20	12.35 ±1.52	18.67
P	>0.1	>0.1	>0.1	>0.1	
48 hr.	20.20 ±2.01	3.14 ±0.40	3.11 ±0.29	13.45 ±1.39	19.70
P	>0.1	>0.1	>0.1	>0.1	

been previously shown [13], 3 hr. after the injection of maleate the content of protein SH groups of the kidney homogenate augmented and reached values almost equal to the normal ones. In mitochondria, however, the SH groups were still lower by 35%.

The content of non-protein SH groups in the kidney of maleate--treated rats decreased distinctly and more or less uniformly in all fractions, the decrease being maintained for over 3 hr. after the injection (Table 3). This indicates that, in the kidney, maleate binds first of all the non-protein SH groups, and that this reaction occurs in all fractions to the same extent. On the other hand, as the protein SH groups were

Table 3

The content of protein and non-protein SH groups in cellular fractions of the kidney of maleate-treated rats

Conditions as in Table 2. Mean values from 10 experiments ± S.D. and *P* values are given.

Time after maleate injection	Sulfhydryl groups (μmoles per g. tissue)							
	Protein SH				Non-protein SH			
	Homogenate	Nuclei	Mitochondria	Cytoplasm	Homogenate	Nuclei	Mitochondria	Cytoplasm
Control	10.15 ±0.91	1.86 ±0.18	1.40 ±0.21	7.03 ±0.79	3.83 ±0.46	0.32 ±0.07	0.31 ±0.07	3.30 ±0.40
15 min.	7.75 ±0.93	1.28 ±0.10	0.76 ±0.39	6.08 ±0.80	2.18 ±0.33	0.23 ±0.02	0.17 ±0.07	1.93 ±0.41
P	<0.001	<0.001	<0.001	>0.1	<0.001	⩽0.02	<0.001	<0.001
3 hr.	9.83 ±1.12	1.55 ±0.30	0.90 ±0.21	7.28 ±0.91	1.98 ±0.32	0.20 ±0.07	0.15 ±0.09	1.85 0.33
P	>0.1	⩽0.05	<0.001	>0.1	<0.001	⩽0.01	<0.001	<0.001
48 hr.	10.25 ±1.22	1.91 ±0.36	1.53 ±0.23	7.10 ±0.88	4.35 ±0.52	0.24 ±0.07	0.27 ±0.08	3.98 ±0.59
P	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1

Table 4

The content of protein and non-protein SH groups in cellular fractions of the liver of maleate-treated rats

Conditions as in Table 2. Mean values from 10 experiments ± S.D. and *P* values are given.

Time after maleate injection	Sulfhydryl groups (μmoles per g. tissue)							
	Protein SH				Non-protein SH			
	Homogenate	Nuclei	Mitochondria	Cytoplasm	Homogenate	Nuclei	Mitochondria	Cytoplasm
Control	15.49	2.84	2.51	9.24	5.10	0.35	0.55	4.41
	±1.51	±0.31	±0.18	±1.12	±0.52	±0.07	±0.16	±0.27
15 min.	15.10	2.86	2.49	9.24	4.90	0.37	0.61	4.26
	±1.30	±0.23	±0.27	±1.22	±0.87	±0.09	±0.14	±0.78
P	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1
3 hr.	15.61	2.80	2.65	9.44	3.81	0.35	0.52	2.91
	±1.41	±0.27	±0.15	±0.98	±0.51	±0.06	±0.14	±0.46
P	>0.1	>0.1	>0.1	>0.1	<0.05	>0.1	>0.1	<0.001
48 hr.	15.06	2.82	2.62	9.20	5.14	0.32	0.49	4.25
	±1.56	±0.19	±0.20	±1.11	±0.81	±0.08	±0.09	±0.75
P	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1

bound significantly, and more firmly, only in the mitochondrial fraction, it appears that the affinity of maleate towards the two kinds of SH groups differs in particular cell fractions of the kidney. The reactivity of non-protein SH groups in all fractions and the reactivity of mitochondrial protein SH is the same, in the cytoplasmic fraction, however, the reactivity of the protein SH groups is almost 3 times lower.

In the liver of maleate-treated rats no significant changes in the SH content were observed (Table 4), only the cytoplasmic non-protein SH groups diminished 3 hr. after the injection. This seems to be caused by the reaction of maleate with glutathione of blood and tissues.

DISCUSSION

Our previous results [4, 15] indicated that in maleate intoxication the selective inhibition of ketoglutarate oxidation may be of considerable importance. A transitory inhibition of this fundamental reaction leads to secondary disturbances in the kidney metabolism, which may result in aminoaciduria. The simultaneous decrease of protein SH groups in kidney mitochondria may suggest a connection between the inhibition of ketoglutarate oxidation, fall of SH content and disorders of kidney function. Previously [3] we believed that the reaction of maleate with the SH groups of the cytoplasmic fraction may be of importance. Now, however, it seems that the binding of the mitochondrial protein SH groups is more significant.

The great differences in the behaviour of non-protein SH groups in the kidney and liver after the intoxication with maleate may be explained by the presence of glutathionase in the kidney [2, 14]. *In vitro* the cysteine liberated from glutathione by glutathionase reacted with maleate faster than glutathione itself. Perhaps this phenomenon may occur also *in vivo* in maleate-treated rats.

This study was partially supported by a grant from the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

SUMMARY

1. The content of both protein and non-protein SH groups in cellular fractions of rat kidney and liver was examined.

2. In rat kidney a significant decrease of mitochondrial protein SH groups, and to a lesser degree, of nuclear protein SH occurs 15 min. after the injection of maleate.

3. In the liver of maleate-treated rats no changes in the content of SH groups in any particular fraction were observed.

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GRUPY SULFHYDRYLOWE FRAKCJI KOMÓRKOWYCH NERKI SZCZURÓW ZATRUTYCH MALEINIANEM

Streszczenie

1. Zbadano rozmieszczenie białkowych i niebiałkowych grup SH we frakcjach komórkowych nerki i wątroby szczurów.

2. Obniżenie poziomu białkowych grup SH w nerce szczurów zatrutych maleinianem występuje już w 15 min. po zastrzyku i to przede wszystkim we frakcji mitochondrialnej oraz w mniejszym stopniu w jądrowej.

3. We frakcjach komórkowych wątroby po zatruciu maleinianem nie stwierdza się żadnych zmian w ilości grup SH.

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PREPARATION AND PROPERTIES OF RIBOFLAVIN FLAVOPROTEIN OF SOLUBLE FRACTION OF EGG YOLK

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It was previously found that flavoprotein with free riboflavin as prosthetic group occurs in the avian eggs [28, 26]. It resembles the avidin-biotin system and probably constitutes a storage form of vitamin B_2 for the developing embryo. As large amounts of pure preparation of flavoprotein are easy to obtain, it may serve as a model for studies on the binding of flavins with proteins. In this paper, a simplified method of isolating homogeneous flavoprotein from hen egg yolk and some of its physico-chemical properties are described.

METHODS

Riboflavin was recrystallized from a commercial preparation (Light & Co., England), and fresh solutions were made each day. Concentration of riboflavin in standard solutions was determined by measurements of extinction at 450 mμ [38] with a Hilger & Watts Uvispec UV spectrophotometer. Assays of riboflavin in flavoprotein preparations were also made by the microbiological method according to Roberts & Snell [29] with *Lactobacillus casei* as test organism. Lactic acid produced by the organism in the medium was estimated potentiometrically [23]. Preparations of flavin nucleotides (FMN and FAD) were obtained from the Sigma Chemical Co., USA.

Vitamin B_{12} was assayed microbiologically with *Euglena gracilis* "z" [15] and *Ochromonas malhamensis* [11]. Growth of the organism was determined by measuring the extinction of chlorophyll extracted from the disintegrated cells, as previously described [25].

Chromatography on diethylaminoethyl cellulose. DEAE-cellulose (Serva Co., Heidelberg, West Germany), 0.60 mEq/g., before use was prepared by the following procedure. About 100 g. was suspended in 4 liters of 0.1 N-HCl containing 0.9 M-NaCl, stirred for 20 min. and the fine particles were decanted. The sediment was washed with water to neutral reaction on a sintered glass filter. The washed cellulose was suspended in 4 liters of 1 M-$NaHCO_3$, shaken overnight, washed with

water, then suspended in 4 liters of 1 M-Na_2CO_3, and again washed with water. After resuspension in 0.1 N-NaOH for several minutes, it was washed on the glass filter with water, 95% ethanol, and then with water. DEAE-cellulose treated as described above was suspended in a suitable buffer solution and packed under hydrostatic pressure about three times greater than the height of the column.

Electrophoresis. Paper electrophoresis was performed on Whatman no. 1 paper in a horizontal cell. Electrophoresis on starch gel was done according to Smithies [32]. Sucrose density gradient electrophoresis was carried out in an apparatus similar to the one described by Cramer & Svensson [7]. The capacity of the separating column was about 270 ml. The buffer in the column was stabilized with sucrose solution employing a gradient from 40 to 0%. After separation, the fractions were collected with a fraction collector. One-ml. fractions were diluted to 3 ml. volume and extinction was determined at 280 and 450 mμ, and riboflavin and vitamin B_{12} were assayed microbiologically.

Paper chromatography. Chromatographic separation of the flavins was carried out by ascending technique in two solvent systems: *A*, isoamyl alcohol saturated with water [14]; *B*, 5% Na_2HPO_4 [16]. Whatman no. 1 and 3 MM papers were used. The spots were localized by a UV lamp.

Ulracentrifugation. Density gradient ultracentrifugation was carried out according to Martin & Ames [22], using a Martin-Christ preparative ultracentrifuge, Omega type (Osterode, West Germany) with swinging bucket rotor no. 001. The sucrose gradient from 20 to 5% was prepared as described by Britten & Roberts [3].

Polarographic determinations. The polarograph type PO3 (Radiometer, Copenhaven) and a 2 ml. cell adapted for deaeration with N_2 were used. The potential of the dropping electrode was referred to a saturated calomel electrode. The riboflavin solution in 0.05 M-Na-phosphate buffer, pH 7.0, was polarographed in the range from -0.2 to -0.8 V and the diffusion current was measured [19].

Fluorometric titrations. Fluorescence determinations were carried out with a spectrofluorometer constructed by Theorell [34]. All determinations were done at 23.5° in 0.05 M-Na-phosphate buffer of pH 7.0.

Infrared absorption spectra. The samples pressed into discs with KBr were analysed in a Unicam SP-100 infrared spectrometer (Unicam Instr. Ltd., Cambridge, England), with NaCl prism grating.

Other determinations. Protein was determined by the microbiuret method of Kirk [18]. Nitrogen was determined by the micro-Kjeldahl method, and inorganic phosphate was estimated according to the procedure of Fiske-Subbarow [9]. Total phosphorus was estimated after digestion with H_2SO_4 and subsequent clarification with hydrogen peroxide. Carbohydrates were analysed as follows: a sample of flavoprotein

or apoprotein was hydrolysed in 1 N-H_2SO_4 [17], dried in vacuum and chromatographed in *n*-butanol - acetic acid - water (4:1:5, by vol.) [1]. The sugars were detected with aniline-hydrogen phthalate spray reagent [27] and with Elson-Morgan reagent for aminosugars [12]. The total neutral sugars in the flavoprotein was determined by the phenol-sulphuric acid method [8].

Purification of flavoprotein

All the procedures were carried out at 3° under reduced illumination.

Yolks from 100 fresh eggs were washed three times with 0.9% NaCl solution to remove egg white, and briefly homogenized in a waring-blendor. Then approx. 1.8 liter of yolk was made up with saline solution to 4.5 liter and extracted with cold ether until a colourless and fat-free ether layer was obtained. The defatted suspension was left overnight in a separating funnel; the clear aqueous layer was then collected, and the dense suspension was discarded. The aqueous solution of livetin proteins was centrifuged for 30 min. at 3 000 r.p.m. and to the clear liquid, solid ammonium sulphate was added in portions with continual stirring to 0.55 saturation. After 12 hr. the precipitate was separated by centrifugation and discarded, to the solution ammonium sulphate was added to 0.80 saturation, and the mixture was left overnight in the cold room. The precipitate was collected by centrifugation, dissolved in a small volume of distilled water (approx. 150 ml.) and dialysed, first against water and then against 0.05 M-phosphate buffer of pH 5.9. The small amount of precipitate formed during dialysis was centrifuged off. The solution was then adsorbed on a DEAE-cellulose column (4 × 30 cm.) equilibrated with the same buffer solution. The column was washed with 0.1 M-phosphate buffer of pH 5.8 till the eluate contained no protein, usually with approx. 800 ml. An intensively yellow flavoprotein zone remained at the top of the column. It was found that under these conditions free riboflavin added in excess to the protein adsorbed, is eluted from the column. Flavoprotein was eluted from the column with about 500 ml. of 0.1 M-phosphate buffer of pH 5.5 containing 0.35 M-NaCl. The intensely yellow, nonfluorescent eluate of flavoprotein was dialysed against distilled water for 24 hr. and then lyophilized. From yolk of 100 eggs about 300 mg. of a product was obtained containing over 60% pure flavoprotein calculated on the basis of riboflavin content after complete saturation with the vitamin.

Further purification was carried out on a DEAE-cellulose column (2.7 × 40 cm.) with linear gradient of pH and ionic strength. The lyophilized flavoprotein, 400 mg., was dissolved in 5 ml. of 0.1 M-Na-phosphate buffer, pH 5.8, and adsorbed slowly on a column previously equilibrated with the same buffer. The column was washed with 500 ml. of

the buffer, and gradient elution was started, the mixing chamber containing 500 ml. of 0.1 M-phosphate buffer, pH 5.8, and the reservoir 500 ml. of 0.1 M-phosphate buffer, pH 5.5, in 0.5 M-NaCl. Elution of flavoprotein was checked by measuring the extinction of protein at 280 mμ and at 450 mμ (Fig. 1). A small amount of impurities, consisting of

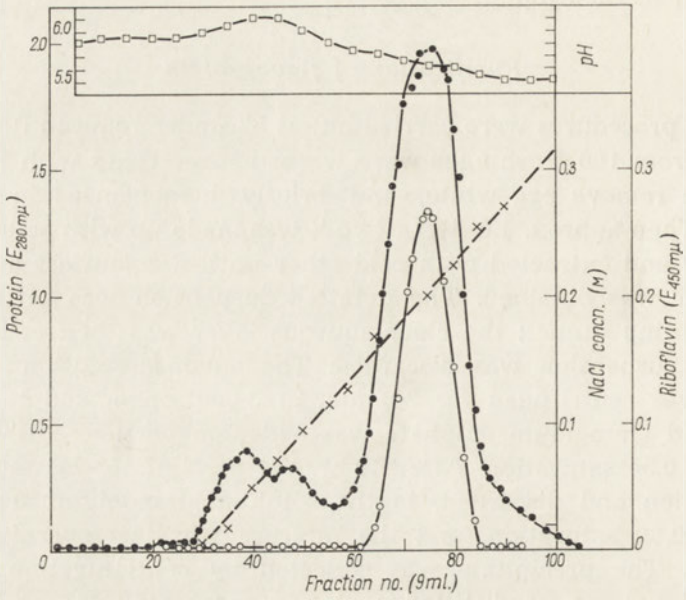

Fig. 1. Chromatography of flavoprotein on DEAE-cellulose. Column, 2.7 × 40 cm., initial buffer, 0.1 M-phosphate, pH 5.8; final buffer, 0.1 M-phosphate - 0.5 M-NaCl, pH 5.5. Sample applied, 5 ml., contained 400 mg. protein. Elution was performed with a linear gradient with 500 ml. each of initial and final buffer. Flow rate, 40 ml. per hour, fraction volume, 9 ml.; (●), E_{280}; (○), E_{450}; (×), NaCl gradient; (□), pH gradient.

a protein not binding riboflavin, was eluted just before the main peak. In the main peak the extinctions at 280 and 450 mμ were parallel. Flavoprotein was eluted within the range of 0.15 and 0.25 M-NaCl concentrations and 5.7 - 5.5 pH values. Fractions with the highest extinction at 450 mμ (nos. 65 to 82, Fig. 1) were pooled and dialysed against water to remove salts, and then lyophilized. From 400 mg. of chromatographed preparation usually 270 to 280 mg. of pure flavoprotein containing 6.5 to 7.3 μg. of riboflavin per mg. protein was obtained.

Preparation of apoprotein by gel filtration. It was found that complete splitting off of the prosthetic group takes place at pH of about 3. Attempts to remove flavin by dialysis, even for several days, at this pH were unsuccessful, due to an equilibrium formed between the dissociated and undissociated forms. Filtration of flavoprotein on a column with Sephadex G-25 (Pharmacia, Uppsala) proved particularly convenient in this case. Results of separation of apoprotein from flavin

on dextran gel are illustrated in Fig. 2. The column (2 × 34 cm.) was equilibrated with 0.1 M-citric acid - NaOH buffer, pH 3.0, and 50 mg. of flavoprotein dissolved in one ml. of the same buffer was applied and eluted at 3°. Apoprotein eluted from the column by the buffer of volume V_0 [10] was completely colourless and was recovered quantitatively. After an additional volume, V_1, of buffer was passed through the

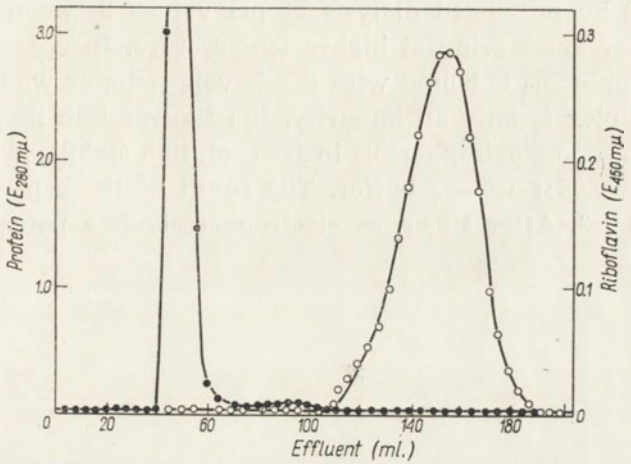

Fig. 2. Gel filtration of the flavoprotein on Sephadex G-25; 50 mg. of protein in 1 ml. was applied to the column, 2 × 34 cm., equilibrated with 0.1 M-citric acid - NaOH buffer, pH 3.0, and eluted with the same buffer solution at a rate of 30 ml. per hour. Fraction volume, 4 ml. (●), E_{280}; (○), E_{450}.

column, riboflavin was eluted. When filtration of flavoprotein was carried out over the range of pH from 3.5 to 8.0, only undissociated flavoprotein appeared after volume V_0 of buffer was passed through the column. The apoprotein solution was dialysed against distilled water and lyophilized. The apoprotein retained the ability of binding riboflavin after repeated filtration.

Isolation of flavin. Fractions with the highest extinction at 450 mμ obtained by filtration on Sephadex G-25 were combined, extracted with butanol and dried in vacuum. The obtained yellow powder was dissolved in a small volume of water and chromatographed on Whatman 3 MM paper in solvent *A*. The spot in the position corresponding to riboflavin was eluted with water and used for further experiments.

The quantitative extraction of riboflavin from flavoprotein was carried out with phenol [39]. The solution of flavoprotein was adjusted with HCl to pH 2.0 and heated for 5 min. at 100°, then neutralized and saturated with ammonium sulphate. The precipitate was removed by centrifugation and the solution was extracted with phenol. Riboflavin was reextracted from the phenol into water in the presence of ether, and dried in vacuum.

RESULTS

Electrophoresis of flavoprotein in sucrose-density gradient. As previously reported [26] the isolated flavoprotein always contained bound vitamin B_{12} in amounts of about 20 μg. per gram of protein determined microbiologically with *E. gracilis* and *O. malhamensis*. Vitamin B_{12} was not released by prolonged dialysis at pH values between 3.0 and 8.6, but only after autoclaving at higher temperature. In order to ascertain whether vitamin B_{12} is bound with the flavoprotein, or whether the B_{12}-protein complex is only an impurity, the flavoprotein preparation was submitted to zone electrophoresis in free solution stabilized with sucrose at pH 5.0 in 0.05 M-acetate buffer. The result of the separation is presented in Fig. 3. After 19 hr. of electrophoresis at a potential gradient

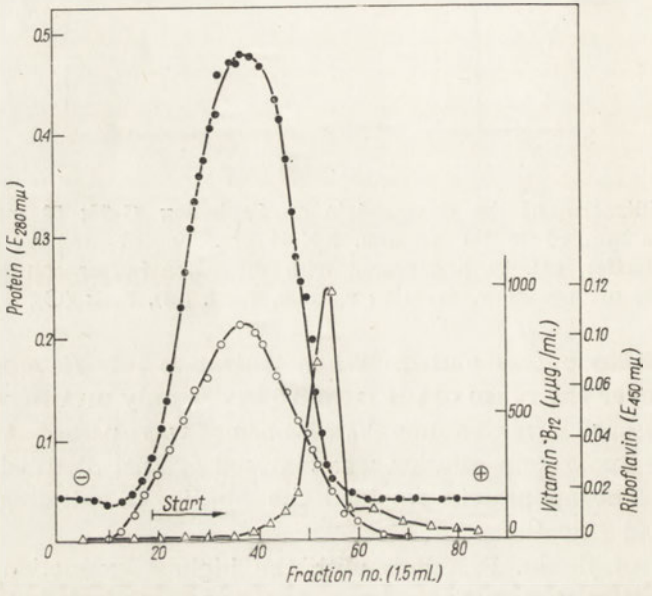

Fig. 3. Electrophoretic separation of the purified flavoprotein in sucrose-density gradient electrophoresis at pH 5.0. Fractions of 1.5 ml. were assayed for extinction (●), at 280 mμ; (○), at 450 mμ; and (△), for their vitamin B_{12} content. Sample, 25 mg. protein in 1 ml. was applied; time 19 hr. at 400 V. and 12 mA. Migration was from higher to lower sucrose gradient (ascending boundary).

of about 4 v/cm. the vitamin B_{12}-protein complex was separated from the flavoprotein. The vitamin B_{12} complex moved toward the anode as a sharply defined band faster than the flavoprotein, whereas free vitamin B_{12} under these conditions moved toward the cathode. These findings show that vitamin B_{12} combined with a specific protein forms a very slight impurity of flavoprotein.

Starch gel electrophoresis. Electrophoretic separation on starch gel over the range of pH 4.0 to 8.6 showed homogeneity of the flavoprotein regardless of the nature of the buffer solutions. The tests were carried out in veronal, tris-HCl and acetate buffer solutions at 0.05 M-concentration. In all of these buffers flavoprotein migrated to the anode as a homogeneous band. Examples of separation at pH 5.0 and 8.5 are illustrated in Fig. 4; at pH 8.5 rat serum albumin moved toward the

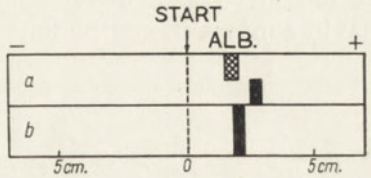

Fig. 4. Starch gel electrophoresis of the purified flavoprotein. (a), 0.05 M-veronal buffer, pH 8.5; (b), 0.05 M-acetate buffer, pH 5.0. Time, 9 hr. at 5 V./cm. and 4.5 mA. Alb., rat serum albumin migration at pH 8.5 in the same conditions as for flavoprotein.

anode more slowly than the flavoprotein. The results show that the isoelectric point of flavoprotein must be below pH 4.5. Under these conditions apoprotein exhibited the same electrophoretic mobility as flavoprotein completely saturated with riboflavin.

Sedimentation in sucrose-density gradient. Preliminary determinations of sedimentation of flavoprotein [24] gave a homogeneous, symmetrical peak, and indicated a molecular weight of about 37 000 in phosphate buffer of pH 7.0 and ionic strength 0.2. This result is in agreement with the minimum molecular weight of about 36 000 which was calculated on the assumption that 1 mg. of completely saturated protein contains 10 μg. of flavin. Further studies on the sedimentation of flavoprotein were performed by sucrose-gradient ultracentrifugation. A sample of flavoprotein with maximum riboflavin content (10.5 μg./g. protein) was dissolved in 0.05 M-tris-HCl buffer, pH 7.0, and dialysed against the same buffer for 20 hr. Then 100 μl. of 0.5 to 3.0% flavoprotein solution containing standard proteins was layered over the sucrose gradient in lusteroid ultracentrifuge tubes which were placed in a precooled swinging bucket rotor. Bovine liver catalase (Light & Co., England) and horse HbO_2 were used as standards. Catalase was assayed on the basis of the drop in extinction of hydrogen peroxide at 240 mμ [22], and HbO_2 by measuring extinction at 417 mμ. Centrifugation was carried out at 38 000 to 39 000 r.p.m. for 8 to 11 hr. at 2°. The bottom of each tube was punctured with a device similar to the one described by Szybalski [33], and 52 fractions were collected. Each tube yielded 365 to 369 drops. The fractions were collected into 1 ml. glass tubes and each fraction was examined for standard proteins and bound riboflavin [37]. Apoprotein was centrifuged in the same way, and its presence in the different fractions was determined at 280 mμ.

The flavoprotein was located between fractions nos. 31 through 50 with the peak at the tube no. 42 (Fig. 5). By comparison of the sedimentation of the flavoprotein with the sedimentation of standard proteins (catalase and HbO_2) a relative sedimentation coefficient was calculated. This value for the flavoprotein (S_{20}) was 3.1, which corresponds to a molecular weight of about 36 500. This result is a mean value of several determinations at different concentrations of flavoprotein and apoprotein between 0.5 and 3% and approaches the values obtained by means of the analytical ultracentrifuge and calculated on the basis of riboflavin content in completely saturated flavoprotein.

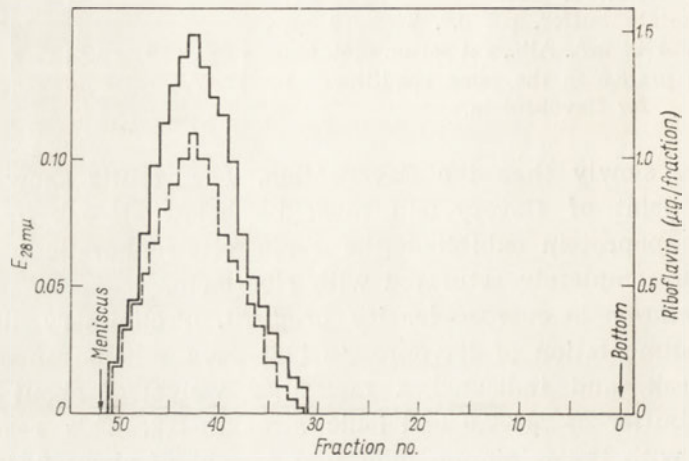

Fig. 5 Sedimentation of flavoprotein and apoprotein in sucrose-density gradient. 100 μl. of the sample in 0.05 M-tris-HCl buffer, pH 7.0, containing 0.5 mg. flavoprotein or 3 mg. apoprotein, was layered over sucrose gradient and centrifuged at 39 000 r.p.m. at 2° for 8.1 hr. Fractions of the gradients were collected as described in the text and analysed for riboflavin content (———) or by extinction at 280 mμ (- - - - -).

Further properties of flavoprotein. Pure flavoprotein is a yellow powder, readily soluble in water and in salt solutions over a wide range of pH. In neutral solution it is highly stable to heating; boiling for 30 min. does not precipitate it from solution or cause dissociation of flavin. Flavoprotein completely saturated with riboflavin contains 13% N, 0.2% P and 8% hexoses calculated as galactose. The hydrolysate of flavoprotein does not contain aminosugars, pentoses or ketoses. Preliminary studies on the identification of the sugar component indicate presence of 16 molecules of galactose to each molecule of protein.

The amino acid composition of the pure flavoprotein was kindly determined by Dr. A. Åkeson, Stockholm, according to the procedure of Moore, Spackman & Stein, using an automatic amino acid analyser. In 20 and 70 hr. hydrolysates of the preparation, 17 amino acids were

found, of which glutamic acid, aspartic acid and serine were present in greater amounts. A preliminary study of the terminal amino acids was also performed. Determination of *N*-terminal amino acids was carried out by the 1-fluoro-2,4-dinitrobenzene (FDNB) method of Sanger & Thompson [31]. The DNP-derivative of flavoprotein showed presence only of α-mono-DNP-arginine in the aqueous phase. No significant amount of any of the amino acids was found in the ether phase. The *C*-terminal amino acids were determined by the method of hydrazinolysis described by Bürgi & Schmid [4] and the results showed that it is probably tyrosine. Full data on the amino acid composition of flavoprotein will be reported in a separate communication.

When added to the synthetic medium for *L. casei*, flavoprotein did not stimulate the growth of the microorganism. A growth response was obtained, however, if the complex was first heated at 105° for 30 min. at pH 4.5 and the solution then adjusted to pH 6.8 and centrifuged [37]. Growth of the organism assessed on the basis of the amount of lactic acid produced, was proportional to the amount of decomposed flavoprotein added (Fig. 6). It can be calculated from the curve that one mg.

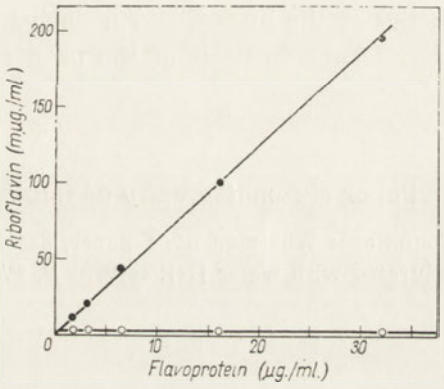

Fig. 6. Microbiological estimation of riboflavin in flavoprotein with *L. casei*. Samples of flavoprotein with concentrations as indicated were heated at pH 4.5, 105°, for 30 min., neutralized and assayed as described in Methods; (●), heated samples, (○), unheated samples.

of the protein complex contains 6.4 μg. of riboflavin, which is in good agreement with the results of spectrophotometric assay for native flavoprotein showing 6.5 μg. of riboflavin per mg. of protein.

Prosthetic group of flavoprotein. Flavin released and isolated from native flavoprotein by filtration on Sephadex at pH 3.0 or by phenol extraction (see Methods) was identified as free riboflavin. The identification of riboflavin was made by absorption spectra, paper electrophoresis and paper chromatography. The spectrum of the isolated flavin was that of riboflavin with maxima at 266, 373 and 445 mμ in neutral solution. The maximum fluorescence absorption at 373 mμ of exciting light, was 530 mμ. The infrared spectrum was essentially that of synthetic riboflavin, showing characteristic absorption bands at frequencies 1248, 1181, 1080 and 851 $cm.^{-1}$.

Fig. 7 shows the electrophoretic patterns of isolated flavin in 0.05 M-acetate buffer of pH 5.1, and 0.05 M-phosphate buffer of pH 8.0 [5]. In both buffers the spots of the studied flavin and of the standard moved toward the cathode. Under these conditions FMN and FAD moved either toward the cathode (pH 8.0) or toward the anode (pH 5.1). The R_F values

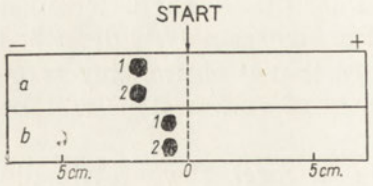

Fig. 7. Paper electrophoresis of flavin. Whatman no. 1 paper, (37 × 4 cm.), 480 Volts for 8 hr. (*a*), in 0.05 M-acetate buffer, pH 5.1; (*b*), in 0.05 M-phosphate buffer, pH 8.0. (*1*), Sample; (*2*), standard of riboflavin.

in two different solvents (Table 1), of the tested and standard riboflavin were in agreement. FMN and FAD under these conditions exhibited much higher partition coefficients. When a larger amount of the tested substance was separated in solvent *A* on Whatman no. 3 MM paper, in addition to the spot corresponding to riboflavin, a weak spot showing yellow fluorescence with R_F 0.63 was obtained, which did not correspond to any of the known flavin derivatives. Its identification will be possible after larger amounts of the preparation have been obtained.

Table 1

Paper chromatography of flavin isolated from purified flavoprotein

Conditions: Whatman no. 1 paper, ascending technique. Solvent *A*, isoamyl alcohol saturated with water [14]; solvent *B*, 5% Na_2HPO_4 [16]. Localization of spots in UV.

Flavin	R_F	
	Solvent *A*	Solvent *B*
FAD	0.86	0.41
FMN	0.81	0.58
Riboflavin	0.37	0.29
Isolated flavin	0.39	0.28

Spectral properties of the flavoprotein. The absorption spectrum of flavoprotein completely saturated with riboflavin is shown in Fig. 8. Three absorption maxima at 276, 375 and 457 - 459 mμ and a shoulder at 485 mμ may be seen. The absorption bands at 375 and 458 mμ were shifted toward the longer wavelengths in relation to free riboflavin. The ratio of the extinction at 276 mμ to that at 458 mμ was 6.5. The apoprotein exhibited only one maximum at 280 mμ with an extinction coefficient about 45% lower than that of the flavoprotein.

The infrared absorption spectra of flavoprotein and apoprotein are illustrated in Fig. 9. Apoprotein exhibited absorption bands at frequencies characteristic of most proteins: at 1110, 1390, 1450, 1540 and 1670 cm.$^{-1}$ [6].

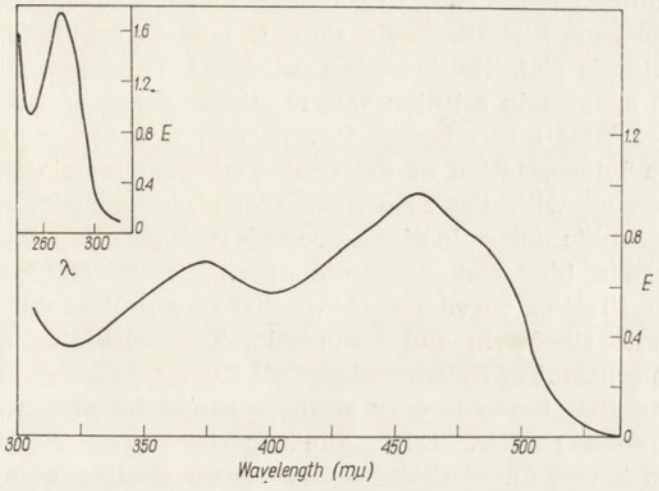

Fig. 8. Absorption spectra of the purified flavoprotein saturated with riboflavin, in 0.05 M-Na-phosphate buffer at pH 7.0. Protein concentrations were 3.0 and 1.0 mg. per 1 ml. respectively, for the visible and ultraviolet regions.

In addition, in the band at about 1050 cm.$^{-1}$ there was a wide peak, presumably attributable to the sugar component bound with protein. Absorption in this range was mainly due to C—O stretching and the O—H bending vibrations at the sugar-protein linkage [13]. The spectrum of flavoprotein differed from the one described mainly in showing wider

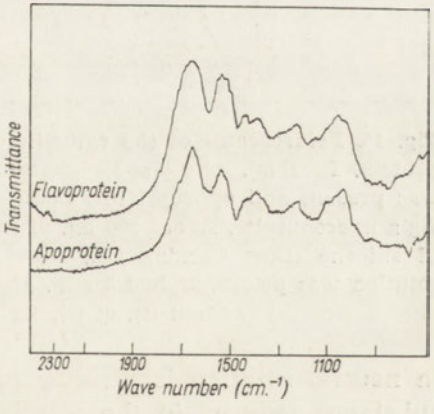

Fig. 9. Infrared spectra of flavoprotein (10 μg. riboflavin per mg. protein) and of apoprotein run in KBr pellets.

peaks at 1670 and 1540 cm.$^{-1}$, which might be due to the presence of riboflavin, which gives strong absorption in this region [30]. Also the peak at about 1050 cm.$^{-1}$ was wider, which might be due to ribitol as a component of riboflavin [2]. Moreover, the spectrum of flavoprotein,

at variance with that of apoprotein, contained a small peak at about 1170, a distinct peak at 1460, and a lowered peak at 1390 cm.$^{-1}$.

Fluorescence properties. In solutions of pH 4.0 to 8.5 flavoprotein did not exhibit fluorescence in ultraviolet light and caused quenching of the fluorescence of riboflavin until it became completely saturated with the vitamin [26]. The breaking point of the titration curve of riboflavin with apoprotein solution was relatively sharp at pH 7.0, so that the point of total saturation of flavoprotein was easily obtained. The quantitative interpretation of the results of fluorometric titration is in good agreement with the results of spectrophotometric, microbiologic and polarographic determinations (see below), indicating that one molecule of protein binds one molecule of riboflavin. The course of the fluorometric titration curve also shows that flavoprotein underwent slow dissociation to riboflavin and apoprotein. The calculated value of the dissociation constant of flavoprotein at pH 7.0 and 23.5° was 2.65×10^{-9} M [24] showing that flavin is very strongly bound by protein.

Polarographic studies. During the experiments on the properties of flavoprotein it was found that riboflavin after binding with the protein loses its reducing ability on the dropping electrode. Furthermore, the stoichiometry of the reaction riboflavin-protein can be easily followed by polarographic titration of riboflavin, since cathode diffusion current

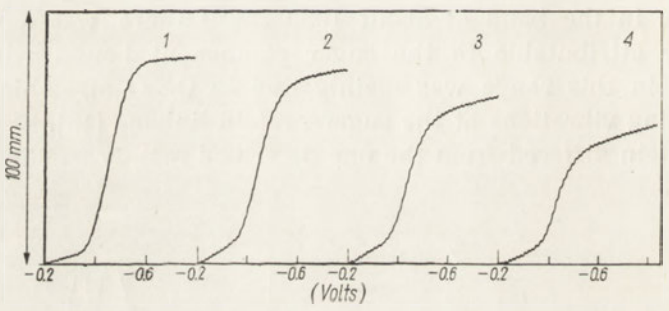

Fig. 10. Polarograms of the cathodic current of riboflavin in the presence of apoprotein. In 2 ml. of 0.05 M-Na-phosphate buffer, pH 7.0, 0.21 μmoles of riboflavin was present and appropriate amounts of apoprotein were added, by means of an Agla microburette. About 200 ml. of nitrogen was passed through the solution, trace of silicone (Dow Corning Co., USA) was added as an antifoam agent, and the solution was polarographed 5 min. after adding the apoprotein solution. Apoprotein added: (1), 0; (2), 1.2 mg.; (3), 2.4 mg.; (4), 4.0 mg.

in neutral solution is directly proportional to the concentration of the substance reduced in the solution [21]. A typical set of polarographic curves of riboflavin after adding different amounts of apoprotein at pH 7.0 is illustrated in Fig. 10. In proportion to saturation of riboflavin with increasing amounts of apoprotein in the polarographic cell, the height of the polarographic wave diminished, while the halfwave po-

tential remained unchanged (-0.47 V). After an equivalent amount of apoprotein had been added, the riboflavin wave disappeared altogether. From the decrease of the diffusion current of riboflavin after adding appropriate amounts of apoprotein the amount of riboflavin-binding sites could be calculated. It was found that one mg. of apoprotein combines with 10 μg. of riboflavin, which gives the molar ratio of protein to flavin as 1:1.

DISCUSSION

From the findings of Rhodes *et al.* [28] and from the presented results it can be concluded that flavoproteins isolated from egg protein are specific compounds with riboflavin as a prosthetic group. Their physico-chemical properties, stability in solutions of different pH, resistance to heating, and the dissociation constant of the complex indicate a very strong linkage between protein and flavin. The aforementioned authors also showed that riboflavin can replace FMN and FAD in combinations with apoprotein from egg white [28], which possesses properties very similar to those of the preparation herein described.

Like other flavoproteins, flavoprotein from egg yolk completely quenches fluorescence of riboflavin and causes a shift of the absorption bands of riboflavin from 373 and 445 mμ toward longer wavelengths. The bond with apoprotein also abolishes the reducing ability of riboflavin on the dropping electrode. This property of flavoprotein is probably due to the formation of several linkages between the isoalloxasine ring and the protein molecule, as it was shown by several authors [20, 36, 35, 28].

The isolated flavoprotein appears to be a homogeneous glycoprotein with a molecular weight of about 36 000 - 37 000 and isoelectric point at acid pH. Native flavoprotein is saturated with riboflavin only to 60 - 70%, as determined microbiologically, fluorometrically and polarographically. It should be mentioned that the state of saturation with riboflavin of the flavoprotein is not due to a loss of flavin during purification, and that both forms, flavoprotein and apoprotein are present in equilibrium under natural conditions in the livetin fraction of yolk proteins. When all the binding sites are saturated with riboflavin, the maximum content of flavin is about 1%, which corresponds to a molar ratio of flavin to protein of 1:1.

The biological role of riboflavin flavoproteins is unknown. They represent a system similar to the avidin-biotin complex in the egg white of hen, and are probably a storage form of vitamin B_2 for the developing embryo.

The authors wish to express their gratitude to Professor J. Supniewski and Dr. J. Vetulani for the use of the infrared spectrometer.

SUMMARY

From the water-soluble fraction of proteins of hen egg yolk a flavoprotein with free riboflavin as a prosthetic group was isolated. The flavoprotein was purified by ammonium sulphate fractionation, and repeated chromatography on DEAE-cellulose. This protein appeared homogeneous in ultracentrifugal and electrophoretic analyses over a pH range from 4.0 to 8.5, and in determinations of *N*- and *C*-terminal amino acids. The molecular weight of 36 000 to 37 000 was obtained from sedimentation in sucrose-density gradient and calculated on the basis of the riboflavin content. The riboflavin content was about 1%. The molar ratio of riboflavin to protein was 1:1. The flavoprotein does not fluoresce in UV and abolishes the reducing ability of riboflavin on the dropping electrode. The chemical composition of flavoprotein is: 13% N, 0.2% P and 8% hexoses. Protein free of riboflavin can be obtained by filtration on dextran gel at pH 3.0.

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OCZYSZCZANIE I WŁASNOŚCI RYBOFLAWINOWEGO FLAWOPROTEIDU ROZPUSZCZALNEJ FRAKCJI ŻÓŁTKA JAJA

Streszczenie

Z rozpuszczalnej frakcji białek żółtka jaja kury otrzymano flawoproteid z wolną ryboflawiną jako grupą prostetyczną. Flawoproteid oczyszczono przez frakcjonowanie siarczanem amonu oraz chromatografię na DEAE-celulozie. Proteid jest jednorodny w ultrawirówce i podczas elektroforezy na żelu skorbiowym w zakresie pH od 4.0 do 8.5; jednorodność preparatu potwierdzono również przez oznaczenie aminokwasów końcowych. Ciężar cząsteczkowy oznaczony na podstawie sedymentacji w gradiencie sacharozy oraz zawartości ryboflawiny wynosi 36 000 do 37 000. Zawartość ryboflawiny wynosi ok. 1%. Molarny stosunek ryboflawiny do białka jest jak 1:1. Flawoproteid nie fluoryzuje w świetle pozafiołkowym i powoduje utratę zdolności redukcji ryboflawiny na elektrodzie kroplowej; zawiera on 13% N, 0.2% P i 8% heksoz. Apoproteid wolny od ryboflawiny można otrzymać przez filtrację na żelu dekstranowym przy pH 3.

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CHEMICAL EQUILIBRIUM BETWEEN ORNITHINE AND ITS LACTAM

I. SYNTHESIS OF β-AMINOPIPERIDON AND PRELIMINARY INFORMATION ABOUT ITS BEHAVIOUR IN AQUEOUS SOLUTIONS

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In the previous communication [4] we reported that a compound with strong basic properties was found as one of the products of hydrolysis of basic peptides which were obtained by hydrazinolysis of conglutin β. This compound in neutral or weakly alkaline solutions was decomposed into ornithine and its lactam, β-aminopiperidon, the latter compound being identified by direct comparison with the ornithine lactam obtained by the method of Fischer & Zemplen [2]. This method, to our knowledge the only one reported in the literature, does not give a high yield of the product and offers no information concerning the transition products; β-aminopiperidon is characterized only as its hydrochloride and chloroplatinate. No data were found on the behaviour of β-aminopiperidon in aqueous solutions, or on the equilibrium between ornithine and its lactam, which may be of biological importance. Recently van der Horst [3] reported the presence of β-aminopiperidon in the liquid from the rumen of cattle, and found that this compound was formed also when ornithine was heated with water, ammonia, or 0.1 N-sodium hydroxide.

As the method of Fischer & Zemplen is not entirely satisfactory, a more convenient method of synthesis of β-aminopiperidon was sought. The final procedure consists in neutralization of salts of ornithine methyl ester by sodium methoxide, and gives a quantitative yield of β-aminopiperidon. The details of the procedure are given under Synthetic procedures. Every step of the synthesis was checked by high-voltage paper electrophoresis and IR spectra. We found that during the saturation of ornithine in methanol with dry hydrogen chloride the dichloride of ornithine methyl ester was formed. It was an amorphous compound, m.p. 192 - 194° (decomp.). It exhibited greater electrophoretic mobility than

ornithine and β-aminopiperidon (Fig. 1), resembling one of the products obtained by hydrolysis of peptides from hydrazinolysis of conglutin β [4].

The infrared spectrum of dihydrochloride of ornithine methyl ester (Fig. 2) shows the maximum absorption of the ester group at 1765 cm.$^{-1}$

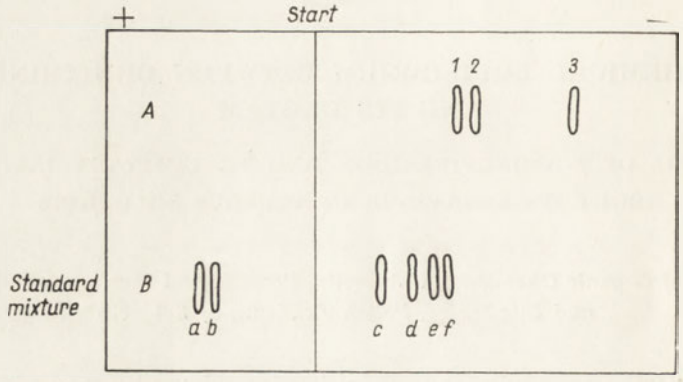


Fig. 1. Paper electrophoresis in pyridine buffer, pH 5.9; 80 v/cm.; 1 hr. (*A*), *1*, Ornithine; *2*, β-aminopiperidon; *3*, ornithine methyl ester. (*B*), Standard mixture of amino acids: *a*, aspartic acid; b, glutamic acid; *c*, histidine; *d*, arginine; *e*, lysine; *f*, ornithine.

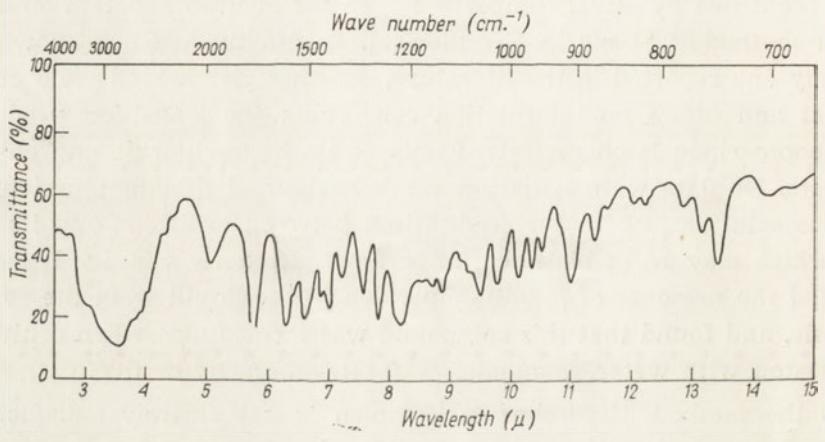


Fig. 2. IR spectrum of dihydrochloride of ornithine methyl ester in KBr disc.

and complex bands of $\upsilon^{+}NH_3$.....X in the region of 3200 - 2600 cm.$^{-1}$, and a band at about 2000 cm.$^{-1}$ which according to Cabana & Sandorfy [1] is a complex band derived from $\delta^{+}NH_3$ (wagging).

As it could be anticipated, ornithine methyl ester can exist only as a dihydrochloride. In weakly alkaline aqueous solution it is in part immediately condensed to β-aminopiperidon and in part hydrolysed to ornithine.

To transfer the dihydrochloride of ornithine methyl ester quantitatively into β-aminopiperidon a stoichiometric amount of sodium methoxide for 2 moles of HCl was added. The reaction was carried out in dry methanol. β-Aminopiperidon appeared to be a crystalline compound (m.p. 42°), very hygroscopic, and turning yellow on storage. It can be kept without decomposition in vacuum in a sealed tube. β-Aminopiperidon could be purified by vacuum distillation (b.p. 185 - 190° at 0.1 mm. Hg pressure).

IR spectrum of the free β-aminopiperidon (Fig. 3) shows the presence of associated NH_2 group (2 bands in the region of 3200 cm.$^{-1}$) and the carbonyl of lactam group (band at 1650 cm.$^{-1}$).

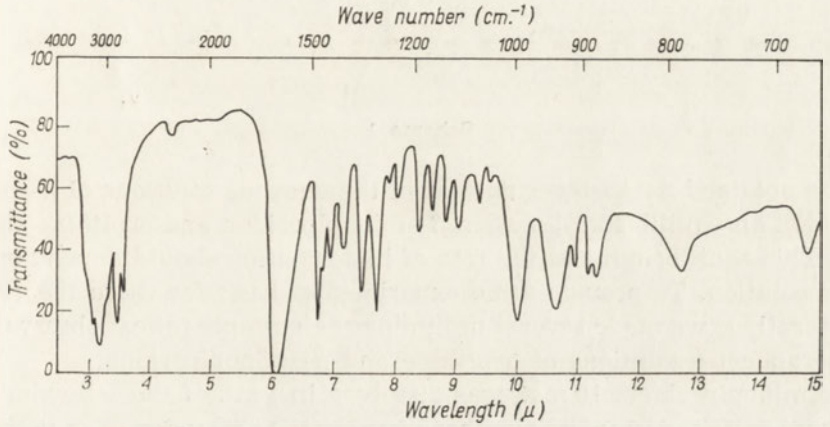
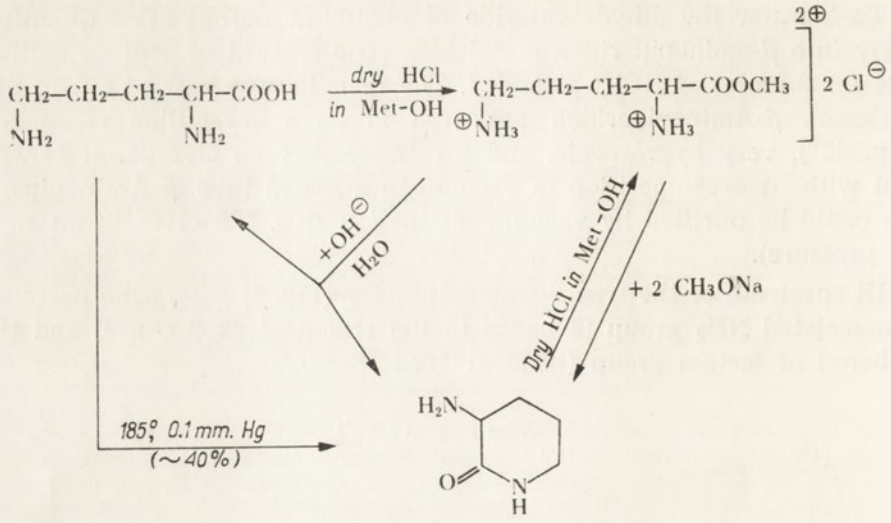


Fig. 3. IR spectrum of β-aminopiperidon (film). The Perkin-Elmer infracord was used and the positions of some bands were additionally checked on UR-10 Zeiss spectrophotometer.

β-Aminopiperidon was also obtained by thermal dehydration of ornithine at 185° at the pressure of 0.1 mm. Hg, with the yield of about 40%.

β-Aminopiperidon saturated in methanol with dry hydrogen chloride was transferred quantitatively and very easily into dihydrochloride of ornithine methyl ester.

The above-mentioned reactions are summarized in the scheme 1. It indicates that the most favourable grouping leading to the formation of $N_{(5)} - C = O$ bond is δ-amino group and esterified carboxylic group. Alcoholic condensation of ornithine ester in anhydrous medium leads to formation of pure β-aminopiperidon, while in water solution a large part of ornithine appears simultaneously. Free carboxyl group also shows a tendency to condensation with β-amino group but higher temperature is then required. The very fast alcohololysis of β-aminopiperidon in the presence of dry hydrogen chloride indicates the attenuation of the lactam bond after protonization of two nitrogen atoms.



Scheme 1

The obtained data suggested that in the aqueous solutions of β-aminopiperidon an equilibrium between β-aminopiperidon and ornithine should exist. This equilibrium and the rate of its formation should depend on pH of the solution. To provide some experimental basis for these theoretical considerations we made several preliminary electrophoretical observations on the aqueous solutions of ornithine and β-aminopiperidon.

β-Aminopiperidon, 10 mg., was dissolved in 1 ml. of the following solvents: 0.1 N-HCl; 0.02 N-HCl; water; 0.1 N-NaOH; 0.02 N-NaOH; 2 N-NaOH. Ornithine, 10 mg., was dissolved in 1 ml. of the following solvents: water + 2 eq. of HCl; water + 1 eq. of HCl; water; 0.1 N-NaOH. These samples were kept for 7 days at 20°, then heated to 50° for 20 hr. and to 60° for 20 hr. The electrophorograms in pyridine buffer, pH 5.9, 80 v/cm., were made every 10 hr. It was found that: (1), β-aminopiperidon is hydrolysed virtually completely in alkaline solution (2 N-NaOH) as well as in acidic solution which is more acidic than the water solution of dihydrochloride of β-aminopiperidon; (2), the equilibrium ornithine $\rightleftharpoons$ β-aminopiperidon is formed over the range of pH from about 3 to about 13; the greater is the pH, the more the equilibrium is shifted toward ornithine; (3), the rate of hydrolysis is greater than the rate of lactamization, and both processes are catalysed by hydroxyl ions. This is the reason why β-aminopiperidon is formed more easily in the solution of ornithine in 0.1 N-NaOH than in the water solution or with the addition of 1 eq. of HCl. In the latter case the formation of β-aminopiperidon is especially slow but it seems that the equilibrium is shifted toward β-aminopiperidon.

The above mentioned results prompted us to perform kinetic studies of the reaction ornithine $\rightleftharpoons$ β-aminopiperidon, which will be presented in the next report.

Synthetic procedures

Dihydrochloride of ornithine methyl ester. Ornithine dihydrochloride, 2 g., was dissolved in 25 ml. of dry methanol and the solution was saturated with dry hydrogen chloride. The solvent was evaporated and the dry residue was kept for 30 min. at 50° and 0.1 mm. Hg pressure. Then 25 ml. of anhydrous methanol was added and the saturation with dry HCl was repeated. After evaporation of the solvent in vacuum a white amorphous hygroscopic substance was obtained in theoretical yield, m.p. 192 - 194°. Calculated for $C_6H_{16}N_2O_2Cl_2$: N, 12.78; Cl, 32.42; found: N, 12.87; Cl, 31.92%.

Dihydrochloride of ornithine methyl ester from β-aminopiperidon. β-Aminopiperidon, 20 mg., was dissolved in anhydrous methanol and the solution was saturated with dry hydrogen chloride. Then the solvent was evaporated in vacuum and the white residue was identified as dihydrochloride of ornithine methyl ester by IR spectrum and electrophoresis.

β-Aminopiperidon from dihydrochloride of ornithine methyl ester. Dihydrochloride of ornithine methyl ester, 200 mg., was dissolved in 16 ml. of anhydrous methanol and 42 mg. of sodium dissolved in methanol was added dropwise with vigorous stirring. The solvent was evaporated in vacuum and the residue extracted with chloroform. The extract was evaporated and β-aminopiperidon was obtained in almost theoretical yield. It was purified by distillation at 185 - 190° and at 0.1 mm. Hg pressure. The melting point determined in a sealed capillary was 42°.

β-Aminopiperidon from ornithine. Ornithine dihydrochloride, 205 mg., was dissolved in 10 ml. of methanol, and 5 ml. methanolic solution of sodium (43 mg.) was added. The solvent was evaporated in vacuum and the residue extracted with chloroform. The chloroform was evaporated and the residue was distilled at 185° at 0.1 mm. Hg pressure, yielding about 40% of β-aminopiperidon which was identified by IR spectrum and electrophoresis.

SUMMARY

1. A new, very effective method of lactamization of ornithine is described.

2. The physical properties of free β-aminopiperidon were determined.

3. Preliminary observations were made on the conditions and the rate of formation of the equilibrium between ornithine and β-aminopiperidon in aqueous solutions.

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O RÓWNOWADZE CHEMICZNEJ MIĘDZY ORNITYNĄ I JEJ LAKTAMEM

I. SYNTEZA β-AMINOPIPERIDONU I WSTĘPNE DANE O JEGO ZACHOWANIU SIĘ W WODNYCH ROZTWORACH

Streszczenie

1. Opracowano b. wydajną metodę laktamizacji ornityny.
2. Określono własności fizyczne wolnego β-aminopiperydonu.
3. Dokonano wstępnych obserwacji nad warunkami i przebiegiem ustalania się równowagi: ornityna $\rightleftharpoons$ β-aminopiperydon w wodnych roztworach tych związków.

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PECTINASE INHIBITOR IN RED CURRANT LEAVES

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The presence of a pectinolytic inhibitor in the juice of pears was reported by Weurman [5] in 1953. A similar inhibitor was described in 1958 by Bell *et al.* [1] and Etchells *et al.* [3] in the leaves of the grape, which are occasionally employed by some producers of salted cucumbers as an anti-softening agent in the process of fermentation. The softening of cucumbers during fermentation is caused by maceration of the tissue, which in turn is catalysed by the pectinolytic enzyme complex, commonly named pectinase (polygalacturonases and pectinesterases).

In this paper we report the presence of the pectinase inhibitor in the leaves of the red currant as well as in the leaves of other plants. An extremely active inhibitor has been found in some red currant varieties and the process of its isolation and partial purification is described.

MATERIAL AND METHODS

Pectinase. A commercial liquid pectinase (Pectinex A. G. Basel, Switzerland), 0.3 ml., was diluted with distilled water to 50 ml. The diluted enzyme solution should be prepared freshly for each series of determinations.

Buffer solution. Citric acid, 3.53 g., and sodium hydroxide, 1.333 g., were dissolved in 1000 ml. of distilled water. The pH of the solution was 5.0.

Sodium polypectate. Sodium polypectate (Sunkist Growers, Ontario, USA), 1.2 g., were dissolved in 100 ml. of the buffer solution previously warmed to 50°, and mixed for 2 min. in a waring blendor. This solution may be kept in the refrigerator for several days.

Determination of enzyme activity and of inhibition. The viscosimetric method of Bell and Etchells [2, 4] was employed with the time of incubation shortened from 2 hr. to 1 hr. Each assay consisted of the following 3 determinations: (I), 1 ml. of the enzyme solution, 3 ml. water and 20 ml.

of the polypectate solution; (II), 1 ml. of the enzyme solution, appropriate amount of the inhibitor, 20 ml. of the polypectate solution and water to 24 ml.; (III), 1 ml. of the heat-inactivated enzyme (15 min. at 100°), 3 ml. of water and 20 ml. of the polypectate.

The samples were well mixed and kept for 1 hr. at 30°. Viscosity was then measured in 10 ml. aliquots and expressed in seconds required for the flow through the viscosimetric capillary pipette. From the loss in viscosity the pectinolytic units were read (Table 1) and the percent of enzyme activity inhibition calculated.

$$\text{Inhibition } (\%) = \frac{A_1 - A_2}{A_1} = \times 100$$

(A_1, pectinolytic units in the proper sample I; A_2, pectinolytic units in the sample with the inhibitor added, II).

Example: Viscosity after 1 hr. incubation for the sample I was 38 sec., for the sample II (with the inhibitor) 62 sec., for the sample III (with inactivated enzyme), 72 sec., and the viscosity of distilled water was 7 sec.

$$A_1 = \frac{72 - 38}{72 - 7} \times 100 = 52.3\% = 110 \text{ units}$$

$$A_2 = \frac{72 - 62}{72 - 7} \times 100 = 15.4\% = 19 \text{ units}$$

$$\text{Inhibition } (\%) = \frac{110 - 19}{110} \times 100 = 82.7\%$$

Table 1

Conversion of losses in viscosity into pectinolytic units according to Bell et al. *[2]*

Visc. loss (%)	Units	Visc. loss (%)	Units	Visc. loss (%)	Units	Visc. loss (%)	Units	Visc. loss (%)	Units
1	1	12	15	23	31	34	52	45	82
2	2	13	16	24	32	35	55	46	86
3	3	14	17	25	34	36	57	47	90
4	4	15	18	26	36	37	60	48	93
5	6	16	20	27	38	38	63	49	96
6	7	17	22	28	40	39	65	50	100
7	8	18	23	29	42	40	68	55	122
8	9	19	25	30	44	41	71	60	146
9	10	20	26	31	46	42	74	65	182
10	12	21	28	32	48	43	77	70	225
11	13	22	29	33	51	44	80	75	280

RESULTS AND DISCUSSION

The following extracts from fresh leaves were investigated for the presence of the pectinase inhibitor: 12 varieties of grapes, 17 varieties of currants (black, white and red) from the Plantation in Warka, 4 varieties of raspberries from the Institute of Horticulture in Skierniewice, as well as leaves from wild growing plants such as horse radish, oak, maple tree, plane tree, sorb tree, mulberry, willow and horse chestnut. On the basis of results obtained, the variety "Heros" of the red currant was selected for further work. The "Houghton Castle" red currant and the "Seedling" variety of the raspberry have also shown high inhibitor activity in their leaves.

Preparation and purification of extracts containing the inhibitor. Fresh leaves harvested in August, 40 g., were washed with tap water, air dried, cut into small strips and homogenized with 533 ml. of anhydrous ethanol for 3 min. The homogenate was filtered through a double layer of gauze and centrifuged for 10 min. at 3000 r.p.m. The supernatant was then concentrated under reduced pressure in a dessicator immersed in a hot water bath. The weight of the dry preparation, contaminated with fats, waxes and chlorophyll was equal to 4 g., amounting to 27.7% of the total dry weight of the leaves. One g. of this

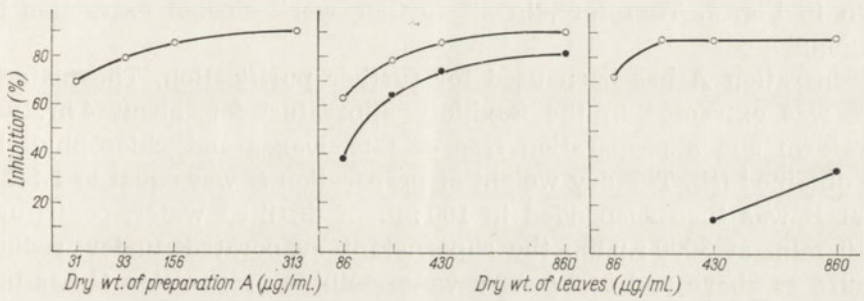

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Effect of red currant leaves preparation *A* on pectinase activity.
Fig. 2. Effect of (○), alcohol pre-treated extract and (●), direct water extract from mature red currant leaves on pectinase activity.
Fig. 3. Effect of (○), alcohol pre-treated extract and (●), direct water extract from young red currant leaves on pectinase activity.

preparation (*A*) was then suspended in 133 ml. of distilled water and 0.1, 0.3, 0.5 and 1 ml. of the solution were assayed for pectinase inhibition. The results (Fig. 1) demonstrate the effect of the inhibitor in various concentrations upon pectinase activity. It is evident that the inhibitor is easily soluble in ethanol and in water and that preparation *A* shows considerable inhibiting activity. Ethanol extracts from leaves are more

active than extracts prepared directly with distilled water (Fig. 2). This was demonstrated on very young leaves harvested in May. Fresh leaves, 20 g., were cut lengthwise into equal halves; 10 g. were extracted directly with 133 ml. of distilled water and the other 10 g. were treated with 133 ml. of anhydrous ethanol as above and the dry matter then

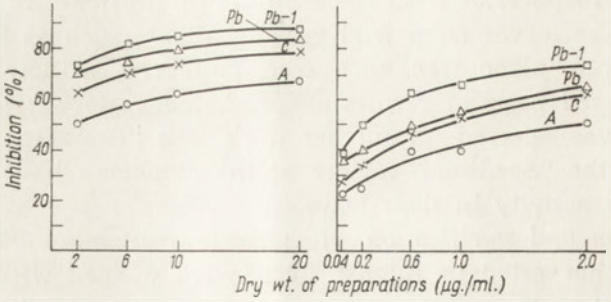

Fig. 4 Fig. 5

Fig. 4. Effect on pectinase activity of preparations from red currant leaves at concn. 2 - 20 µg./ml.

Fig. 5. Effect on pectinase activity of preparations from red currant leaves at concn. 0.04 - 2 µg./ml.

extracted with 133 ml. of distilled water. The inhibition curves are shown in Fig. 3. Therefore, in all further work alcohol extraction has been employed.

Preparation *A* has been used for further purification. The material, 1.5 g., was extracted in the Soxhlett apparatus for about 4 hr. with chloroform and a preparation free of fats, waxes and chlorophyll has been obtained (*B*). The dry weight of preparation *B* was equal to 1.145 g.; 1 g. of *B* was then suspended in 100 ml. of distilled water, centrifuged for 10 min. at 3000 r.p.m., the supernatant evaporated under reduced pressure as above and 0.82 g. of a water-soluble preparation *C* has been obtained. The dry weight proportions of preparations *A*:*B*:*C* were 100:76:62, and when the above proportions (25:19:15 mg., respectively, per 40 ml. of water) were applied for the assays no significant differences of pectinase activity inhibition could be detected.

A very useful observation was then made, namely that the inhibitor could be precipitated with neutral lead acetate. Therefore, 1.6 g. of the preparation *C* was dissolved in 100 ml. of water and precipitated with 50 ml. of a 1% solution of lead acetate. The precipitate was collected by centrifugation (10 min. at 3000 r.p.m.), washed with 10 ml. of a 0.1% solution of lead acetate and centrifuged again. The precipitate was then treated with 25 ml. of 0.1 N-H_2SO_4 and adjusted to pH 7.0 with a 2% solution of barium hydroxide. Barium sulphate was centrifuged off and the supernatant concentrated under reduced pressure as above and

0.50 g. of powder was obtained (preparation *Pb*), amounting to 31% of dry weight of the preparation *C*. When the two preparations were assayed in the above mentioned proportions there were no significant differences in pectinase inhibition, thus showing that the lead acetate treatment caused a further threefold purification of the inhibitor preparation.

Table 2

Inhibition of pectinase activity on fractional precipitation with neutral lead acetate

Preparation *C*, 5 g., dissolved in 300 ml. of water was submitted to lead acetate fractionation. For inhibition assays, the incubation mixture contained 10.4 μg./ml. of the preparation.

Fraction no.	Dry weight (mg.)	Inhibition (%)
1	635	68
2	375	65
3	180	51
4	193	39
5	95	18

Fractional precipitation with lead acetate was tried next. Preparation *C*, 5 g., was dissolved in 300 ml. of water and the inhibitor precipitated with 50 ml. of a 1% solution of lead acetate (1/3 of the amount required to precipitate all lead acetate-insoluble substances). The precipitate was treated as described previously and 0.635 g. of dry powder was obtained (preparation *Pb-1*). The supernatant from *Pb-1* was again precipitated with 25 ml. of the lead acetate solution and on further treatment as above 0.373 g. of *Pb-2* was obtained. Fractional precipitation was continued from the respective supernatants three more times and 0.180 g. of *Pb-3*, 0.193 g. of *Pb-4* and 0.095 g. of *Pb-5* were obtained. The five fractions were then assayed for pectinase inhibition, 10 mg. of powder from each fraction having been dissolved in 40 ml. of distilled water and inhibition determined in incubates containing 1 ml. of the inhibitor. As can be seen from Table 2, the preparation *Pb-1* was showing the highest inhibitory activity. The results presented in Figs. 4 and 5 show the relative inhibitions of the preparations *A*, *C*, *Pb* and *Pb-1*; 20 mg. per 40 ml. (Fig. 4) and 2 mg. per 40 ml. (Fig. 5), respectively, were assayed for pectinase inhibition, using 0.02, 0.1, 0.3, 0.5 and 1.0 ml. of the solutions in each assay with a total volume of 24 ml. Thus the concentration of the inhibitor applied varied from 0.04 to 20 μg./ml. of incubation mixture.

It is evident that considerable purification of the inhibitor has been achieved and that *Pb-1*, thus far the purest preparation shows an inhibition of about 38% at a concentration of 0.04 μg./ml. According to Bell & Etchells [1] the acetone powder preparations from grape leaves inhibit pectinolytic activity by 57% in a concentration of 111 μg./ml. In comparison, therefore, our *Pb-1* preparation is over 100 times more active.

However, the evidence obtained on preliminary purification by means of ultrafiltration through a Sephadex-G-25 column, as well as indirect evidence on dry weights, suggest that even *Pb-1* is still contaminated with inactive material. From 100 g. of dry red currant leaves as much as 740 mg. of *Pb-1* were obtained, which is several times more than should be expected.

Further work on the purification of the inhibitor, on its identification, its kinetic properties, as well as on its effect on the softening activity of cucumber saltstock during fermentation is in progress.

SUMMARY

1. Red currant leaves contain a very active inhibitor of pectinase activity.

2. A method of partial purification of the inhibitor has been described.

3. The partially purified inhibitor shows considerable activity even at a dilution of 0.04 μg./ml.

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INHIBITOR PEKTYNAZY Z LIŚCI CZERWONEJ PORZECZKI

Streszczenie

1. Liście czerwonej porzeczki odmiany „Heros" zawierają bardzo czynny inhibitor aktywności pektynazy.

2. Opisano metodę częściowego oczyszczenia inhibitora.

3. Częściowo oczyszczony inhibitor hamuje aktywność pektynazy jeszcze w stężeniu 0.04 μg./ml.

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ON THE SPECIFICITY OF CYTIDINE COENZYME IN THE INCORPORATION OF PHOSPHORYLCHOLINE INTO PHOSPHOLIPIDS BY TISSUE HOMOGENATES OF VARIOUS ANIMAL SPECIES

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The role of cytidine nucleotides in phospholipid biosynthesis was discovered by Kennedy & Weiss in 1955 [11], and a number of observations were reported for the occurrence of various reactions of the formation of natural phospholipids in which the cytidine nucleotides were found to be precursors [9]. In the transfer of the phosphorylated choline onto the D-α,β-diglyceride, the specificity of CMP-PC [1] was proved; AMP-PC, GMP-PC, and UMP-PC were inactive [12]. It was further shown that the incorporation of PC into lecithin by the enzyme preparation from chicken liver was not lower when CMP-PC was replaced by dCMP-PC [10]. However, the results obtained with rat tissue homogenates [1] in different experimental conditions showed that dCMP-PC was about three times less effective than CMP-PC.

In the present paper the incorporation of PC into phospholipids was studied on varied biological material and the rates of the incorporation were compared using AMP-PC, CMP-PC, dCMP-PC and the unnatural analogue of CMP-PC, 6-*N*-monomethylcytidine diphosphate choline (MeCMP-PC). By the use of the last compound we expected to obtain some information concerning the basis of the specificity of cytidine nucleotide in the formation of lecithin.

MATERIAL AND METHODS

Animals and tissues. For the experiments rats 6 weeks old, chickens about 3 months old, frogs (*Rana temporaria*) and snails (*Helix pomatia*) were used. Adult locusts (*Locusta migratoria*) and silkworm (*Bombyx mori*) caterpillars of the last instar were bred in our laboratory. The

[1] Abbreviations used: PC, phosphorylcholine; AMP-PC, adenosine diphosphate choline; CMP-PC, cytidine diphosphate choline; CMP-PE, cytidine diphosphate ethanolamine; dCMP-PC, deoxycytidine diphosphate choline; GMP-PC, guanosine diphosphate choline; MeCMP, 6-*N*-monomethylcytidine-5′-phosphate; MeCMP-PC, 6-*N*-monomethylcytidine diphosphate choline; UMP-PC, uridine diphosphate choline.

neoplastic tissue was the Crocker Mouse Sarcoma 180 grown on mice (this was obtained from the Institute of Antibiotics, Warszawa). The reaction of PC incorporation was studied in tissue homogenates as previously described [3]. Some experiments were performed on the mitochondrial fraction obtained from the chicken liver [12].

Special reagents. AMP and CMP were Sigma, USA, products; dCMP, Light, England; dicyclohexylcarbodiimide, BDH, London; 6-*N*-monomethylcytidine-5′-phosphate has been synthesised and kindly given by Prof. Dr. D. Shugar and Dr. W. Szer from our Institute (the data on this synthesis will be published separately). The ^{32}P-labelled orthophosphoric acid was of French origin.

The radioactive nucleotides: AMP-^{32}PC, CMP-^{32}PC, dCMP-^{32}PC and MeCMP-^{32}PC were prepared from ^{32}PC [14] and the respective nucleoside-5′-monophosphates by the method of Kennedy [8] as previously described [3]. The efficiency of the coupling was about 30 - 40%. The molar ratio of the nucleotide base to phosphorus in the synthesised radioactive compounds was: CMP-PC, 1:1.84; dCMP-PC, 1:2.08; MeCMP--PC, 1:1.90 (calc. 1:2). The paper chromatograms of these compounds and of the products of their acid hydrolysis are shown in Fig. 1. On acid

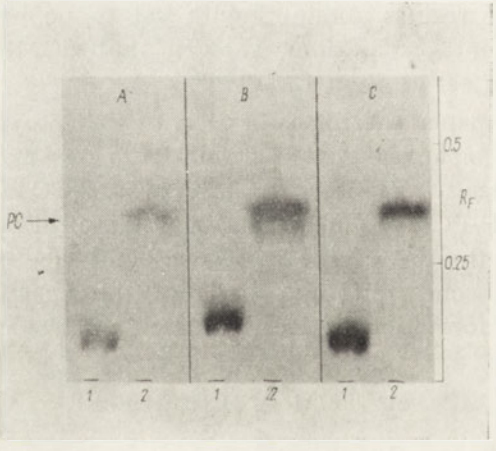

Fig. 1. Autoradiogram of paper chromatogram of (*A*), CMP-^{32}PC; (*B*), dCMP-^{32}PC; and (*C*), MeCMP-^{32}PC; (*1*), before and (*2*), after hydrolysis in 1 N-HCl, 1 hr., 100°. The ascending technique was used with the mixture: propan-2-ol, 75 ml.; water, 25 ml.; trichloroacetic acid, 5 g.; conc. ammonia (d_{20} = 0.895), 0.3 ml. [4].

hydrolysis (1 hr., 100°, 1 N-HCl) they were cleaved with the formation of ^{32}PC and the unlabelled nucleoside-5′-phosphate. The AMP-^{32}PC had also similar R_F values in this solvent system and on acid hydrolysis gave rise to the formation of ^{32}PC.

Estimation of nucleoside bases. This was made spectrophotometrically in acid medium and calculated from the milimolar extinction values: CMP, ε_{281}, 13.6; dCMP, ε_{280}, 13.8; MeCMP, $\varepsilon_{281\cdot5}$, 15.7.

Phosphorus and ^{32}P *determination.* The content of phosphorus in nucleotides, in ^{32}PC and in phospholipids was estimated according to Strickland *et al.* [16] after digestion of the samples with perchloric acid. The amount of ^{32}P was assayed with a VA-Z 410 VEB Vacutronic liquid counter. In each lipid sample the specific activity of phospholipids (counts/min./μg. P) was determined.

The molar proportion of the newly synthesised ^{32}P-labelled phospholipids per 1000 molecules of phospholipids (considered as monophospholipids) in the incubate was calculated by multiplying by 1000 the ratio of specific activity of total phospholipids to that of ^{32}PC present in CMP-PC.

RESULTS

The data on ^{32}PC incorporation into phospholipids from the four nucleotide derivatives are presented in Table 1. They are expressed as percentages of ^{32}P incorporated from 0.05 μmole of the precursor added and as the molar proportion of the newly synthesised ^{32}P-labelled phospholipids per 1000 phospholipid molecules of the incubate.

As it can be seen from Table 1 the reaction of PC transfer from CMP-PC occurs in tissues of all the species studied; the highest rate was found in liver tissue of the rat, frog and chicken and in the brain cortex of the rat. There was also a high incorporation in the Crocker

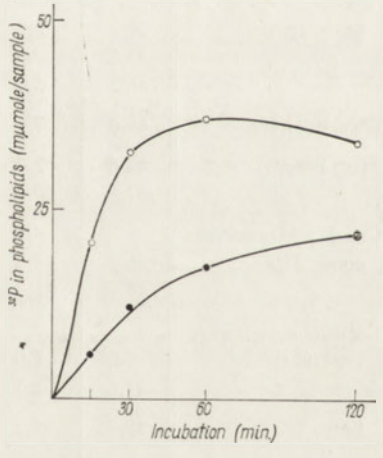

Fig. 2. Incorporation of ^{32}P into phospholipids from (○), CMP[^{32}P]phosphorylcholine; and (●), dCMP[^{32}P]phosphorylcholine in chicken liver mitochondrial fraction. Composition of the incubation mixture as in Table 1, except that instead of the tissue homogenate it contained the dialysed mitochondrial fraction corresponding to about 50 μg. of lipid P. To each sample 50 mμmoles (4600 counts/min.) of substrates was added.

Mouse Sarcoma 180 neoplastic tissue. Lower incorporation was found in tissues of *Locusta migratoria* and *Bombyx mori*. In the hepatopancreas of *Helix pomatia* the incorporation was about 50 times less than in rat, chicken or frog.

Table 1

CMP-PC, dCMP-PC, MeCMP-PC, and AMP-PC as precursors of phospholipids in homogenates of tissues of various species

Each incubation sample contained 100 mg. of homogenized tissue in a medium containing: 0.0316 M-KCl; 0.0095 M-NaF; 0.02 M-$MgCl_2$; 0.004 M-Na_2HPO_4; 0.0266 M--tris - HCl buffer, pH 7.4. 0.05 μmole of CMP-^{32}PC, dCMP-^{32}PC, MeCMP-^{32}PC or AMP-^{32}PC were added as indicated. The final volume was 1.5 ml. Incubation period 1 hr. at 37°. The phospholipids were extracted according to Folch *et al.* [5] and the radioactivity measured as described in Methods. Relative activity × 1000 (Rel. act. × 1000) is the molar proportion of the newly synthetized ^{32}P-labelled phospholipids per 1000 phospholipid molecules in the incubate, calculated from the ratio:

$$\frac{\text{counts/min./μg. P in total phospholipids}}{\text{counts/min./μg. P in } ^{32}\text{PC}} \times 1000$$

Tissue	CMP-^{32}PC		dCMP-^{32}PC		MeCMP-^{32}PC		AMP-^{32}PC	
	^{32}P incorporated (%)	Rel. act. ×1000	^{32}P incorporated (%)	Rel. act. ×1000	^{32}P incorporated (%)	Rel. act. ×1000	^{32}P incorporated (%)	Rel. act. ×1000
Rat								
liver	46.6	7.7	—	—	0.4	0.1	—	—
	40.1	7.0	—	—	0.7	0.2	—	—
	58.4	7.5	22.4	2.8	—	—	1.2	0.1
brain cortex	29.2	2.6	—	—	0.9	0.1	—	—
	33.2	3.0	—	—	0.4	0.1	—	—
	46.2	5.0	17.7	2.2	—	—	0	0
Chicken liver	48.0	7.6	30.7	5.8	0.4	0.1	0.2	0.1
Frog liver	42.6	12.4	14.0	4.0	0.6	0.2	—	—
	67.5	20.9	14.5	4.9	—	—	0	0
Crocker Mouse Sarcoma 180	36.4	17.6	7.8	3.7	0.9	0.4	—	—
	47.7	21.7	7.8	3.7	0.9	0.4	—	—
Locusta migratoria								
fat-body	5.0	1.0	1.0	0.2	—	—	0.1	0.1
	11.6	2.3	4.8	0.9	0.5	0.1	—	—
brain	18.9	5.3	10.4	3.0	2.4	0.7	—	—
Bombyx mori,								
fat-body	17.5	10.8	6.5	3.9	—	—	0.5	0.3
brain	3.0	2.3	1.1	0.8	—	—	0.1	0.1
Helix pomatia								
hepatopancreas	1.2	0.5	0.1	0.1	—	—	0.1	0.1

When AMP-PC or MeCMP-PC were added to the homogenate instead of cytidine diphosphate choline the incorporation of PC into phospholipids was very low. However, in brain tissue of *Locusta migratoria* the differences in the incorporation from CMP-PC and MeCMP-PC were not so distinct. In all tissues tested the incorporation from deoxycytidine nucleotide was smaller than from cytidine compound, but the difference varied from one tissue to another. In the chicken liver homogenate dCMP-PC was only slightly less effective, while in other tissues 1 hr. incubation with dCMP-PC led to considerably smaller labelling. When the time curves of PC incorporation were made using cytidine and deoxycytidine derivatives and chicken liver mitochondrial fraction (Fig. 2) it was found that after longer periods of time only slightly less PC was incorporated from deoxy compound (43%) than from cytidine precursor (68%). After shorter incubation (15 min., 30 min.) the incorporation was 3 - 4 times smaller from deoxy compound than from CMP-PC.

DISCUSSION

A good labelling of phospholipids was obtained in this study by using relatively small amounts of the nucleotide precursor (0.05 μmole of CMP-^{32}PC per 100 mg. of fresh tissue). In the previous studies of Ansell & Chojnacki [2] four times larger amount of the precursor was used but the labelling of phospholipids was only slightly better. This could probably be due to the limited amount of the diglyceride acceptor present in tissue homogenate.

The presented experiments indicate that the biosynthesis of phospholipids *via* cytidine intermediate may be fairly common in nature as it can also be inferred from the fact of the wide occurrence of cholinephosphate cytidylyltransferase [12]. Even in *Helix pomatia* where only small incorporation of PC from cytidine precursor was noted, the rate of labelling was higher than when adenosine and deoxycytidine derivatives were used.

The possible role of dCMP-PC and dCMP-PE in lecithin and cephalin biosynthesis was suggested by the fact that compounds of this type are also present in organisms besides the CMP-PC and CMP-PE [17, 13], and especially in neoplastic tissues they were found in greater amounts [15]. It was shown by Kennedy *et al.* [10] that in the chicken liver dCMP-PE was a less effective precursor of cephalin, while dCMP-PC was not a worse precursor of lecithin, than CMP-PC. Ansell & Chojnacki [1] found that in rat tissues dCMP-PC is less effective and these data were confirmed presently on larger animal material. The identical rates of lecithin formation from cytidine and deoxycytidine precursors in the experiments of Kennedy *et al.* [10] might have been due to the experimental conditions in which large amounts of diglyceride and long incu-

bation period were used. When studying the rates of ^{32}PC incorporation after shorter time intervals a distinct difference between the labelling of phospholipids from dCMP-^{32}PC and CMP-^{32}PC was observed.

The smaller effectiveness of the deoxy compound may not be related only to the difference in the sugar moiety of the nucleotide, but it may result from the difference in cytosine. As it was shown by nuclear magnetic resonance studies [6], deoxycytidine and cytidine differ in respect to the pyrimidine as well, cytidine having a true amino group at $C_{(6)}$, while an imino group at this position is present in deoxycytidine. If the same is true for nucleoside phosphates the smaller efficiency of deoxycytidine compounds as phospholipid precursors may be due also to this difference. This suggests that the $C_{(6)}$ amino, but not $C_{(6)}$ imino, group plays a role in the enzymic transfer of phosphorylcholine. The ineffectiveness of UMP-PC supports the above supposition [12]. The observation that also MeCMP-PC is ineffective indicates that the reaction can occur only when the $C_{(6)}$ amino group of cytosine is not substituted.

It should be emphasized that different 6-*N*-acylated cytosine derivatives are found among substances endowed with antibiotic properties (amicetin, bamicetin and plicacetin [7]).

We are indebted to Prof. Dr. D. Shugar and Dr. W. Szer from the Institute of Biochemistry and Biophysics, Warszawa, for the gift of a sample of 6-*N*-methylcytidine-5′-phosphate and to Dr. A. Ukleja-Bortkiewicz from the Institute of Antibiotics for supplying the neoplastic tissue of the Crocker Mouse Sarcoma 180.

SUMMARY

The incorporation of ^{32}P-labelled phosphorylcholine (PC) from its nucleotide derivatives was studied in tissue homogenates of different species. In all tissues tested the CMP-PC was found to be the most effective precursor of phospholipids; dCMP-PC was less effective. Neither AMP-PC nor 6-*N*-methylcytidine diphosphate choline served as precursors in this process.

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O SPECYFICZNOŚCI KOENZYMU CYTYDYNOWEGO DLA REAKCJI WBUDOWYWANIA FOSFOCHOLINY W FOSFOLIPIDY W HOMOGENATACH TKANEK U RÓŻNYCH GATUNKÓW

Streszczenie

Badano wbudowywanie fosfocholiny (PC) znakowanej ^{32}P z jej nukleotydowych pochodnych w fosfolipidy w homogenatach tkanek u różnych gatunków. We wszystkich badanych tkankach CMP-PC była najlepszym prekursorem fosfolipidów. Mniej efektywną była dCMP-PC. AMP-PC ani 6-*N*-metylocytydynodwufosfocholina nie spełniały roli prekursorów w tym procesie.

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RECENZJE KSIĄŻEK

Engelberg H.: HEPARIN, METABOLISM, PHYSIOLOGY AND CLINICAL APPLICATION. C. C Thomas Publ. Springfield (Ill.), 1963; str. 218, cena $ 8.50.

Krótka monografia Engelberga o przemianie, fizjologii i klinicznych zastosowaniach heparyny składa się z dwóch części. Pierwsza została poświęcona wynikom podstawowych badań nad heparyną, druga omawia kliniczne jej zastosowania. Opis chemicznej budowy heparyny opracował K. D. Brown w osobnym rozdziale, w którym uwzględniono literaturę przedmiotu do końca 1961 roku. Wyniki omówiono krytycznie zwracając uwagę na te, które są dobrze doświadczalnie uzasadnione, jak i na te, co do których istnieją rozbieżności. Mimo że niemal pół wieku upłynęło od odkrycia heparyny, istnieje jeszcze dość dużo niejasności co do jej budowy chemicznej. Wynika to w znacznej mierze z faktu niejednorodności preparatów, z którymi różni autorzy mieli do czynienia. Dr Engelberg jest lekarzem szpitala w Los Angeles. Lekarskie wykształcenie autora nadaje książce lekarsko-fizjologiczny charakter. Rozdział „Metabolism of Heparin" można by raczej zatytułować „Fizjologia heparyny". W rozdziale tym omówiono centralną rolę komórek tucznych w produkcji heparyny, wpływ hormonów, napromieniowania, zawartość heparyny we krwi i wreszcie jej wydalanie. W rozdziale o działaniach heparyny zgromadzono dużą ilość danych opartych na 263 pozycjach literatury obejmującej okres do 1960 roku. Podstawowemu działaniu heparyny, działaniu na układ krzepnienia, poświęcono zaledwie pięć stron. Chociaż autor podaje, że dyskusja nad krzepnieniem krwi wykracza poza ramy monografii, to jednak wydaje się, że poświęcono temu zagadnieniu zbyt mało miejsca. Mimo, że podano szczegółowe dane o tych fazach procesu krzepnienia, w których heparyna interferuje, to jednak przedstawienie jednego schematu krzepnienia (wg Monkgouse'a, *Am. J. Clin. Nutr.* **8**, 1, 1960) bez wyjaśnienia użytych skrótów (AHF, PTC itd.) i bez podania nomenklatury (numeracji) Międzynarodowego Komitetu Czynników Krzepliwości Krwi nie wydaje się dostateczne. Stosunkowo dużo miejsca poświęcono wpływowi heparyny na usuwanie trójglicerydów z krwi, co tłumaczy się osobistym dorobkiem autora w doświadczalnych pracach w tej dziedzinie. W wykazie bibliograficznym tego rozdziału, obejmującym 239 pozycji, wymieniono 13 publikacji autora, a w tekście umieszczono także jego niepublikowane wyniki eksperymentalne. Rozdział ten napisano krytycznie i zreferowano zagadnienie wszechstronnie.

W drugiej, klinicznej części pracy najwięcej miejsca poświęcono terapii heparynowej przy zakrzepach. Omówiono wyniki stosowania heparyny przy ostrym zawale sercowym, zakrzepach żylnych, przypadkach chirurgicznych zabiegów na naczyniach i sercu, podano także dawkowanie. Następny rozdział zawiera dane o zastosowaniu heparyny w zapobieganiu i leczeniu miażdżycy, a także w wielu innych jednostkach chorobowych, jak przy stanach zapalnych, oparzeniach itd. W końcowym rozdziale omówiono reakcje uboczne po podaniu heparyny.

Książkę zaopatrzono w indeks autorów i indeks przedmiotowy. Pierwszy odsyła czytelnika zarówno do tych miejsc tekstu, w których nazwisko zostało wymienione, jak również do tych, gdzie zamieszczono tylko kolejny numer pozycji bibliogra-

ficznej, co wobec niealfabetycznego układu literatury sprawia czytelnikowi pewną trudność. Drugą usterką redakcyjną jest to, że tzw. żywa pagina w całej książce jest jednakowa i jest identyczna z tytułem książki, a nie informuje o treści poszczególnych rozdziałów.

Książka Engelberga stanowi dobre źródło informacji o heparynie we wszystkich aspektach, może być z pożytkiem użyta i przez badacza doświadczalnego i przez klinicystę.

Tadeusz Korzybski

ALLERGOLOGY. Proceedings of the IVth International Congress of Allergology (E. A. Brown, ed.). Pergamon Press, Oxford - London - New York - Paris, 1962; str. 455, cena £ 5.05.

Omawiana książka stanowi sprawozdanie z IV Międzynarodowego Kongresu Alergologii, który odbył się w Nowym Jorku w październiku 1961 r. Zawiera ona przemówienia wstępne i referaty wygłoszone na siedmiu sesjach Kongresu.

W części wstępnej m.in. Pasteur Vallery-Radot przedstawił naukową biografię pioniera badań nad alergią, R. A. Cooke'a, zmarłego w 1960 r. Właściwa treść książki podzielona jest na siedem części, odpowiadających sekcjom Kongresu. Biochemika zainteresują przede wszystkim referaty I sekcji, obradującej pod przewodnictwem Pasteura Vallery-Radot, a więc wykłady M. Heidelbergera o jednorodności i różności antyciał, S. Cohena i R. R. Postera o strukturze i metabolizmie antyciał, J. H. Humphrey'a o biochemicznych pośrednikach reakcji między antygenem a antyciałem.

Sekcje od II do V zajmowały się przeważnie praktyczną stroną zagadnienia, tj. profilaktyką i leczeniem alergii. Poszczególne referaty, zwłaszcza referaty zagajające obrady danej sekcji, dają szerszą biologiczną podbudowę i są interesujące także dla niespecjalisty. Sekcja VI zajmowała się metodyką badań, zaś sekcja VII — astmą u dzieci.

Jak wynika z przeglądu treści, książka omawia poszczególne strony zagadnienia alergii, stanowi więc cenne źródło informacji w tej dziedzinie, w której niewiele jeszcze mamy systematycznych i źródłowych opracowań. W każdym razie nawet pobieżne zapoznanie się z treścią poszczególnych wykładów pozostawia wrażenie, że alergologia jest dziedziną, której ciężar gatunkowy rośnie szybko z roku na rok i będzie nadal wzrastał w miarę postępów lecznictwa i eliminowania innych źródeł zachorzeń. Książka nie ma jednak charakteru podręcznika ani monografii, a brak skorowidza rzeczowego nie ułatwia znalezienia określonej informacji.

Józef Heller

Lapin V. A. and Jakovleva L. A., COMPARATIVE PATHOLOGY IN MONKEYS (tłumaczenie z rosyjskiego). C. C. Thomas Publ., Springfield (Ill.) 1963; str. 272, rys. 114, cena $ 10.00.

Książka stanowi przegląd głównych objawów anatomopatologicznych i histopatologicznych najczęstszych schorzeń występujących u małp. Materiał stanowiło 1000 małp z największego na świecie ośrodka badań nad małpami w Suchumi

w ZSRR. Autorzy podkreślają krytycznie, że schorzenia i opisywane objawy dotyczą zwierząt przebywających w niewoli. Objawy anatomopatologiczne są bardzo zbliżone do obrazów znanych z patologii człowieka, choć wykazują pewną swoistość. Częstość występowania schorzeń infekcyjnych jest przez autorów szczególnie podkreślana; prawdopodobnie wynika ona z kontaktów z ludźmi. Poza tym opisane są i inne schorzenia wraz ze schorzeniami nowotworowymi i wadami wrodzonymi występującymi u małp. Książka zawiera szereg czytelnych makro- i mikrofotografii.

Kazimierz Ostrowski

von Kaulla K.: CHEMISTRY OF THROMBOLYSIS, HUMAN FIBRINOLYTIC ENZYMES. Charles C. Thomas, Publ., Springfield (Ill.) 1963; str. 333; cena $ 12.75.

Fibrynoliza czyli proteolityczne trawienie fibrynogenu i fibryny należy do podstawowych zjawisk fizjologicznych w ustroju ludzkim. Zaburzenia fibrynolizy leżą u podstaw takich procesów patologicznych, jak skazy krwotoczne i sprawy zakrzepowe. Istnieją również powiązania między fibrynolizą a zapaleniem, miażdżycą, zmianami łącznotkankowymi typu zwyrodnienia włóknikowatego.

Z punktu widzenia biochemicznego złożony układ fibrynolityczny, składający się z prekursorów enzymów, aktywatorów różnego typu i inhibitorów przedstawia instruktywny przykład reakcji łańcuchowej pozwalający na studiowanie powiązań między poszczególnymi członami tego łańcucha. Wzajemne zależności pomiędzy układem fibrynolitycznym i układem krzepnięcia są pięknym przykładem istnienia mechanizmów samoregulujących na poziomie molekularnym w żywym ustroju. Większość reakcji zachodzących w tych układach jest natury proteolitycznej. Jeden ciąg reakcji — proces krzepnięcia — prowadzi do utworzenia sieci fibryny, drugi — fibrynoliza — do jej rozpuszczenia. Istnieje dobrze kontrolowana dynamiczna równowaga między obydwoma układami, warunkująca utrzymanie krwi w stanie płynnym oraz prawidłową hemostazę.

Pod nazwą trombolizy rozumie się rozpuszczenie już powstałych skrzepów. Może ona zachodzić w ustroju, jeżeli nasila się potencjał enzymatycznych mechanizmów fibrynolitycznych i uzyskuje przewagę nad potencjałem układu krzepnięcia, który poprzednio był przyczyną zakrzepu. Tromboliza może być również sztucznie wywołana jako zabieg leczniczy, bądź bezpośrednio drogą podania enzymów proteolitycznych lub ich aktywatorów, bądź pośrednio przez mobilizację endogennego układu fibrynolitycznego drogą oddziaływania farmakologicznego na ustrój ludzki.

O wszystkich tych sprawach pisze znany amerykański uczony Kurt von Kaulla w książce pt. „Chemistry of thrombolysis, human fibrynolytic enzymes".

V. Kaulla od przeszło 20 lat pracuje eksperymentalnie w tej dziedzinie, a ostatnie lata poświęcił głównie sprawom trombolizy. Książka jest pierwszą próbą tak obszernego, monograficznego przedstawienia tej zawiłej dziedziny wiedzy.

Ocena książki powinna być przeprowadzona z punktu widzenia poszczególnych jej użytkowników. Dla kogo może być ona przeznaczona? Istnieje liczna rzesza pracowników nauki, zajmujących się zagadnieniem fibrynolizy i trombolizy. Najliczniej reprezentowani są lekarze i analitycy lekarscy, dla których nieraz codziennym obowiązkiem może być badanie układu fibrynolitycznego u chorych krwawiących lub u chorych z zakrzepami. W tych okolicznościach ocena aktualnego stanu układu fibrynolitycznego i układu krzepnięcia może być decydującym czynnikiem pozwalającym na ustalenie rozpoznania i właściwego leczenia. Ale mogą to być również

biochemicy lub patolodzy eksperymentalni prowadzący badania podstawowe nad jakimkolwiek aspektem fibrynolizy lub też terapeuci stosujący leczenie trombolityczne będące wciąż jeszcze metodą eksperymentalnej tylko terapii jednej z najbardziej rozpowszechnionych chorób, jaką jest choroba zakrzepowa.

Jako jeden z czytelników tego kręgu, mogę zapewnić, że książka spełnia swoje zadania. Znajduje się w niej krótki i rzeczowy opis faktów, sposób przedstawienia ich jest obiektywny, również gdy nie są one zgodne z osobistymi poglądami autora. A autor posiada osobiste poglądy, które dzielą nie wszyscy „koagulacjoniści" i fibrynolitycy. Do takich należy pogląd uznający istnienie wzajemnej dynamicznej równowagi między układem krzepnięcia i fibrynolizy. Subiektywny jest sposób ujęcia aktywacji układu fibrynolitycznego, a również pogląd o szczególnej roli antyplazminy w układzie fibrynolitycznym. Również stanowisko autora w spornych zagadnieniach trombolizy musi z konieczności być subiektywne. Trudno jest mi polemizować z tymi poglądami autora, gdyż zgadzają się one w dużej mierze z moimi własnymi. Ważne jest to, że również inne poglądy są uwzględnione, przy czym autor zachowuje obiektywność. Spis autorów i literatury obejmuje wieleset pozycji, pomiędzy nimi pocieszająco dużo pozycji polskich. Pod tym względem książka jest prawdziwą kopalnią wiedzy i informacji

Te zalety monografii v. Kaulli usprawiedliwiają jej ocenę ze strony specjalistów w dziedzinie fibrynolizy. Lecz również dla mniej obeznanych z przedmiotem i niezaangażowanych bezpośrednio w te zagadnienia książka może być pożyteczna. Wykład jest przejrzysty, język jasny i prosty, a układ rozdziałów logiczny. Fibrynoliza należy do tych dziedzin medycyny, w których chemia, patologia i klinika krzyżują się i zazębiają jak rzadko w innej. Jakie jest powiązanie pomiędzy aktywacją układu fibrynolitycznego i krwawieniem, między leczeniem choroby zakrzepowej i zawałów a biochemią rozpuszczania fibryny? Niewiele jest dziedzin klinicznej medycyny, w których zagadnienia codziennej praktyki lekarskiej tak ściśle się wiążą z badaniami podstawowymi, gdzie codzienne postępowanie przy łóżku chorego wymaga zrozumienia zjawisk chemicznych leżących u podstaw klinicznych spostrzeżeń.

Z tego też względu należałoby życzyć książce przekładu na język polski i licznej rzeszy czytelników.

Edward Kowalski