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A C T A B I O C H I M I C A P O L O N I C A
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MARIA GUMIŃSKA and J. BORYSIEWICZ

STUDIES ON THE CRABTREE EFFECT

I. GASEOUS METABOLISM OF TISSUE CULTURES

Department of Physiological Chemistry, and Department of Medical Microbiology, Medical School, Kraków

Since the studies of Warburg, the decrease of respiration and increase of glycolysis, especially in aerobic conditions, is considered as one of the principal chemical characteristics of neoplastic material. Another characteristic feature of that material, described in 1929 by Crabtree [7], is the reduction of oxygen consumption after addition of glucose. This phenomenon, called also the "reversed Pasteur effect", is known as the Crabtree effect and was observed only in aerobically glycolysing tissues, e.g. in several tumour slices [8, 14, 20, 27], in experimental tumours such as Ehrlich's ascites cells [15, 19] and in some normal tissues (embryonic tissues, placenta, retina, blood platelets) [2, 6, 16, 25, 21].

Kun, Talalay & Williams-Ashman [19] and El'Tsina & Seits [15] reported in 1951 the occurrence of the Crabtree effect in the ascites tumour cells, and since then that material was the only one in which this phenomenon has been investigated. Nevertheless the problem has not yet been elucidated and there is no agreement as to its interpretation. In comparison with the number of works on respiration and glycolysis in tumours, the number of studies on the Crabtree effect is rather small.

The present communication deals with the occurrence of the Crabtree effect in experimental material other than that which has been used up to now, namely in cells from tissue cultures of human and animal origin, both neoplastic and non-neoplastic.

In recent years, as a result of advances in methods of tissue culture, living human cells cultivated *in vitro* have become a very frequent object of biochemical investigations. They give homogeneous suspensions not contaminated by tissues of the stroma, blood cells or dead cells.

The most commonly used human neoplastic cell culture is the HeLa line of cells from cancer of the uterine cervix, adapted to growth on glass by Gey *et al.* in 1951 [17, 18]. These cells have been used for determinations of requirements for exogenous nutrients and in metabolic studies. Only a small number of reports dealt with their carbohydrate metabolism [5] or respiration and glycolysis [24, 1, 31, 22].

Another human neoplastic tissue being used consists of the cells of cancer of the nasopharynx - KB - isolated by Eagle in 1955 [12]. HeLa, as well as KB cells, despite numerous passages performed during many years, have not lost their malignancy. It may be assumed that the type of metabolism of these tissues approaches that of tumours.

Among normal animal cells, the heart muscle cells of *Macacus cynomolgus* were adapted by Salk & Ward [26] in 1957 to growth on glass. Like the HeLa and KB cells, these cells constitute a stable tissue line which has undergone the process of transformation. The process of transformation, which has been often described in tissue cultures, and which is manifested by a change of the morphologic and biologic properties, leads to the similarity of the normal cells to the neoplastic ones [23]. The metabolism of normal cells after transformation may show more characteristics in common with the neoplastic cells than with the maternal cells. For this reason *Macacus cynomolgus* cells, in spite of their normal origin, are not suitable as a comparison system for true neoplastic cells. Cells from primary cultures of non-neoplastic origin cultivated on glass for only a short time (during two or three passages) are more suitable for this purpose, their metabolism being probably different from that of neoplastic and transformed cells.

MATERIAL AND METHODS

Material. The material used is presented in Table 1. Cells of the HeLa, KB and *Macacus cynomolgus* lines were obtained from the Institute of Marine and Tropical Medicine in Gdańsk. Canine kidney cells were isolated from the cortical part of kidneys of 2 to 4-month-old puppies. Human fibroblasts were secured from embryos removed in the first weeks of pregnancy because of social indications in the Ist Gynaecological and Obstetric Clinic of the Medical School in Kraków. Liver tumours in rats were induced by means of p-dimethylaminoazobenzene [Weber, 30]. Primary cultures were made as described by Youngner [32].

Cells of the tissue lines and primary cultures were grown on the surface of Roux flasks in EEC_{20} nutrient medium composed of Eagle's basal medium (Microbiological Associates, Bethesda) [10, 11], Earle's buffer solution [13], and with 20% of calf serum added. To each 100 ml.

Table 1

Classification of the experimental material

Tissue cultures					Slices	
Transformed tissue lines		Primary cultures			Neoplastic	Non-neoplastic
Neoplastic		Non-neoplastic				
Human		*Macacus cynomolgus*	Canine	Human	Rat	
HeLa cells, cancer of the uterine cervix	KB cells, cancer of the nasopharynx	Myocardium cells	Renal cortex cells	Embryonic fibroblasts	Hepatoma	Normal liver

of the medium 100 units of penicillin, 100 μg. of streptomycin and phenol red as indicator were added. The same Eagle's basal medium solution was employed throughout the experiments, assuring the uniformity of the medium. During incubation of the cultures the medium was replaced in 3-4-day-intervals depending on the change of colour of the indicator from red to salmon or yellow. An 0.25% solution of trypsin (Nutritional Biochemical Co.) [9] was used for isolating the cells from tissues when inoculating the primary cultures, and for separating the cells for subcultures.

Cells were scraped mechanically from the surface of the glass, suspended in Krebs-Ringer phosphate solution, washed three times to remove traces of organic substances, and then used for experiments. Fragments of tumours and normal liver after weighing were cut into thin transparent slices and used for the determinations.

Oxygen consumption. The manometric method of Warburg [28] was used. The determinations were carried out in the air at 38° in the presence of KOH. Two series of determinations were always made. In the first one, glucose dissolved in Krebs-Ringer phosphate solution (0.5 ml.) was added, and in the second series (control), Krebs-Ringer solution alone. The final concentration of glucose was 10 mM except when otherwise stated.

After adjusting the temperature, the readings of endogenous oxygen consumption were made. Then the substrate was added from the side-

-arm and further readings were made in 5 min. intervals. The mean oxygen consumption in µl. per hour was calculated in relation to 1 mg. of dry weight of the material or 10^6 cells.

Table 2

Dry weight of the cells

Material	mg./10^6 cells, ±S.D.
HeLa	1.22±0.1
KB	1.51±0.11
M. cynomolgus	1.20±0.09
Canine kidney cells	0.36±0.05
Embryonic fibroblasts	0.45±0.05

The number of cells in the suspensions was determined with a Buerker haemacytometer. The dry weight of cells was determined after washing the suspensions with physiological saline solution to remove traces of solids dissolved in the medium, and drying to constant weight. The relation between the dry weight and the number of cells is presented in Table 2. The dry weight of the cells of tissue lines, i.e. of the neoplastic and transformed cells, is approx. three times greater than the dry weight of cells in primary cultures.

RESULTS

Table 3 illustrates the average endogenous oxygen consumption, calculated per one million cells per hour, measured during the first 20 min. Calculation in relation to the number of cells showed greater oxygen consumption (12.7 - 17.6 µl. O_2) in the cells of transformed and neoplastic tissue lines in comparison with the non-neoplastic cells of primary cultures (8.5 and 10.2 µl. O_2). However, a different relation was found when the results were calculated in relation to dry weight. As a result of the larger dimensions of the neoplastic cells, cells of the tissue lines consumed less oxygen per g. (10.8 - 14 µl. O_2) than the cells of non-neoplastic origin (18.8, 28.3 µl. O_2). The respiration of tumour slices was also smaller than that of normal liver tissue. As the cells in slices had smaller contact with the incubation medium than cells from tissue lines suspended in the medium, the results may be interpreted only in terms of general trends, and can not be used for direct comparison.

Table 3

Endogenous oxygen consumption

Cell suspensions and tissues in Krebs-Ringer phosphate solution, pH 7.4. The results are expressed as average values of readings during the first 20 min. of the determinations, ± S. D.

Material	Number of determinations	$\mu l.O_2/10^6$ cells/hr.	$\mu l.O_2$/mg. of dry wt./hr.
		Time 0 - 20 min.	
HeLa	16	12.7±3.1	10.8
KB	16	17.6±3.8	11.7
M. cynomolgus	16	16.8±3.3	14.0
Embryo	6	10.2±2.1	28.3
Canine kidney	6	8.5±1.9	18.8
Liver slices	6	—	9.0±1.8
Hepatoma slices	6	—	8.4±1.9

The effect of glucose on respiration is presented in Table 4. All cells cultivated in tissue cultures displayed a diminished uptake of oxygen when glucose was added. A similar phenomenon was observed in slices of hepatoma. Normal liver slices were the only material in which oxygen consumption was not inhibited by glucose. The difference in oxygen uptake before and after addition of glucose amounted to about 40%. However, in control series, to which only Krebs-Ringer phosphate buffer was added from the side-arm, a certain degree of inhibition was also found, due to slower diffusion of gas to the incubated cells as the final volume of liquid was higher by 50%. When corrections in the calculations, based on the control series are taken into account, the glucose--induced Crabtree effect appears to be much smaller. Although the difference between oxygen consumption without glucose and in the presence of glucose was not very large, it was nevertheless always reproducible. The inhibition of oxygen consumption 40 min. after adding glucose was approx. 20% in the neoplastic material (HeLa, KB, and tumour slices), and approx. 10% in the non-neoplastic cells (embryonic fibroblasts, canine kidney cells). The effect of glucose observed in non-neoplastic cells indicates that the Crabtree effect can be observed in tissue cultures already at an early stage, after one or two subcultures of the primary culture.

The course of endogenous oxygen consumption and its inhibition by glucose is illustrated for HeLa cells in Fig. 1. Inhibition of oxygen uptake appears very quickly, already 5 min. after the addition of glucose.

Table 4

Effect of glucose on oxygen uptake

After 20 min. of incubation, and O_2 uptake measurement, the Krebs-Ringer phosphate solution alone (K.R.) was added from the side-arm (control series) or glucose in Krebs-Ringer phosphate solution.

Material	Addition after 20 min.	Number of determinations	μl. $O_2/10^6$ cells/hr.			Inhibition of O_2 uptake 40 min. after addition of glucose
			Time (min.)			
			0-20	20-60	60-120	
HeLa	K.R.	16	12.7±3.1	10.7±2.9	8.8±2.9	
	Glucose	16	12.7±3.1	8.2±2.2	7.6±2.4	23
KB	K.R.	16	17.6±3.8	15.8±3.3	16.1±3.1	
	Glucose	16	17.6±3.8	12.2±2.9	13.5±3.0	23
M. cynomolgus	K.R.	16	16.8±3.3	14.6±2.9	15.9±3.2	
	Glucose	16	16.8±3.3	12.9±2.8	14.0±3.0	11.6
Embryo	K.R.	6	10.2±2.1	8.7±2.0	9.3±2.0	
	Glucose	6	10.2±2.1	7.6±1.8	7.6±1.8	13
Canine kidney	K.R.	6	8.5±1.9	7.5±1.3	7.2±1.6	
	Glucose	6	8.5±1.9	6.4±1.4	6.3±1.3	14.5
			μl.O_2/mg. dry wt./hr.			
Rat hepatoma	K.R.	6	8.4±1.9	6.4±1.3	6.0±1.2	
	Glucose	6	8.4±1.9	4.8±1.1	4.8±1.0	23
Rat liver	K.R.	6	9.0±1.8	7.6±1.3	6.3±1.2	
	Glucose	6	9.0±1.8	7.8±1.3	6.6±1.2	0

The decrease of the values corresponding to the oxygen uptake may be the result of disturbed equilibrium between the liquid and gas phases after adding the content of the side-arm. The disturbed equilibrium is regained after a few minutes, but the inhibition continues. The highest respiration in the studied tissues was observed during the first 20 min.; later, until the end of the first hour, oxygen consumption remained stable, or even declined as a result of exhaustion of endogenous organic substances.

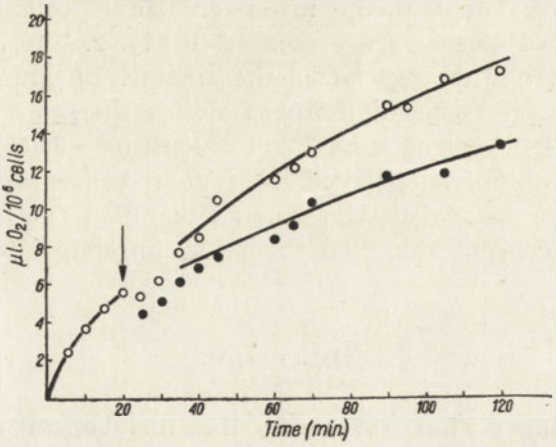

Fig. 1. The effect of glucose on the oxygen uptake by HeLa cells. (O), Endogenous respiration, (●), after adding glucose. The arrow indicates the addition from the side-arm

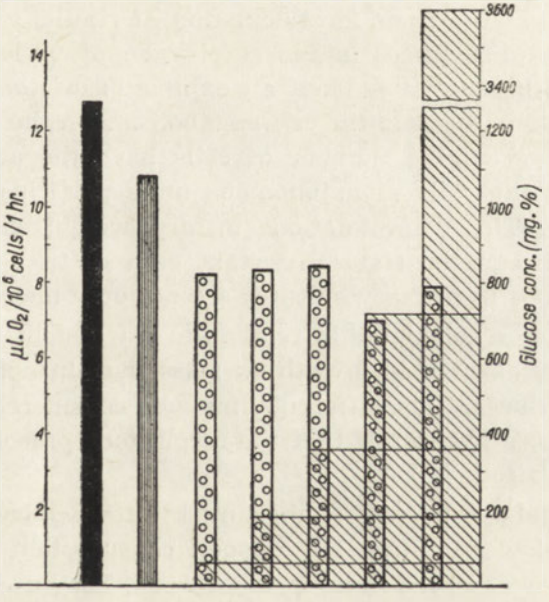

Fig. 2. Inhibition of oxygen uptake by HeLa cells in the presence of different concentrations of glucose. Black column, endogenous oxygen uptake (initial); vertically striated column, endogenous oxygen uptake after adding Krebs-Ringer phosphate solution (control); columns with points, oxygen uptake after adding glucose; diagonally striated area, concentration of glucose in the vessel (mg. %)

The results of experiments made on HeLa cells with different concentrations of glucose are presented in Fig. 2. The intensity of the Crabtree effect did not depend on the amount of glucose added. The inhibition of oxygen consumption was always the same with concentrations of glucose increasing from 3.3 to 200 mM (60 - 3600 mg.%). A somewhat greater inhibition observed at higher concentrations of glucose (40 and 200 mM) may be explained as a side-effect due to osmotic action of high sugar concentrations on the cells, unrelated to the Crabtree effect.

DISCUSSION

Tissue cultures allow to study the metabolism of living cells, including human neoplastic cells. Of course, conclusions from studies of cultivated tissues cannot be applied without restriction to tissues in the living body, since adaptation to growth on glass markedly modifies certain characteristics and properties of the cells. However, comparative studies can demonstrate the analogies or the differences in material derived from cultures or from tumours and normal tissues of the living body.

Dry weight used as the basis for calculation of results gives a more exact picture of the differences in the respiration of various cultures and tissues studied, because it is itself an expression of the content of organic substances taking part in the cell metabolism. On the other hand, the calculations based on the number of cells have the advantage of taking into account the cells as autonomous units participating in the studied phenomena. The determinations of dry weight demonstrated that differences between the stable, resistant cells of tissue lines, and the delicate small cells in primary cultures are not only microscopic.

Oxygen consumption calculated in relation to dry weight was smaller in tissue lines stably adapted to growth on glass than in non-neoplastic cells of primary cultures. This difference may be considered as a confirmation of Warburg's hypothesis that the respiratory processes in neoplastic cells are impaired.

Respiration of HeLa cells was studied by Philips & McCarthy [24], who found much lower values for oxygen consumption (5.0 - 6.3 µl. O_2/mg./hr.) than those observed in the present work. They used, however, cells cultivated in much less favourable conditions. To estimate the respiration they employed cells suspended in an incubation medium which contained organic compounds, and therefore no values for endogenous respiration were given. The respiration undoubtedly was decreased by the presence of glucose, which explains the lower values

observed by these authors. They studied also aerobic and anaerobic glycolysis and did not observe the Pasteur effect in HeLa cells.

The respiration of HeLa cells was studied also by Angelucci & Nava [1], although also in a medium containing organic substrates. Our results in the presence of glucose are in agreement with the respiration values found by these authors. Recently Wu [31] has investigated the respiration and glycolysis of HeLa cells; he differentiated the endogenous respiration and respiration in the presence of substrates. The cells of other tissues studied in the present work have not previously been the object of biochemical observations, especially those pertaining to respiration and the Crabtree effect.

The inhibition of oxygen consumption by glucose was first observed in slices of neoplastic tissue by Crabtree [7] and confirmed by other authors [8, 14, 20, 27]. Since the Crabtree effect has also been found in suspensions of Ehrlich's ascites carcinoma cells, in which it is especially clearly manifested (40 - 50% inhibition of oxygen uptake) [15, 19], it can be considered a characteristic feature of neoplastic cells. The demonstration of the Crabtree effect in tissue cultures of typical neoplastic cells, such as HeLa and KB, is a further confirmation of this effect.

The finding that oxidation is inhibited by glucose also in normal animal cells adapted to growth on glass (such as *M. cynomolgus* cells) was, however, rather unexpected. Altogether unforeseen was the finding of the Crabtree effect in primary cultures of normal animal cells not yet stably adapted to growth on glass. Assuming that the Crabtree effect is a characteristic feature of tumours, it could be concluded that the conditions of primary culture alter rapidly the metabolism of normal cells which acquire the properties of neoplastic cells. Hence, from the biochemical point of view, normal cells after transformation and complete adaptation to growth on glass, are the equivalent of neoplastic cells.

This conclusion coincides with that of Warburg *et al.* [29] who showed that already after 24 - 48 hr. of cultivation of embryonic chick cells on glass, their metabolism displays properties typical for neoplastic cells: their oxygen consumption is diminished and aerobic glycolysis increases.

In experiments on Ehrlich's ascites tumour cells it was observed that the intensity of the Crabtree effect is independent from the glucose concentration changing in a wide range [4, 3]. Our experiments with HeLa cells confirm these observations. Apparently, the degree of the Crabtree effect depends on the quantity of sugar permeating through the cell membrane into the cytoplasm; this in turn is regulated by factors governing the permeability of cells and initiating the first stage of the metabolism of sugar.

The authors are gratefully indebted to Professor Dr. B. Skarżyński for his interest in this work and for valuable advice.

SUMMARY

1. Respiration and the Crabtree effect were studied on human neoplastic cells (HeLa and KB) and non-neoplastic cells of *Macacus cynomolgus* from tissue culture. In addition to these cells, which are stably adapted to growth on glass, primary cultures of canine kidney cells, fibroblasts from human embryos, slices of normal rat liver and from hepatoma were also studied.

2. Endogenous consumption of oxygen per g. of dry weight was less in the neoplastic than in the normal material.

3. The Crabtree effect, which is characteristic of neoplastic material, was observed also in normal transformed cells *(M. cynomolgus)* and in primary cultures (canine kidney cells and embryonic cells) even soon after the initiation of the cultures.

4. Adaptation to growth on glass modifies the metabolism of normal cells into a type characteristic for neoplastic tissues.

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BADANIA NAD EFEKTEM CRABTREE

I. METABOLIZM GAZOWY HODOWLI TKANKOWYCH

Streszczenie

1. Badano oddychanie i efekt Crabtree w komórkach z hodowli tkankowych: w komórkach nowotworów ludzkich linii HeLa i KB oraz w komórkach nienowotworowych linii *Macacus cynomolgus*. Poza tymi trzema typami trwale zaadaptowanymi do wzrostu na szkle badano również hodowle pierwotne komórek nerki psa i fibroblastów zarodka ludzkiego oraz skrawki prawidłowej wątroby szczura i wywodzącego się z niej guza.

2. Endogenne zużycie tlenu w przeliczeniu na jednostkę suchej masy było niższe w materiale nowotworowym niż w normalnym.

3. Efekt Crabtree, jako jedna z cech materiału nowotworowego, obserwowany był nie tylko w typowych tkankach nowotworowych, lecz również w komórkach prawidłowych transformowanych (*M. cynomolgus*) i w hodowlach pierwotnych wkrótce po ich założeniu (komórki nerki psa i zarodka).

4. Adaptacja do wzrostu na szkle zmienia metabolizm komórek prawidłowych na typ przemian cechujący tkanki nowotworowe.

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JADWIGA PURZYCKA

AMP AND ADENOSINE AMINOHYDROLASES IN RAT TISSUES

Department of Biochemistry, Medical School, Gdańsk

AMP aminohydrolase was first prepared from rabbit muscles by G. Schmidt in 1928 [26]. This preparation, however, showed all properties of myosin including ATPase activity. Engelhardt [10] separated partly the AMP aminohydrolase from ATPase, and in 1957 Lee [15] obtained crystalline AMP aminohydrolase from muscles.

Adenosine aminohydrolase accompanies in many tissues the AMP aminohydrolase, it seems, however, to be more abundant in animal tissues than the latter [2, 12]. Both aminohydrolases have been separated from each other by Żydowo [32] in kidney preparations in a relatively simple way.

The present paper is concerned with the intracellular localization of AMP aminohydrolase, adenosine aminohydrolase and 5′-ribonucleotide phosphohydrolase (5′-nucleotidase) in rat tissues; an attempt was also made to separate these enzymes.

METHODS

Biological material. White rats were bled by heart puncture after a short chloroform anaesthesia. The tissues were removed immediately and put into ice-cold 0.1 M-KCl - 0.039 M-borate buffer, pH 7. Then the tissues were weighed, rinsed and homogenized with 7.5 volumes of KCl--borate solution in a Potter-Elvehjem homogenizer.

Whole kidneys, spleen, lungs, brain, testis and pancreas were homogenized, but only a part of liver (about 3 g.). From the heart muscle big vessels were removed. Skeletal muscle was taken from hind legs. The smooth muscle was obtained from stomach of animals fasted for 24 hr. The stomach mucosa was removed with a hard brush and the muscle cut into small pieces and homogenized. The intestine mucosa was also taken from fasted animals. Blood was taken by heart puncture into

a syringe moistened with heparin solution, and separated into plasma and cells by centrifuging.

The subcellular fractions were prepared as described by Żydowo [32]. The mitochondrial fraction sedimenting between 600 *g* and 16 000 *g*, and the supernatant, designated the cytoplasmic fraction, were used after dialysis against distilled water. If during dialysis of the cytoplasm a protein fraction was precipitated it was separated, redissolved in KCl solution and designated cytoplasmic globulins; the proteins remaining in the solution after dialysis were designated cytoplasmic albumins. Some insoluble protein remained after the globulin fractions were dissolved in KCl; this was probably denatured protein.

Enzymic incubations. AMP aminohydrolase and 5′-nucleotidase in subcellular fractions were assayed as described by Żydowo [32] except that 0.025 M-tris buffer, pH 7.2, was used instead of succinate buffer, pH 6.0. The samples containing 10 μmoles AMP and 200 μmoles KCl were incubated at 25° for 1 hr. The activity of adenosine aminohydrolase was determined according to Kalckar [11]. About 65 μmoles adenosine was dissolved per 1 ml. 0.1 M-phosphate, pH 7.0, and 3 ml. was added with a corresponding fraction containing 10 μg. of protein nitrogen. The decrease in extinction at 265 mμ was followed with SF-4 spectrophotometer for half an hour in 5 min. intervals.

Table 1

The recovery of standards after chromatography

Solution of one compound or a mixture of four compounds were chromatographed. Details see Methods. The figures represent the means from seven estimations, ± S. E.

Applied (μmoles)		Recovered (μmoles)	
		From a single standard	From a standard mixture
Adenosine	31	31.6±0.89	33.6±0.73
AMP	38	36.1±0.01	38.6±1.1
Inosine	27	28.4±2.45	27.5±0.56
IMP	23	25.9±0.57	23.6±0.6

Paper chromatography. The nucleosides and nucleotides present in deproteinized incubation mixtures were separated by one dimensional descending paper chromatography using saturated ammonium sulphate - - *iso*propanol - water (79 : 2 : 19, by vol.) according to Deutsch & Nilsson [6]. For quantitative determination of the reaction products the ultraviolet absorbing spots were cut out from the chromatogram, cut into small pieces and eluted with 5 ml. 0.1 N-HCl for 12 hr.; appropriate

blanks were cut out and eluted simultaneously. The extinctions of eluates were read with the SF-4 spectrophotometer at 250 mμ for inosine, and 260 mμ for adenosine derivatives. The amount of compound recovered was calculated by using molar extinction coefficient estimated by Deutsch & Nilsson [7]. The amount of product formed was calculated taking as 100 the sum of nucleotides recovered from the particular sample. This way of calculation assumes that the eventual loss of the separated compounds is proportional for all the nucleotides and nucleosides. The standard experiments presented in Table 1 supported this assumption.

Other analytical procedures. Protein nitrogen of dialysed subcellular fractions was estimated with Kjeldahl method in the Parnas-Wagner apparatus using $CuSO_4$ as catalyst for combustion and boric acid for ammonia binding.

In order to relate the enzymatic activities to the tissue weight, in some experiments the tissues were fractionated quantitatively and the protein nitrogen was estimated as above.

Reagents. AMP, adenosine, IMP, and tris(hydroxymethyl)aminomethane were L. Light & Co. Ltd. products. Tris has been recrystallized from ethanol before use. Other chemicals were obtained from Fabryka Odczynników Chemicznych, Gliwice.

RESULTS

By the determination of chromatographically separated reaction products the direct data on the deaminating enzymes in subcellular fractions of various rat tissues have been obtained. One of the typical chromatographic separations after incubation of cellular fractions is presented in Fig. 1.

Żydowo [32] demonstrated that in kidney AMP aminohydrolase is present in the cytoplasmic globulin fraction, while adenosine aminohydrolase in the albumin fraction. In the present work the same was found (Table 2) in liver, lungs, spleen and heart. The cytoplasm of stomach smooth muscle contained the activities of both aminohydrolases, but did not separate during dialysis into the two protein fractions. Also the cytoplasm of testis, pancreas and intestine mucosa did not separate during dialysis. In the cytoplasm of these tissues, however, only the activity of adenosine aminohydrolase was found, and no AMP aminohydrolase. In the mitochondrial fraction of all tissues investigated, except striated muscle, the activity of 5′-nucleotidase was found (Table 3). In the blood the activity of AMP aminohydrolase was present in cells; the plasma showed a slight 5′-nucleotidase activity.

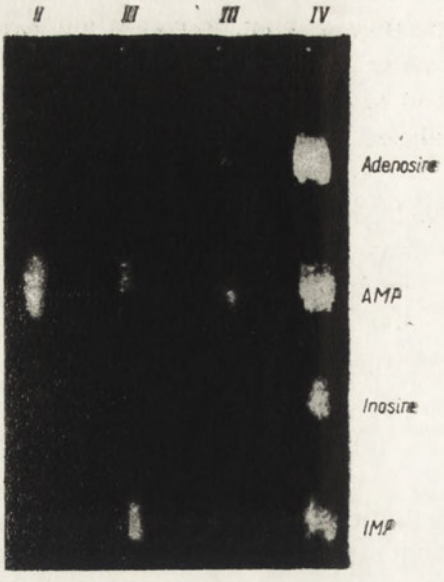

Fig. 1. The chromatogram representing the breakdown of AMP by spleen cell fractions. (I), Cytoplasmic albumins; (II), cytoplasmic globulins; (III), mitochondrial fraction; (IV), standards

Table 2

The deamination of muscle adenylic acid and adenosine by cytoplasmic proteins

The figures represent the means from 2—5 animals, ± S. E. AMP aminohydrolase activity was assayed by chromatography of the products after the incubation of 0.005 M-AMP in tris buffer, pH 7.2, at 25° for 60 min. Adenosine aminohydrolase activity was assayed according to Kalckar [13]

Tissues	Cytoplasmic globulins		Cytoplasmic albumins	
	AMP aminohydrolase (μmoles IMP/mg. protein-N)	Adenosine aminohydrolase (μmoles inosine/mg. protein-N)	AMP aminohydrolase (μmoles IMP/mg. protein-N)	Adenosine aminohydrolase (μmoles inosine/mg.protein-N)
Striated muscle	>110*	0	>110*	0
Smooth muscle (stomach)	no globulins		13±2	27.1 ±5.1
Heart muscle	50±2.8	0	0	5.31±0.37
Kidney	17±2.2	0	0	11.4 ±0.84
Liver	5±0.4	0	0	3.03±0.53
Lungs	22±1.3	0	0	15.4 ±1.64
Spleen	20±0.8	0	0	27.1 ±2.37
Testis	no globulins		0	7.92±0.34
Pancreas	no globulins		0	6.43±1.7
Intestinal mucosa	no globulins		0	31.56±0.82
Red blood cells	13±2.5	0	0	
Brain	undialysed cytoplasm		18±2.5	

* The activity was so high that under experimental conditions the whole AMP in the sample (10 μmoles) has been deaminated to IMP.

It is known from papers of Muntz [17, 18] and Weil-Malherbe [28, 29] that the brain aminohydrolase is active in the presence of catalytic amounts of ATP only. A fresh, undialysed brain cytoplasm split off ammonia from AMP (Table 2), after dialysis, however, no deaminating activity was found.

The skeletal muscle differs very much from other tissues. A very high activity of AMP aminohydrolase was localized in the globulin fraction of the cytoplasm, in the mitochondrial fraction and in myofibrils (sedimenting at 600 *g*). The activity of AMP aminohydrolase in skeletal muscle exceeded markedly that in all other tissues, which is a fact known since a long time [26, 5]. Yet no fraction of the skeletal muscle had any adenosine aminohydrolase activity, or any enzyme splitting off phosphate from AMP.

Table 3

The dephosphorylation of AMP by the mitochondrial fractions

The incubation was carried out at 25° in 0.025 M-tris buffer, pH 7.2, for 60 min. The figures represent the means from 2-5 animals, ± S. E.

Tissue	Adenosine produced (μmoles/mg. protein-N)
Striated muscle	0
Smooth muscle	13.7 ±0.6
Heart muscle	25.4 ±3.99
Kidney	40.4 ±0.35
Liver	16.5 ±3.45
Lungs	23.3 ±1.1
Spleen	7.72±1.25
Testis	> 66.6*
Pancreas	5.67±0.86
Intestinal mucosa	33.0 ±9.3
Plasma	0.7 ±0.05

* The activity was so high that under experimental conditions the whole AMP in the sample (10 μmoles) has been dephosphorylated to adenosine.

The results presented in Tables 2 and 3 are expressed in relation to 1 mg. of protein nitrogen of the fraction in which the enzyme is present. Since in this way the total activity of an organ could not be evaluated, the distribution of protein nitrogen in the subcellular fractions of the tissues has been determined (Table 4). It allowed to calculate the

Table 4

The distribution of protein nitrogen in subcellular fractions

Values in mg. per 1 g. of fresh tissue. ± S. E.

Tissue	Homogenate	Albumin	Globulin	KCl-insoluble globulin	Nuclei	Mitochondrial fraction	Sum of the fractions
Smooth muscle	15.22±1.48	3.79±1.01		—	5.1 ±0.33	1.53±0.29	10.47±1.58
Heart muscle	23.5 ±2.35	3.17±0.1	2.69±1.5	2.03±0.4	11.8 ±2.3	2.44±0.24	21.64±1.84
Kidney	22.8 ±0.66	3.59±0.1	2.46±0.73	2.9 ±0.05	6.66±0.05	3.86±1.57	19.48±0.68
Liver	21.4	5.35±0.1	3.71±0.12	2.38±0.14	6.31±0.63	5.53±0.55	23.29±1.1
Lungs	18.4 ±2.6	3.08±0.8	1.8 ±0.74	2.36±0.68	8.1 ±0.7	1.9 ±0.16	17.75±2.6
Spleen	27.9 ±1.9	3.64±0.6	4.11±0.14	3.27±1.4	6.07±0.46	3.86±0.05	20.96±0.52
Testis	13.4 ±0.05	6.15±0.2		—	3.13±0.07	2.49±0.15	11.78±0.28
Pancreas	22.4 ±1.8	7.4 ±1.6		3.52±1.08	3.69±0.02	6.6 ±1.0	21.2 ±0.51
Intestinal mucosa	6.41±0.56	2.71±0.51		—	1.57±0.45	1.53±0.02	5.82±0.97
Blood, whole	29.24±0.44						
erythrocytes		10.86±0.26	8.25±0.65	7.09±0.49			31.29±0.49
plasma		5.09±0.06					
Brain, undialysed preparations	13.58±2.88	4.16±0.69		—	8.45±0.3	1.3 ±0.13	13.93±1.1

Table 5

The content of the enzymes decomposing adenylic acid in various rat tissues

Details see Methods. The values are calculated from Tables 2, 3 and 4. The figures represent the means from 2-5 animals, ± S.E.

Tissue	AMP aminohydrolase (μmoles IMP/g. of fresh tissue)	Adenosine aminohydrolase (μmoles inosine /g fresh tissue)	5′-Nucleotidase (μmoles adenosine/g. of fresh tissue)
Striated muscle	400.0*	0	0
Smooth muscle	49.3±13.1	102.7±37.3	20.9 ± 3.9
Heart muscle	89.3±30.3	16.8± 0.5	62 ± 6
Kidney	41.8±12.6	41.0± 1.2	155.2 ±62.8
Liver	27.6± 2.2	16.2± 0.3	91.25± 7.25
Lungs	39.6±13.3	47.4±12.3	44.25± 3.75
Spleen	82.2±28	98.7±16.2	29.8 ± 0.01
Testis	0	48.6± 1.6	>166*
Pancreas	0	47.5±10.3	37.3 ± 5.7
Intestinal mucosa	0	85.5±16	50.75± 0.75
Blood	107.2± 8.7	0	3.57± 0.05
Brain, undialysed cytoplasm	75.5±12	0	0

* See the footnotes under Tables 2 and 3.

activities per gram of tissue (Table 5). Skeletal muscle apart, blood contains the highest amount of AMP aminohydrolase. The testis possesses the highest AMP dephosphorylating activity.

DISCUSSION

By differential centrifugation and dialysis it is possible to separate the three enzymes breaking down AMP. This has been done by Żydowo on the kidney [31, 32]; in the present work the same was achieved using heart muscle, liver, lungs and spleen. In these organs AMP aminohydrolase was localized in cytoplasmic globulins, and adenosine aminohydrolase was found in the water soluble part of cytoplasm.

Till now almost all the investigations concerning AMP aminohydrolase were carried out in an indirect way [5, 14] and only indirect conclusions on the sequence of the breakdown of the adenylic acid in the organism could be drawn. Not in every tissue it is easy to separate AMP aminohydrolase from adenosine aminohydrolase, but even where the two enzymes are difficult to separate the chromatographic analysis

of the reaction products shows the presence of the AMP aminohydrolase and may be also used for the quantitative determination of its activity.

The activity of AMP aminohydrolase in skeletal muscle is incommensurably high as compared with all other tissues. It seems that skeletal muscle may be responsible in the highest degree for the production of IMP and ammonia from AMP. On the second place is the blood (Table 5). If we assume that a rat weighing 200 g. contains 20 g. of blood, then the potential ability of the whole blood of the animal to deaminate AMP amounts to about 2000 μmoles per hour. Analogous values for liver, kidney, heart muscle, spleen and lungs show that each of these organs would be able to produce 70 - 100 μmoles of IMP and ammonia from AMP during one hour, that is about 20 times less than the blood.

These comparative calculations were based on rather arbitrary assumptions and did not take into consideration such factors as optimal conditions *in vivo* which may be different from those applied for the determination of enzyme activity *in vitro*. Also the concentration of nucleotide used as substrate was higher than that found in rat tissues [4]. However, a fast incorporation of isotopic nitrogen *in vivo* into the amino group of AMP [20], 5 times faster than the incorporation into glutamic acid [13], permits to suppose that the deamination of AMP *in vivo* is very fast, too.

The distribution of AMP aminohydrolase and adenosine aminohydrolase in rat tissues found in this work does not agree with the results of Conway & Cooke [5] on rabbits. These authors reported that in kidney, liver, intestine mucosa, smooth muscle and heart muscle AMP was deaminated after dephosphorylation only, and that the direct deamination of AMP took place in skeletal muscle, nervous tissue, heart auricle and in red blood cells only. The authors had taken as the measure of the direct deamination the difference between the ammonia produced from muscle AMP and that produced from yeast AMP, or the difference of the amount of ammonia produced by the tissue from the mixture of adenosine and AMP, and the ammonia produced from adenosine under the same conditions. Both ways enabled to draw only indirect conclusions about the sequence of AMP breakdown.

Kutscher & Sarreither [14] have estimated the activity of AMP aminohydrolase in striated muscle, heart, spleen, kidney, liver and lungs, but they did not take into consideration the activity of adenosine aminohydrolase, or 5′-nucleotidase which has been shown by Reis [23] to be present in rat tissues.

5′-Nucleotidase, the third enzyme investigated in this work, dephosphorylated AMP [24, 25, 1]. In all rat tissues studied, this enzyme was present in the mitochondrial fraction in an overwhelming amount

(Table 3). The mitochondrial fractions obtained were certainly not homogeneous and contained not only mitochondria but also lysosomes containing different lytic enzymes. Although Novikoff [21] regards 5′-nucleotidase as a specific enzyme which may be separated from non-specific phosphatases, it is not quite sure whether the dephosphorylating activity observed should not be ascribed, at least partly, to the action of acid or alkaline phosphatases, which still show a low activity at pH 7.2 [32].

The enzymes deaminating adenosine derivatives are present not only in the eleven animal tissues investigated. It is known that they are also present in different parts of bone [3, 16], in chorioid plexus of the eye and of the brain [27], and may be also in other parts of the organism.

Nevertheless our knowledge on the biochemical role of these enzymes is until now rather scarce. Embden [8, 9] and later Parnas and co-workers [22] had suggested that AMP aminohydrolase is responsible for ammonia production in the working muscle. The participation of the adenosine derivatives in the production of ammonia from amino acids seems to be probable as Newton & Perry [19] and Yefimochkina & Braunstein [30] have reported on the reamination of IMP by aspartic acid in skeletal muscle.

The author expresses her gratitude to Doc. Dr. Mariusz Żydowo for suggesting the subject of this work and for helpful discussion, and thanks Mrs. Regina Kaczorowska for her technical assistance.

SUMMARY

1. Activity and intracellular distribution of AMP aminohydrolase, adenosine aminohydrolase and 5′-nucleotidase have been studied in rat tissues.

2. In cytoplasm of kidney, heart muscle, liver, lungs, spleen and smooth muscle (stomach) the activity of both aminohydrolases has been found. The cytoplasm of testis, pancreas and intestine mucosa showed only adenosine aminohydrolase activity. Blood cells contained only AMP aminohydrolase.

3. During dialysis of cytoplasm of kidney, liver, lungs, heart muscle and spleen against distilled water the precipitated globulin fraction contained the AMP aminohydrolase activity. The adenosine aminohydrolase remained in the solution.

4. The greater part of 5′-nucleotidase was found in the mitochondrial fraction. In blood plasma a very low activity was found.

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AMINOHYDROLAZY AMP I ADENOZYNY W TKANKACH SZCZURA

Streszczenie

1. W tkankach szczura badano aktywność i rozmieszczenie wewnątrz komórki aminohydrolazy AMP, aminohydrolazy adenozyny oraz 5′-nukleotydazy.

2. W cytoplazmie nerki, mięśnia sercowego, wątroby, płuc, śledziony i mięśnia gładkiego (żołądek) znaleziono aktywność obu aminohydrolaz.

Cytoplazma jąder, trzustki i śluzówki jelita ma aktywność tylko aminohydrolazy adenozyny. Krwinki czerwone mają jedynie aktywność aminohydrolazy AMP.

3. W czasie dializy wobec wody destylowanej z cytoplazmy nerki, wątroby, płuc, mięśnia sercowego i śledziony wypada frakcja globulinowa posiadająca aktywność aminohydrolazy AMP. Aminohydrolaza adenozyny pozostaje w roztworze.

4. Aktywność 5′-nukleotydazy w przeważającej części znaleziono we frakcji mitochondrialnej. Osocze krwi wykazuje nieznaczną aktywność.

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T. CHOJNACKI and T. KORZYBSKI

BIOSYNTHESIS OF PHOSPHOLIPIDS IN INSECTS

III. THE INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO PHOSPHOLIPIDS OF *ARCTIA CAIA* MOTHS

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

Previous studies on phospholipids [5, 6] led us to the conclusion that the biosynthesis of lecithin and cephalin in insects seems to be similar to the scheme proposed by Kennedy and Weiss [19, 30]. In the present work the rates of incorporation of [^{32}P]orthophosphate into lecithin, cephalin, phosphatidyl serine and inositol phosphatide in *Arctia caia* moths were studied. The rate of biosynthesis of phosphorylcholine was estimated also, this being the main phosphorus compound in the acid soluble fraction in the moth. An attempt was also made to examine *in vivo* the rate of biosynthesis of lecithins differing in fatty acid radicals. Recent papers of Collins [7] and Harris & Robinson [17] suggest different incorporation rates of ^{32}P into lecithins with different fatty acid radicals. In our experiments no such relationship could be found.

EXPERIMENTAL

Materials

Insects. Arctia caia moths were bred in our laboratory from diapausing and non-diapausing caterpillars.

Special reagents. ^{32}P-labelled phosphoric acid or sodium orthophosphate distributed by Nuclear Research Institute (Warszawa) was of Polish or French origin. The solution used contained 30 μg. of P in the volume injected. It corresponded to 2 - 5 μc. The chemical composition of the solution injected was the same as that of 0.1 M-phosphate buffer (17.8 g. $Na_2HPO_4 \cdot 2H_2O$ + 20 ml. 1 N-HCl), pH being about 7.

Aluminium oxide "MFC", 100 - 200 mesh, Brockman activity 1 - 2 (Hopkin & Williams Ltd. England) was used for column chromatography of phospholipids.

o-Silicic acid, pure (Fabryka Odczynników Chemicznych, Gliwice) was used, after heating at 110° for 12 hr., for column chromatography of phospholipids. Silicic acid was mixed with Celite 545 (BDH).

Injection of [^{32}P]orthophosphate

Ten μl. portion of the solution of labelled orthophosphate was injected into the moth's fat-body by means of a needle-like drawn-out pipette made from glass capillary tube. In females the volume introduced was about 1.5% of the body weight and the amount of phosphorus introduced was about 2% of the total phosphorus content of the body. In males the respective percentages were about 1.5 times greater due to the smaller body weight and lower total phosphorus, the amount of phosphorus introduced being the same.

Moths were injected 12 - 24 hr. after eclosion. They were kept without food and water; after predetermined periods of time (up to 10 days) they were killed. The injection was without any detectable deleterious effect, the injected moths being able to live as long as controls, i. e. more than 10 days. The copulation between labelled and unlabelled individuals took place, and the resulting eggs could develop into a new generation.

Extraction of lipids

To obtain lipids freed from water-soluble phosphorus contaminants the extraction and purification procedure of Folch *et al.* [13] was adopted. Each moth (about 600 mg.) was ground in a mortar with 3 ml. of chloroform - methanol, 2 : 1 v/v (in the following, the ratios of solvent systems are always given on the volume base), homogenized with an additional 7 ml. portion of the same solvent in a Struer's blendor for 5 min. and filtered. The clear extract (10 ml.) was shaken with 0.9% NaCl (2 ml.), stored at 5°, or immediately centrifuged. The upper water - methanol layer was discarded. The lower chloroform layer containing lipids was freed from water-soluble, highly radioactive phosphorus compounds by washing with a mixture of chloroform - methanol - water (3 : 48 : 47) containing 0.02% $CaCl_2$, 0.017% $MgCl_2$ and 0.29% NaCl. Three or four washings were sufficient.

Analytical fractionation of phospholipids

The course of fractionation is presented in Table 1. The method of Dawson [10] was adopted for fractionation of phospholipids contained in the chloroform - methanol extracts. It consists (Table 1) in obtaining phosphoric water-soluble degradation products of phospholipids after a mild alkaline (alkali-labile phospholipids, fraction *A*), then a subsequent acid hydrolysis (alkali-stable and acid-labile phospholipids, fraction *B*), and the remaining phospholipids (alkali- and acid-stable, fraction *C*).

Table 1

Analytical fractionation of phospholipids

Chloroform-methanol extracts, washed, evaporated, dissolved in chloroform, petrol ether added, precipitate removed, the solution applied onto silicic acid column

SILICIC ACID COLUMN

$CHCl_3$ → Non-phosphorus lipids

Met-OH → Phospholipids

FRACTIONATION

Mild alkaline hydrolysis → Water-soluble products from **fraction** *A* (alkali-labile)

Paper chromatography, analysis of spots

GPChol from lecithins
GPEt from cephalins
GPSer from phosphatidyl serine
other from other phospholipids

Water-insoluble products

Mild acid hydrolysis → Water-soluble products from **fraction** *B* (alkali-stable and acid-labile)

GPChol from lecithin plasmalogens
GPEt from cephalin plasmalogens
other from other phospholipids

Water-insoluble products, **fraction** *C* (alkali- and acid-stable) e.g. sphingomyelin

Fraction *A* consists of those phospholipids which after mild alkaline hydrolysis give water-soluble phosphoric degradation products. For ex-

ample lecithins, cephalins etc. are thus split to form GPChol [1], GPEt etc., respectively. Fraction *B* consists of those phospholipids which after mild alkaline hydrolysis and the subsequent acid one give water-soluble phosphoric degradation products. In this step the plasmalogens, e. g. lecithin plasmalogens and cephalin plasmalogens, are split to form the corresponding diesters, GPChol and GPEt respectively. Fraction *C* consists of those phospholipids which are stable in mild alkaline and acid conditions, e. g. sphingomyelin.

Removal of non-phosphorus lipids. Before fractionation the non-phosphorus lipids were to be removed (Dawson [10]). For this purpose a modified Borgström [2] method was used. The washed lipid extract was evaporated to dryness. All evaporations were made under reduced pressure and below 50°. The residue was dissolved in 2 ml. of chloroform, then 3 ml. of petrol ether was added to eliminate the precipitated insoluble non-lipid material. The clear solution of lipids (4.6 ml., about 400 μg. of lipid P) was applied onto a column (0.8 × 2.5 cm.) of a mixture of 150 mg. of silicic acid and 100 mg. of Celite 545 previously washed with a few ml. of methanol, acetone, ethyl and petrol ether. Non-phosphorus lipids were removed by elution with 20 ml. of chloroform (Table 1). Thus about 80% of the amount of ester compounds as assayed by $FeCl_3$ - hydroxylamine [25] was removed, whereas total phospholipids remained on the column. The elution of phospholipids with 30 ml. methanol gave 97.4% recovery (average of 12 determinations). It seems that the loss did not result from a preferential adsorption; when, namely, the eluted phospholipids were rechromatographed on a similar column and eluted in the same manner, the recoveries in three successive adsorptions and elutions were the same.

Obtaining of phospholipid degradation products, and their fractionation. The phospholipid methanol eluate (Table 1) containing about 400 μg. of lipid P was evaporated to dryness and the residue dissolved in 0.8 ml. of carbon tetrachloride; then 7.5 ml. of ethanol, 0.65 ml. of water and 0.25 ml. of 0.8 N-NaOH were added and the mixture was placed in a water bath at 37° for 15 min. To neutralize the excess of alkali 0.4 ml. of ethyl formate was added and the mixture left in the water bath for the next 5 min.; then it was evaporated to dryness and the residue dissolved in 1 ml. of the upper and 2 ml. of the lower phase of the system *iso*butanol - chloroform - water (1 : 2 : 1.5). Water-soluble compounds resulting from the alkaline hydrolysis of fraction *A* phospholipids were contained in the upper phase and the unhydrolysed lipids in the lower one. After centrifuging, the upper phase was thor-

[1] Abbreviations used: GPChol, glycerylphosphorylcholine; GPEt, glycerylphosphorylethanolamine; GPSer, glycerylphosphorylserine.

oughly separated, and 1.6 ml. from the lower phase was hydrolysed at 37° with 0.8 ml. of 10% trichloroacetic acid for 30 min. with vigorous shaking. After cooling and mixing with 2 ml. of petrol ether the upper phase containing unhydrolysed lipids (fraction *C*) was pipetted off. The lower phase, containing water-soluble phosphorus products of the degradation of plasmalogens (fraction *B*) was washed twice with 4.5 ml. portions of chloroform - ether (1 : 4). The washings were added to the main petrol ether portion of fraction *C*.

Phosphorus compounds in the alkali hydrolysate of the fraction *A* were separated by two-dimensional chromatography (Dawson [10]) with solvent systems: *I*, phenol saturated with water - acetic acid - ethanol (100 : 10 : 12), *II*, methanol - 98% formic acid - water (80 : 13 : 7). To estimate glycerylphosphorylcholine and glycerylphosphorylethanolamine one-dimensional technique with solvent *I* was sufficient, for the remaining phosphorus compounds of fraction *A*, however, the respective chromatogram segments from solvent *I* had to be rechromatographed with solvent *II* as may be seen from Fig. 1.

The amount of plasmalogen phospholipids (fraction *B*) was so small that it prohibited any fractionation.

Preparative fractionation of phospholipids

For preparing choline-, ethanolamine-, and inositol phosphatide fractions the column chromatography on aluminium oxide was used (Table 2). The chloroform solution of lipids was applied onto a column (1 × 6 cm., 5 g.). Non-phosphorus lipids were removed by passing through 20 ml. of chloroform. Choline lipids were eluted with 40 ml. of chloroform - - methanol mixture (1 : 1) [26], cephalins with ethanol - chloroform - - water (5 : 2 : 1), and inositol phosphatides with ethanol - chloroform - - water (5 : 2 : 2) according to Hanahan [14].

To free the lecithins from other choline phospholipids and to fractionate them according to unsaturation degree, the choline lipid eluate from aluminium oxide column was evaporated under nitrogen, dissolved in chloroform and applied onto a silicic acid column [27] (0.8 × 8 cm., filled with 500 mg. of silicic acid mixed with 250 mg. of Celite 545). After passing through 20 ml. of chloroform, lecithins were eluted with chloroform - methanol (3 : 2); 2 ml. portions were collected at the rate of about 30 ml. per hour. The absence of phospholipids other than lecithins was checked chromatographically according to Lea *et al.* [21] on Whatman no. 3 MM paper impregnated with silicic acid and chloroform - methanol (4 : 1) as solvent, and according to Hörhammer *et al.* [18] on Whatman no. 1 paper impregnated with formaldehyde, the solvent

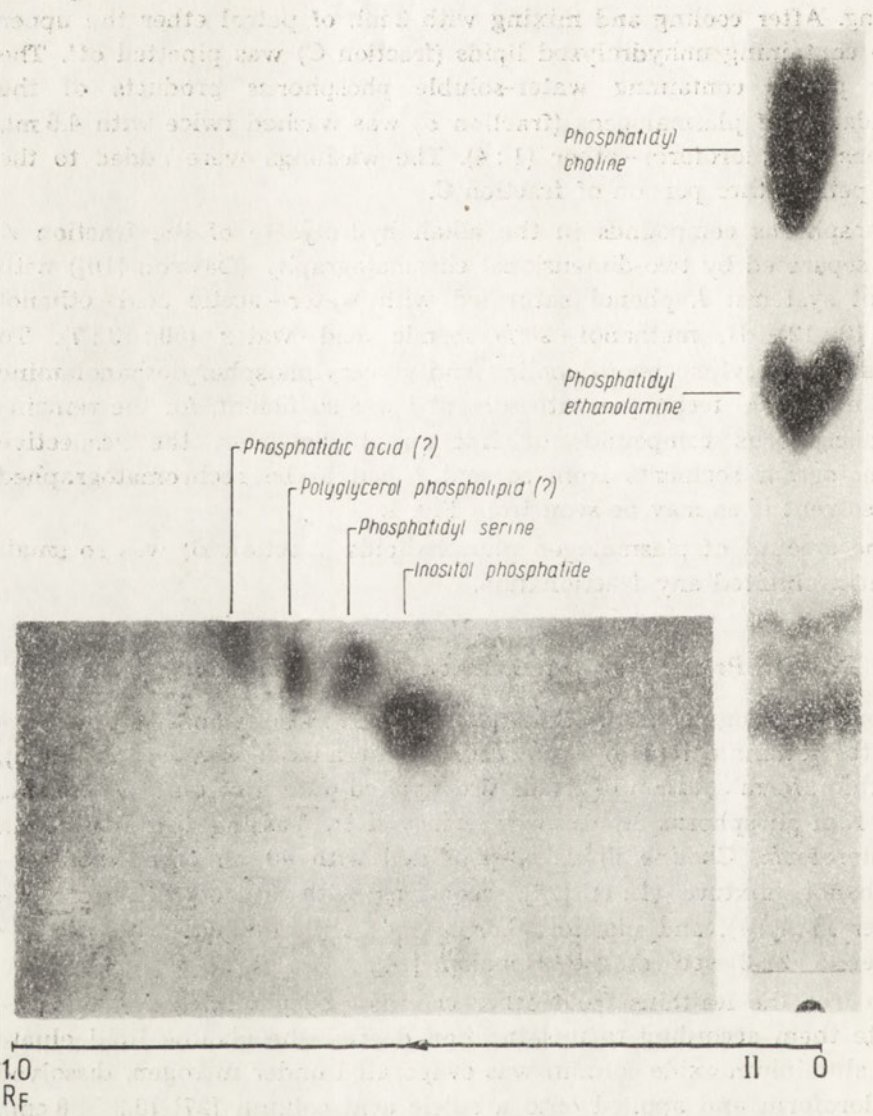

Fig. 1. Autoradiograms of paper chromatograms of water-soluble phosphorus derivatives of alkali-labile phospholipids (fraction *A*) of an *Arctia caia* male moth, 10 days after injection of [^{32}P]orthophosphate. On the Figure the names of parent phospholipids are marked. The ascending chromatogram (*I*) was developed with phenol saturated with water - acetic acid - ethanol (100 : 10 : 12). The lower segment of one-dimensional chromatogram was developed in the second dimension (*II*) with methanol - 98% formic acid - water (80 : 13 : 7)

being the upper phase of the mixture butanol - acetic acid - water (4 : 1 : 5). Phospholipid spots were detected by autoradiography and with Rodamine G solution according to Marinetti & Stotz [23]. The chromatographic analyses according to Dawson [11] were also performed.

Table 2

Preparative fractionation of phospholipids

Chloroform - methanol extracts, washed, evaporated, dissolved in chloroform, applied onto

ALUMINIUM OXIDE COLUMN

Solvent	Lipid fraction
$CHCl_3$	Non-phosphorus lipids
$CHCl_3$ - Met-OH (1:1)	Choline lipids [26]
Et-OH - $CHCl_3$ - H_2O (5:2:1)	Cephalins [14]
Et-OH - $CHCl_3$ - H_2O (5:2:2)	Inositol phosphatides [14]

↓

Choline lipids in $CHCl_3$ - Met-OH (1:1) evaporated (in N_2), dissolved in chloroform, applied onto

SILICIC ACID COLUMN

Solvent	Lipid fraction
$CHCl_3$ - Met-OH (3:2)	Lecithins* [15]
$CHCl_3$ - Met-OH (4:1)	Other choline lipids**

* Fractionated further according to the unsaturation degree [27], see Fig. 6.
** Traces only of lecithins were found.

The amounts of silicic acid used for chromatography of phospholipids were 2 - 3 times smaller than those given by other authors [2, 21, 15]. In our experiments the usual load was 2 mg. of phospholipid P per g. of silicic acid; a 100% adsorption of phospholipid has always been achieved, and 97% recovery was obtained.

Analytical procedures

Assay of phosphorus and ^{32}P. Phosphorus was assayed according to Strickland *et al.* [29], and the amount of ^{32}P with a VA-Z 410 VEB Vacutronic liquid counter. Samples of washed lipid extracts were di-

gested with 0.6 ml. of 60% perchloric acid. For assaying phosphorus and ^{32}P in lecithin, cephalin, phosphatidyl serine and inositol phosphatide the chromatographic spots of their hydrolytic degradation products were cut out, digested as above, and phosphorus and ^{32}P assayed. Samples with greater amounts of phosphorus were digested with 0.8 ml. of perchloric acid and phosphorus assayed with King's method [20].

Estimation of specific activity of phosphorylcholine. The fraction of water-soluble phosphorus compounds obtained with the method of Dawson [8] was passed through a column of Amberlite IRC 50 (H^+) and shaken with three volumes of *iso*butanol; the lower phase was chromatographed on paper in a two-dimensional system: *I*, phenol saturated with 0.1% NH_3, *II*, ethanol - conc. ammonia (d = 0.88) - water (61 : 29 : 10) [9]. The phosphorylcholine spot was cut out and its phosphorus and ^{32}P content determined.

Estimation of double bonds. This was estimated according to Yasuda [32] in purified lecithins obtained from silicic acid column.

Identification of phospholipids

Lecithin. In the chromatogram of alkali-labile fraction *A* the spot of glycerylphosphorylcholine was found in the presence of lecithin in the fraction. The eluate of the spot after 15 min. hydrolysis in 1 N-HCl contained free choline on chromatograms developed with water-saturated phenol [28]. The phospholipid of *Arctia caia* which gave glycerylphosphorylcholine after mild alkaline hydrolysis, did not exhibit any difference from hen's egg lecithin when chromatographed according to Lea *et al.* [21] or to Hörhammer *et al.* [18]. When eluted from silicic acid or aluminium oxide columns it behaved in a way similar to lecithin.

Cephalin. The glycerylphosphorylethanolamine spot in the chromatogram of alkali-labile fraction *A* indicated the presence of cephalin in the fraction. In the eluate of the spot after 15 min. hydrolysis in 1 N-HCl free ethanolamine was detected with 0.2% acetone solution of ninhydrin on chromatograms developed with water-saturated phenol. The phospholipid which after mild alkaline hydrolysis gave glycerylphosphorylethanolamine behaved in the same way as cephalin in Hanahan's [14] procedure of column chromatography on aluminium oxide.

Phosphatidyl serine. In examining the products of mild alkaline hydrolysis of fraction *A* a ninhydrin positive spot, R_F 0.54, in the system methanol - 98% formic acid - water (80 : 13 : 7) indicated the presence of phosphatidyl serine in the extracts. R_F was identical with that observed by Dawson [10] for glycerylphosphorylserine. In the eluate of this spot after 1 hr. hydrolysis in 1 N-HCl free serine was detected with 0.2%

acetone solution of ninhydrin on chromatograms developed with water-saturated phenol.

Inositol phosphatide. In examining the products of mild alkaline hydrolysis of fraction *A*, the chromatographic spot of glycerylphosphoryl-inositol + phosphorylinositol indicated the presence of inositol phosphatide. Inositol was found chromatographically in the eluate of the spot after 12 hr. hydrolysis in 1 N-HCl; the hydrolysate was neutralized by passing it through a column with Dowex-1 (OH^-), chromatographed on Whatman no. 1 paper with propanol - ethanol - water (50 : 30 : 20) [16], and free inositol detected with the method of Trevelyan *et al.* [31]. The elution of inositol phosphatide from an aluminium oxide column was similar to that of phosphatidyl inositol from rat liver in the study of Hanahan *et al.* [15].

RESULTS

Incorporation of ^{32}P into different phospholipids

In 10-day experiments with injected moths the amount of body lipid phosphorus decreased (Fig. 2a). Apparently it was proportional to the concomitant decrease of body weight which was observed during the experiment. The specific activity of lipid phosphorus increased slowly (Fig. 2b).

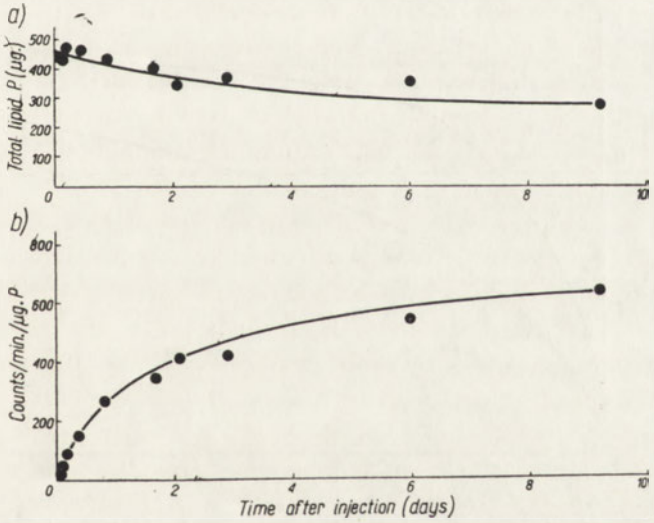

Fig. 2. Phospholipid phosphorus (*a*), and the incorporation of ^{32}P into phospholipids (*b*), in *Arctia caia* female moths, injected with [^{32}P]orthophosphate. Each point represents the average of three moths handled separately

Table 3

Phospholipids of Arctia caia *female moths*

The averages of 10 determinations are given

	P (% of total lipid phosphorus)	
Fraction *A*. Alkali-labile		83.7
Phosphatidyl choline (lecithin)	47	
Phosphatidyl ethanolamine (cephalin)	24	
Phosphatidyl serine	2	
Inositol phosphatide	1.8	
Other* and lost	8.9	
Fraction *B*. Alkali-stable, acid-labile (plasmalogens)		1.3
Fraction *C*. Alkali- and acid-stable (e.g. sphingomyelin)		10.7
Lost		4.3
		100

* Traces only of phosphatidic acid and polyglycerol phosphatide were found.

The results of fractionating phospholipids of moths are presented in Table 3.

The changes of specific activities of fraction *A* and *C* in females injected with [^{32}P]orthophosphate are presented in Fig. 3. The rate of incorporation of ^{32}P into the fraction *C* is lower than into the fraction *A*.

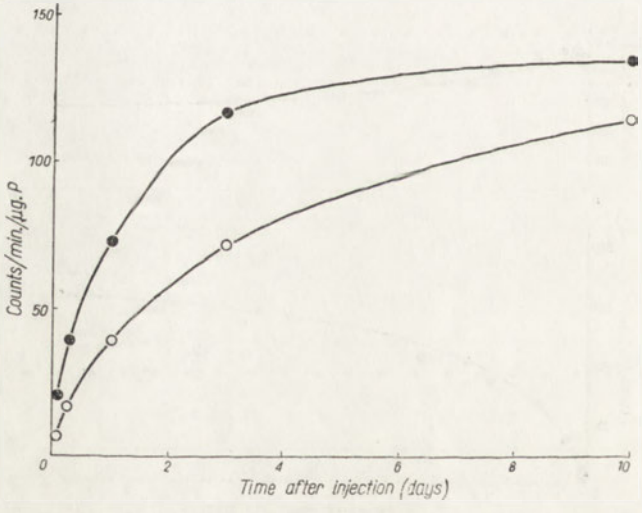

Fig. 3. Specific activities of phospholipid fractions of *Arctia caia* female moths, injected with [^{32}P]orthophosphate. (●), fraction *A*, alkali-labile; (○), fraction *C*, alkali- and acid-stable. Each point represents the average of three moths handled separately

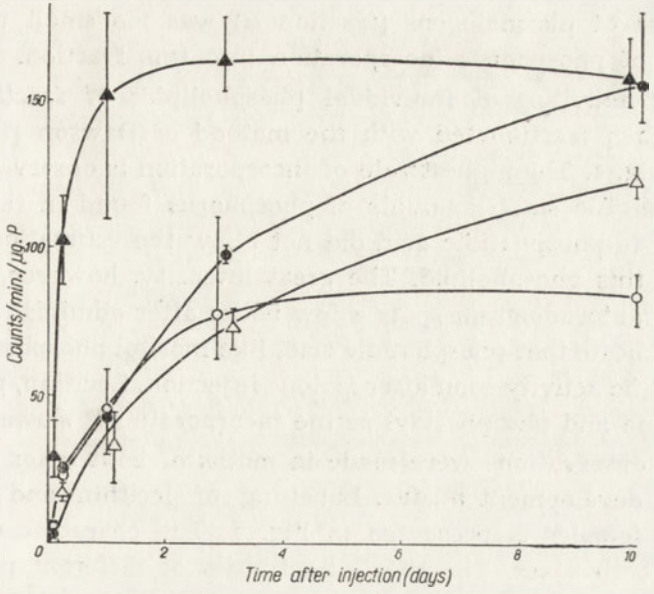

Fig. 4. Specific activities of phospholipid fractions in *Arctia caia* female moths, injected with [^{32}P] orthophosphate. (●), lecithin; (O), cephalin; (△), phosphatidyl serine; (▲) inositol phosphatide. Each point and line represent the average and the range, respectively, for three moths handled separately

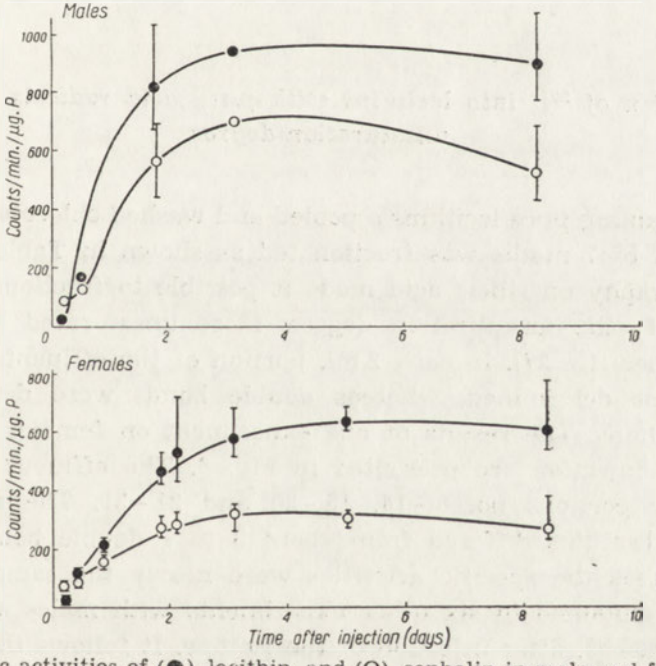

Fig. 5. Specific activities of (●), lecithin, and (O), cephalin in male and female *Arctia caia* moths, injected with [^{32}P]orthophosphate. Points with lines represent the average and the range, respectively, for three moths handled separately

The amount of plasmalogens (fraction *B*) was too small to allow the estimation of phosphorus incorporation into this fraction.

Specific activities of individual phospholipids of fraction *A* from females, when fractionated with the method of Dawson [10], are presented in Fig. 4. The highest rate of incorporation is observed in inositol phosphatide. Too small amounts of phosphorus found in the spots corresponding to phosphatidic acid did not allow the estimation of specific activity of this phospholipid. The great intensity, however, of the corresponding autoradiogram spots a few hours after administration of ^{32}P, seems to indicate that phosphatidic acid, like inositol phosphatide, reaches a high specific activity soon after isotope injection. Lecithin, phosphatidyl ethanolamine and phosphatidyl serine incorporate ^{32}P slower.

Similar observations were made in moths of both sexes and regardless of the development *modus*. Labelling of lecithin and cephalin in males and females is presented in Fig. 5. The character of curves is similar in both sexes. The specific activities of different phospholipids was higher in males due to the same amount of ^{32}P injected and a lesser amount of total phosphorus in males. In the first hours after the injection the increase of specific activity of cephalin was greater than that of lecithin.

Incorporation of ^{32}P into lecithins with fatty acid radicals of different unsaturation degree

For obtaining pure lecithins a pooled and washed chloroform - methanol extract of 7 moths was fractionated as shown in Table 2. Column chromatography on silicic acid made it possible to fractionate lecithins according to the unsaturation degree, more unsaturated having been eluted sooner [15, 27]. In each 2 ml. portion of the effluent the specific activity was determined, whereas double bonds were determined in pooled portions. The results of one experiment on females in the 18th hour after injection are presented in Fig. 6. The effluent was pooled as follows: portions no. 8 - 14, 15 - 20 and 21 - 31. The unsaturation degree of lecithins differed from about 3 to 1 double bond per atom of P, whereas the specific activities were nearly the same. A similar result was obtained in six other experiments with males and females, 18, 40 and 96 hr. after injection of the isotope. It follows that in *Arctia caia* the rate of phosphorus incorporation into lecithins does not seem to be dependent on the unsaturation degree of acyl moieties.

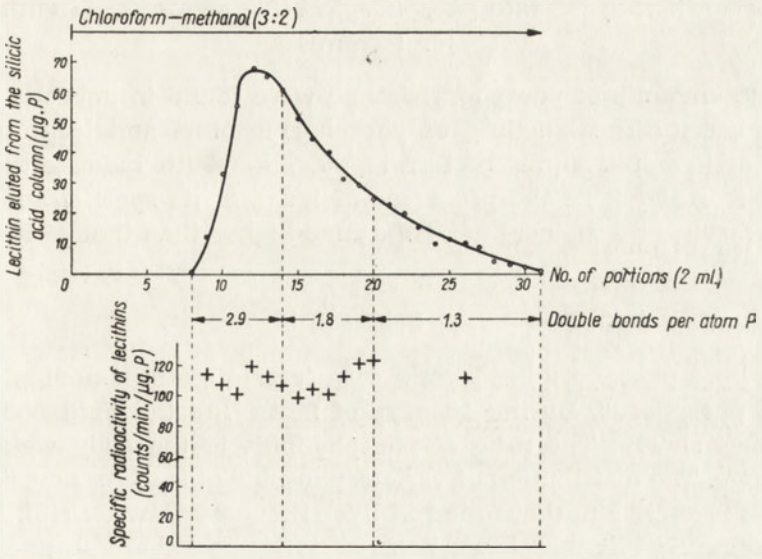

Fig. 6. Column chromatography of lecithins on silicic acid, and specific activities of lecithin fractions differing in unsaturation degree. *Arctia caia* female moths, 18 hr. after injection of [^{32}P]orthophosphate

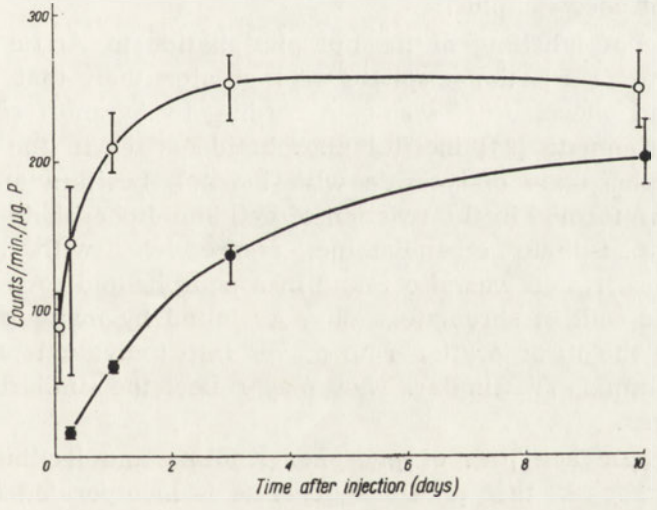

Fig. 7. Specific activities of phosphorylcholine and lecithin of *Arctia caia* female moths, injected with [^{32}P]orthophosphate. (O), phosphorylcholine, (●), lecithin. Each point and line represent the average and the range, respectively, for three moths handled separately

Incorporation of ^{32}P into phosphorylcholine as compared with that for lecithins

Large amounts of phosphorylcholine were found in aqueous extracts of *Arctia caia*. The labelling of phosphorylcholine and lecithin in the same female moths is presented in Fig. 7. The results indicate that phosphorylcholine may be the precursor of lecithin; its specific activity increases faster, and its level is all the time higher than that of lecithin.

DISCUSSION

The lipid phosphorus of *Arctia caia* female moths amounts to 25% of total phosphorus. During 10 days of moth life the lipid phosphorus diminishes slowly. The ratio of phospholipids to the body weight does not change. The distribution of various phospholipids in *Arctia caia* moths (Table 3) is similar to that found in liver tissue by Dawson [10]. Lecithin phosphorus represents about half of total phospholipid phosphorus, cephalin about a quarter, and sphingomyelin fraction about 10%. Phosphatidyl serine and inositol phosphatide are present in minute amounts only. Traces only of compounds corresponding to phosphatidic acids and polyglycerol phospholipids were found by chromatography. The plasmalogen phosphorus amounts to about 1%. Plasmalogen estimation when made with fuchsin method [12, 22] gave similar results. The phospholipid pattern in *Arctia caia* eggs was similar; eggs did not contain polyglycerol phospholipids.

The rate of labelling of inositol phosphatide in *Arctia caia* moths injected with [^{32}P]orthophosphate was greater than that of lecithin, cephalin and phosphatidyl serine. According to Agranoff *et al.* [1] and Paulus & Kennedy [24] inositol phosphatide arises in the reaction of cytidine diphosphate diglyceride with inositol. Lecithin and cephalin, however, are formed in the reaction of cytidine diphosphate choline and cytidine diphosphate ethanolamine, respectively, with diglycerides. A high incorporation was also found in a phospholipid corresponding to phosphatidic acid in chromatography. As found by many authors, differences in the incorporation rate of ^{32}P into individual phospholipids in higher animals are similar. They may reflect the similarity of metabolic patterns.

The specific activities of phosphorylcholine and lecithin in *Arctia caia* moths suggest that phosphorylcholine is incorporated *in toto* into lecithin as was observed in *Celerio euphorbiae* in a previous work [6]. The higher position of the curve of specific activities of lecithin as compared with that of cephalin (Fig. 4 and 5) seems to exclude the possibility of arising of lecithin from cephalin by methylation as described by

Bremer & Greenberg [4]. Only up to 6 hr. after isotope administration, the specific activities of cephalin were a little higher than those of lecithin. The same but more accentuated fact (twice as great specific activity of cephalin as that of lecithin up to 12 hr.) was observed [5] in *Celerio euphorbiae*, a species which belongs to the same *ordo Lepidoptera*. Both moths, *Arctia caia* and *Celerio euphorbiae* were kept without food, the second ones, however, belong to *Sphingidae* which are known to be the most active moths.

There is no reason for supposing that in *Arctia caia* phosphatidyl serine arises from cephalin. Such a biosynthetic pathway, however, was recently discovered by Borkenhagen, Kennedy & Fielding in rat liver [3]. It consists in replacing ethanolamine of cephalin by serine. The higher specific activity of phosphatidyl serine than that of cephalin, 10 days after isotope injection, is not consistent with that possibility in *Arctia caia*.

The *in vitro* experiments on lecithin formation from cytidine diphosphate choline and diglycerides showed that it depends on fatty acid composition of diglycerides [30]. Collins [7] in *in vivo* experiments on rat liver found that the rate of incorporation of ^{32}P into lecithins (fractionated in counter-current distribution or in silicic acid chromatography) depends on the character of their fatty acid moieties. In our experiments the specific activities of lecithins with 3, 2 and 1 double bond per atom P were the same in moths 18, 40 or 96 hr. after isotope injection. However, from the 18th to the 96th hour about 4-fold increase of specific activity of lecithin was observed.

SUMMARY

The [^{32}P]orthophosphate incorporation into phospholipids of *Arctia caia* moths was studied. Inositol phosphatide showed the highest incorporation rate. Lecithin, phosphatidyl ethanolamine and phosphatidyl serine incorporated ^{32}P more slowly. The course of isotope incorporation did not substantiate the possibility of lecithin or phosphatidyl serine arising from phosphatidyl ethanolamine. The course of incorporation of ^{32}P into phosphorylcholine indicated that it might be a precursor of lecithin.

There was no difference in the incorporation rate of ^{32}P into lecithins differing in unsaturation degree of fatty acid radicals.

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BIOSYNTEZA FOSFOLIPIDÓW U OWADÓW

III. WŁĄCZANIE [^{32}P]ORTOFOSFORANU W FOSFOLIPIDY U MOTYLI *ARCTIA CAIA*

Streszczenie

Badano *in vivo* włączanie [^{32}P]ortofosforanu w fosfolipidy motyli *Arctia caia*. Inozytofosfatyd wykazywał największą szybkość włączania izotopu. Lecytyna, fosfatydyloetanoloamina i fosfatydyloseryna włączały ^{32}P wolniej. Przebieg włączania izotopu nie wskazuje na możliwość powstawania lecytyny lub fosfatydyloseryny z fosfatydyloetanoloaminy. Porównanie włączenia ^{32}P w lecytynę i fosfocholinę wskazuje, że fosfocholina może być prekursorem lecytyny.

Nie stwierdzono różnic w szybkości włączania ^{32}P w lecytyny różniące się stopniem nienasycenia kwasów tłuszczowych.

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KONSTANCJA RACZYŃSKA-BOJANOWSKA and KRYSTYNA BEŁŻECKA

TRANSAMINATION IN INSECTS

III. THE EFFECT OF METAL IONS ON ASPARTATE: α-KETOGLUTARATE AMINOTRANSPHERASE IN *CELERIO EUPHORBIAE**

Department of Physiological Chemistry, Medical School, Warszawa

According to Snell [12] metal ions participate in the pyridoxal phosphate-catalysed non-enzymatic transamination reactions. Much controversy, however, exists between authors studying this problem in various enzymatic preparations [5, 7, 8]. Our previous studies [1, 2] proved marked stability of aspartate : α-ketoglutarate aminotranspherase in *C. euphorbiae* as compared with corresponding systems in animal and plant tissues. Neither inactivation of the enzymatic protein nor splitting of the coenzyme occurred upon a 48 hr. dialysis against phosphate buffer, pH 7.4. Therefore this system seemed to be a suitable one for studying the role of metallic ions in transamination mechanism.

MATERIALS AND METHODS

5% homogenates in 0.01 M-phosphate buffer, pH 7.4, from isolated muscles of pupae and moths of *C. euphorbiae* were centrifuged at 6 000 r.p.m. for 15 min.; the dialysis of the supernatant against 0.01 M-phosphate buffer was performed during 48 hr. at 4° [2]. The pupae were used at various periods of diapausis, which may have contributed to the variance of the activity. The moth homogenates were prepared from moths stored after death at 4° for about 3-5 months.

α-Ketoglutaric acid (Nutritional Biochemicals Corporation U.S.A.), L-aspartic acid (B.D.H.); pyridoxal phosphate (Fluka, Switzerland); sodium versenate (EDTA), ammonium *N*-nitrosophenylhydroxylamine

* Supported partially by a grant from the Biochemical and Biophysical Committee of the Polish Academy of Sciences.

(Cupferron), and α,α′-dipyridyl (Biuro Odczynników Chemicznych, Gliwice); copper sulphate and ferrous sulphate (Fabryka Odczynników Chemicznych, Gliwice); tris(hydroxymethyl)aminomethane (Sigma 121).

The activity of aspartate : α-ketoglutarate aminotranspherase (A-KG aminotranspherase) was determined by the spectrophotometric method of Cammarata & Cohen [3]. The following system was used: aliquots of the investigated enzymatic preparations (0.15 - 3.0 mg. of protein), 20 μmoles of sodium L-aspartate and 20 μmoles of sodium α-ketoglutarate in 0.05 M-phosphate buffer, pH 7.4; final volume 3.2 ml. Oxaloacetate formed during transamination from aspartate was measured at 280 mμ spectrophotometrically. The rate of the reaction was graphically established by plotting values from three subsequent determinations.

Protein concentration in dialysed preparations was determined by the absorption measurements at 260 and 280 mμ [14] and by the tannin method [10]. In undialysed preparations the results obtained by the former method are not reliable because of the absorbance in this region of low molecular compounds, mainly uric acid, present in the pupal tissues. This was responsible for the erroneous calculation of the specific activity of aminotranspherase in undialysed preparations and lead previously [2] to a wrong conclusion on activation of aminotranspherase in pupal tissue during dialysis.

All spectrophotometric measurements were made with the Unicam spectrophotometer, type SP 500.

RESULTS

The dialysis carried out alternatively against 0.01 M-phosphate and 0.01 M-tris buffers, pH 7.4, had no effect upon the activity of A-KG aminotranspherase in spite of reported differences in their chelating properties.

Among metal ions that were found to participate in transamination Fe and Cu ions seemed to be of special interest in connection with the results obtained by Canellakis & Cohen [5] and Patwardhan [11]. The content of these ions in the undialysed and dialysed 5% homogenates of pupal muscles was practically the same. Total content of Fe ion was estimated by the thiocyanate method and found to be 5 and 7 μg per ml., and Cu^{2+} ion as determined by the colorimetric carbamate method was 10 μg. per ml.

The addition of Fe^{2+} and Cu^{2+} ions as sulphates in final concentration of 10^{-4} M in 0.05 M-tris buffer, pH 7.4, had no effect on the activation of pupal muscle aminotranspherase.

Further study on the role of metal ions in A-KG transamination

Table 1

The effect of chelating agents on the activity of aspartate: α-ketoglutarate aminotranspherase in C. euphorbiae

0.05-1.2 mg. of undialysed protein and 0.20 mg. of dialysed protein of pupal muscles and 0.10 mg. of protein of undialysed and dialysed moth muscles were preincubated with chelating agents for 15 min. at 37.5° in concentrations given in the Table. PalP, pyridoxal phosphate. Details see Methods. The activity is expressed in μmoles of oxaloacetate formed/100 mg. of protein/min.

Tissue	Preparation	Addition	Activity
Pupal muscles	Undialysed	None	4.0
		EDTA, 10^{-3} M	4.1
		None	3.3
		α,α′-dipyridyl, 6×10^{-4} M	2.6
		None	5.4
		Cupferron, 10^{-4} M	2.6
	Dialysed	None	4.6
		Cupferron, 5×10^{-5} M	3.6
Moth muscles	Undialysed	None	35.5
		Cupferron, 10^{-4} M	17.6
		Cupferron, 10^{-4} M + PalP, 2×10^{-5} M	25.0
		Cupferron, 10^{-4} M + PalP, 10^{-4} M	35.7
	Dialysed	None	36.0
		Cupferron, 5×10^{-5} M	27.0

system included metal chelating agents such as: Cupferron, α,α′-dipyridyl and EDTA. The effect of these agents is shown in Table 1. EDTA in final concentration of 10^{-3} M is without effect. Differences in the activity of control samples and those treated with α,α′-dipyridyl in 6.10^{-4} M concentration are not convincing: higher concentrations of this agent could not be used in spectrophotometric studies because of high absorption at 280 mμ. Cupferron in 10^{-4} M concentration inhibits the aminotranspherase activity in 50%. This inhibition, however, is totally overcome by pyridoxal phosphate added in stoichiometric amounts. It indicates that the inhibition is due to the reaction between coenzyme

and Cupferron. The latter, being a hydroxylamine derivative, may bind pyridoxal phosphate in the same way as that reported for hydroxylamine [6].

DISCUSSION

Studies on participation of metal ions are complicated by the susceptibility of SH-groups of aminotranspherase protein and by the indirect effects of antagonism existing among various ions [13]. Besides, the requirement for metal ions was proved mainly in the labile transamination systems which are easily dissociated on dialysis. In many such instances changes occurring herewith were found to be irreversible despite the pyridoxal phosphate added [4, 9].

The mechanism of inactivation and restoration in such cases is not clearly understood and may require agents for instance metal ions that are not needed in the native state. A stable system of A-KG aminotranspherase in *C. euphorbiae* used in our studies excluded possible side effects of resolution.

A special emphasis in our experiments was made to Fe and Cu ions as Snell included participation of these particular divalent ions in the general transamination mechanism. Besides, Fe^{2+} according to Patwardhan [11] is prerequisite for the activity of A-KG aminotranspherase in green beans and *Dolichos lablob*, and Cu^{2+} is a component of tyrosine: α-ketoglutarate aminotranspherase purified by Canellakis & Cohen [5].

Our results seem to exclude the participation of metal ions in the A-KG aminotranspherase system in *C. euphorbiae*, it may be however that ions so firmly bound with protein as to withstand dialysis do not chelate with the metal binding agents. The attempt to remove metal ions by more drastic measures resulted in partial inactivation and dissociation of the aminotranspherase components [2].

The authors are very much indebted to Z. Marczenko M.S. from the Laboratory of Analytical Chemistry of the Institute of General Chemistry, Warszawa, for determinations of Fe and Cu ions.

SUMMARY

1. A 48 hr. dialysis in phosphate and tris buffers does not affect the activity of aspartate:α-ketoglutarate aminotranspherase in *C. euphorbiae*.

2. Fe^{2+} and Cu^{2+} ions although firmly bound with the protein probably do not participate in the transamination system in *C. euphorbiae*.

3. Cupferron inhibits the aminotranspherase activity because of its reaction with pyridoxal phosphate.

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TRANSAMINACJA U OWADÓW

III. WPŁYW JONÓW METALI NA CZYNNOŚĆ AMINOTRANSFERAZY KWAS ASPARAGINOWY: KWAS α-KETOGLUTAROWY U *C. EUPHORBIAE*

Streszczenie

1. Dializa 48-godzinna w buforze tris lub fosforanowym, pH 7.4, nie ma wpływu na czynność aminotransferazy kwas asparaginowy:kwas α-ketoglutarowy u *C. euphorbiae*.

2. Jony Fe^{2+} i Cu^{2+} prawdopodobnie nie biorą udziału w badanym procesie transaminacji. Możliwe jednak, że ze względu na ścisłe powiązanie z białkami jony te nie są wiązane przez użyte związki chelatujące.

3. Kupferron hamuje transaminację przez wiązanie fosforanu pirydoksalu.

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M. ŻYDOWO, JADWIGA PURZYCKA and L. ŻELEWSKI

THE ADAPTATION OF AMP AMINOHYDROLASE IN RAT KIDNEY TO PROLONGED ACIDOSIS

Department of Biochemistry, Medical School, Gdańsk

Several years ago it has been suggested [9] that the enzymes deaminating adenosine derivatives might participate in the production of urine ammonia. No evidence supporting this view has been presented so far. The experiments which showed that glutamine and certain other amino acids may be the source of urine ammonia were of two types. Either the concentration of a compound had been estimated in the blood entering the kidney and leaving it [11], or the conditions of increased ammonia excretion had been produced by acidification, and the activity of ammonia producing enzymes in the kidney of acidotic animals compared with that of normal animals [3, 4]. Some authors [11] regard an increased ammonia excretion after ingestion of a substance supposed to liberate ammonia as an evidence of the participation of this substance in the direct ammonia production in the kidney.

In the present work "adaptation to acidosis" procedure was applied to answer the question whether the enzymes deaminating adenylic acid in rat kidney may participate in the production of urine ammonia.

MATERIALS AND METHODS

Albino rats of both sexes aged 8 weeks were divided into three groups. One group was given 0.05 N-HCl instead of drinking water, the second one 0.1 N-$NaHCO_3$ and the third (control) group received tap water to drink. All animals were kept on a standard laboratory diet. The acidification and alkalization of animals lasted for 6 months in the first series of experiments and for 3 months in the second.

After 6 or 3 months of this regime the rats were placed in metabolic cages, the urine collected, and the daily ammonia excretion estimated

by the Conway microdiffusion technique [2]. For enzyme assays the rats were killed by decapitation, the kidneys removed immediately, placed in the ice-cold 0.1 M-KCl - 0.039 M-borate buffer, pH 7, weighed and 5% homogenate was prepared. A part of homogenate was kept for glutaminase assay and nitrogen estimation, the remainder was centrifuged at 16 000 *g* for 15 min. The supernatant cytoplasm which contained no appreciable phosphatase or 5'-nucleotidase activity [12] was used for estimation of AMP aminohydrolase and adenosine aminohydrolase activities.

L-Glutamine aminohydrolase (glutaminase) activity was estimated by incubating the kidney homogenate with a mixture containing 0.01 M-L-glutamine, 0.247 M-NaCl, 0.05 M-phosphate and 0.025 M-tris buffer, pH 7.7. Final volume 2 ml. The incubation was carried out for 15 min. at 25° with three different quantities of homogenate (0.2, 0.3 and 0.4 ml.) and the average value has been calculated. A sample containing all the components, but deproteinized prior to incubation, served as blank. The reaction was stopped by adding 1 ml. 15% trichloroacetic acid, the sample diluted to 10 ml. and ammonia content estimated both in the incubation mixture and in the blanks by the microdiffusion technique of Brown *et al.* [1] using either Coleman-14 spectrophotometer or Leitz-Leifo E colorimeter for the determination of the colour intensity of phenol-ammonia complex.

AMP aminohydrolase activity was estimated from the ammonia liberated during the incubation of the cytoplasm with AMP in a mixture containing 0.005 M-AMP, 0.1 M-KCl and 0.025 M-tris buffer, pH 7.2. Final volume 2.0 ml. The incubation was carried out at 25° for 15 min. with 0.4 ml. of cytoplasm and for 30 min. with 0.2 ml. The reaction was stopped and ammonia estimated as above. In each sample orthophosphate was estimated by the Gomori method [5]. The AMP aminohydrolase activity was calculated with a correction for the activity of adenosine aminohydrolase present in the cytoplasm.

Adenosine aminohydrolase activity in cytoplasm was estimated according to Kalckar [7] by following the decrease of extinction of adenosine at 265 mμ with a SF-4 spectrophotometer.

Total nitrogen of the homogenates and of the cytoplasmic fractions was estimated by the Kjeldahl procedure using boric acid for binding of ammonia.

Reagents. AMP and glutamine were Light & Co. products. Other reagents were obtained from Fabryka Odczynników Chemicznych, Gliwice. Phenol was distilled before use, sodium hypochloride was prepared from calcium salt.

RESULTS

When rats had been kept on hydrochloric acid for six months the urinary ammonia increased markedly. It can be seen from Table 1 that the increase in female rats was about two-fold as compared with the control group, whereas in males it did not exceed 50%. The alkalization caused a decrease of ammonia excretion.

Table 1

Ammonia excretion in the urine of acidotic, alkalotic and control rats

Each figure represents the mean daily excretion by three animals in 12 successive days, in mg. per kg. body weight, ± S. E.

Rats	Acidified	Control	Alkalized
Males	60.2±4.0	41.2±2.4	16.2±1.5
Females	82.5±4.7	38.3±2.1	24.9±2.2

The results of enzyme activities measurements are summarized in Table 2. It can be seen that there was an increase of AMP aminohydrolase activity in the kidneys of acidotic female rats as compared with the control group. This increase was greater than the analogous increase of glutaminase activity. It was surprising enough to find the increase of AMP aminohydrolase activity only in acidotic females but not in males.

There were no changes in the activity of adenosine aminohydrolase in either group of rats.

The sex-dependent differences in the enzymatic response to acidosis suggested a control experiment in which a larger group of rats (6 females and 6 males) had been acidified for 3 months before kidney enzymes were estimated. An equal group of rats served as control. The results obtained agreed with the first series. It can be seen from Table 3 that here again there was an increase of AMP aminohydrolase activity in acidified females but not in males. There was no difference in activities of adenosine aminohydrolase between acidotic and control rats, and only insignificant difference in glutaminase activities.

Table 2

The activities of glutaminase, AMP aminohydrolase and adenosine aminohydrolase in the kidneys of acidotic, alkalotic and control rats

Glutaminase was assayed in homogenates, aminohydrolases in cytoplasm. Each number represents the mean value from 3 animals, ± S. E. For experimental conditions see Methods

Rats		Glutaminase		AMP aminohydrolase		Adenosine aminohydrolase	
		μmoles NH_3/ mg.N	μmoles NH_3/ 100 mg. of fresh tissue	μmoles NH_3/ mg.N	μmoles NH_3/ 100 mg. of fresh tissue	μmoles inosine/ mg.N	μmoles inosine/ 100 mg. of fresh tissue
Acidotic	Males	5.90±0.94	16.8±2.69	5.06±1.18	6.8±1.37	5.38±0.19	2.34±0.10
	Females	5.69±0.63	13.9±1.68	4.80±0.42	4.8±0.27	5.13±0.11	2.06±0.06
Alkalotic	Males	5.36±1.49	14.6±3.56	5.81±0.86	7.6±0.81	5.23±0.47	2.27±0.23
	Females	4.92±0.49	12.5±1.36	3.13±0.42	3.9±0.53	5.55±0.24	2.30±0.15
Control	Males	4.91±1.20	13.4±3.09	5.97±1.05	6.7±0.83	6.27±1.69	2.68±0.42
	Females	4.89±0.13	12.4±0.93	2.61±0.51	3.0±0.66	5.68±0.85	2.42±0.36

Table 3

The activities of glutaminase and AMP aminohydrolase in the kidneys of rats acidified for 3 months and in control rats

Glutaminase was assayed in homogenates and AMP aminohydrolase in cytoplasm. Each number represents a mean value from 6 animals, ± S. E. For experimental conditions see Methods

Rats		Glutaminase		AMP aminohydrolase	
		μmoles NH_3/mg.N	μmoles NH_3/100 mg. of fresh tissue	μmoles NH_3/mg. N	μmoles NH_3/100 mg. of fresh tissue
Acidotic	Males	4.6±0.25	13.7±0.86	4.2±0.52	5.5±0.56
	Females	5.2±0.62	14.5±1.61	3.7±0.37	4.8±0.57
Control	Males	4.1±0.38	11.8±1.25	4.2±0.56	5.3±0.75
	Females	5.1±1.24	14.1±1.95	2.4±0.34	2.9±0.468

DISCUSSION

Since van Slyke *et al.* [11] have shown that the increased ammonia excretion in the urine is accompanied by faster removal of glutamine from the blood passing the kidney, many experiments have been made to correlate the urine ammonia output with kidney glutaminase activity. Although some authors [6] did not find any increase of kidney glutaminase activity in acidified animals, it is generally accepted that kidney glutaminase produces ammonia which is subsequently excreted in the urine. Goldstein & Kensler [4] have shown that the increase of kidney glutaminase activity in acidotic guinea pig is the true enzyme induction connected with the increased rate of enzyme synthesis. Davies & Yudkin [3] have found increased deamination of several amino acids in the kidneys of rats submitted to prolonged acidosis. These authors have studied glutaminase in females only and they have found the increase of activity of this enzyme. The presented data support this finding and also show that in male animals the kidney glutaminase may be induced by acid intake lasting for 6 months.

Another possible origin of urine ammonia is the deamination of AMP [12]. However, van Slyke *et al.* [11] have shown that the whole amount of adenosine and AMP disappearing from the blood passing through the kidney appears in the urine. On the other hand the AMP aminohydrolase is present in many rat tissues [10], in the kidneys of

many animal species (W. Makarewicz, unpublished data), and as Kalckar & Rittenberg [8] demonstrated the 6-amino group of AMP is very rapidly renewed *in vivo*.

The presented results seem to support the view that the kidney AMP aminohydrolase may participate in the production of urine ammonia. It is difficult at present to say whether the increase of the AMP aminohydrolase activity found in the kidney of acidotic female rats is due to the true enzyme induction. It is possible that in the kidney of normal female rats an inhibitor is present the action of which might be overcome by acidosis. This inhibitor would have to be connected with the female sex since both normal and acidotic male rats have the same AMP aminohydrolase activity in the kidney.

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SUMMARY

1. The activities of glutaminase, AMP aminohydrolase and adenosine aminohydrolase have been estimated in the kidneys of the rats submitted to prolonged acidosis or alkalosis.

2. Kidney glutaminase increases in both sexes after 6 months of 0.05 N-HCl intake but not after 3 months.

3. AMP aminohydrolase activity increases in females both after 6 months acid intake and after 3 months, but not at all in male animals.

4. Adenosine aminohydrolase activity remains unchanged during acidosis and alkalosis.

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ADAPTACJA AMP AMINOHYDROLAZY W NERCE SZCZURÓW PODDANYCH ZAKWASZANIU

Streszczenie

1. Oznaczano aktywności glutaminazy, aminohydrolazy AMP oraz aminohydrolazy adenozyny w nerkach szczurów obojga płci poddanych długotrwałemu zakwaszaniu lub alkalizacji.

2. Aktywność glutaminazy wzrasta w nerkach szczurów obu płci po sześciu miesiącach spożywania 0.05 N-HCl, ale nie wzrasta po trzech miesiącach zakwaszania.

3. Aminohydrolaza AMP wzrasta w nerkach samic szczurów zarówno po sześciu, jak i po trzech miesiącach zakwaszania, ale nie zmienia się u samców.

4. Aktywność aminohydrolazy adenozyny w nerkach szczurów pozostaje niezmieniona zarówno w czasie zakwaszania, jak i alkalizacji.

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K. OSTROWSKI, J. KOMENDER, HANNA KOŚCIANEK and K. KWARECKI

ELUTION OF SOME SUBSTANCES FROM THE TISSUES FIXED BY THE "FREEZE-SUBSTITUTION" METHOD

Department of Histology and Embryology, Medical School, Warszawa

In the previous communication [12] it was shown that in tissues fixed by the freeze-substitution (F - S) method of Simpson [17], the solubility of proteins remains nearly the same as in the fresh or lyophilized material. Freeze-substitution consists in freezing the tissues to the temperature of liquid gases and then replacing the tissues' water by organic solvent at -20° to -79°. During this procedure and the histological treatment some losses of the tissue substances may occur. The determination of these losses is of importance for the quantitative histochemical estimations, and many papers have been concerned with the losses involved in the most frequently used fixation methods. Sylven [19, 20] has noticed significant losses of nitrogen, lipids and nucleic acids after fixation of the tissues in Carnoy's solution, in formalin or in alcohol. His results were not confirmed by Sandritter & Hartleib [16] nor by Hartleib *et al.* [5]. On the other hand Harbers & Neumann [4] have reported the losses of polypeptides and proteins amounting to about 1% of their content in the fresh tissue. Merriam [10] has shown that during the histological treatment of liver fixed with formalin 2.4% of protein was lost. Stenram [18] has observed that the denaturating effect of Carnoy's solution on the proteins was less marked in the lyophilized tissues than in the fresh ones. Lagerstaedt has described significant losses of nucleic acids after fixation of the tissues in formalin [7] and in Carnoy's solution [8].

The F - S method is very convenient and has many advantages [2]; its application in histochemistry gives good results in enzymatic studies [1, 3] as well as in estimations of nucleic acids [14], glycogen [9] and proteins [12]. The results, however, obtained in different laboratories are often non-comparable because the elution of the tissue substances may vary with the different dehydration media and different temperatures used.

In the present paper the losses of nitrogen and phosphorus have been examined, as well as the decrease of dry weight of rat liver during substitution in most frequently used organic solvents.

MATERIAL AND METHODS

For the experiments, the livers of 9 female Wistar rats weighing about 150 g. were used. The samples of 30 - 100 mg. were weighed on thin aluminium plates, frozen in *iso*pentan at -105° and then placed in 5 ml. of the substitution medium at -79°. From each liver 8 samples were taken; 5 of them were substituted with absolute ethanol, methanol, butanol, propanol and acetone, respectively; 3 samples were not treated by the F - S method and served as controls. The substitution lasted for 6 - 9 days, the material being kept frozen in the mixture of dry ice with ethanol. The end of the process was established by observing the cross--section of a tissue sample in butanol, which is the least penetrating alcohol. When the substitution was terminated the samples were dried at 100° to constant weight and were compared with the dry weight of the controls. Then the samples were digested with the selenic mixture [15], transferred into 50 ml. volumetric flasks and the content of phosphorus and nitrogen was estimated. The amount of phosphorus eluted into substituting media was also determined.

Determination of nitrogen. The Kjeldahl micromethod was used, with the apparatus of Parnas-Wagner for distillation [13]. From the digested and diluted samples, amounts corresponding to 6 - 12 mg. of the fresh tissue were analysed. The amount of nitrogen found varied from 0.2 to 0.5 mg. The error of the method was found to be 1.7%.

Determination of phosphorus. Fiske-Subbarow method with the modification of Horecker, Ma & Haas [6], and photocolorimeter FEKN-2M were used. Samples of solutions corresponding to 1.0 - 1.5 mg. of the fresh tissue containing from 4 to 13 μg. P were analysed. The error of the method was found to be 2.5%.

RESULTS AND DISCUSSION

The obtained results are presented in Table 1 and Fig. 1. Average percentage values of phosphorus recovered in tissues and in substituting media are shown in Table 2. The losses of nitrogen were found to be quite small and therefore similar calculations were not carried out.

From the data given in Table 1 it can be concluded that the changes in the dry weight due to the elution of tissue compounds into the organic solvents were insignificant; these changes were most marked when

Table 1

Changes in the dry weight, and nitrogen and phosphorus content of rat liver after substitution with different media

Values of nitrogen and phosphorus in controls are given in relation to fresh tissue

Experiment No.	Controls			Ethanol			Methanol			Butanol			Propanol			Acetone		
	Dry wt. (%)	mg.N/ 100mg.	µg.P/ 100mg.	Dry wt. (%)	N recov-ered (%)	P recov-ered (%)	Dry wt. (%)	N recov-ered (%)	P recov-ered (%)	Dry wt. (%)	N recov-ered (%)	P recov-ered (%)	Dry wt. (%)	N recov-ered (%)	P recov-ered (%)	Dry wt. (%)	N recov-ered (%)	P recov-ered (%)
1	28.4	2.84	380.6	25.1	95.8	74.5	23.2	94.7	49.9	26.6	97.8	74.8	27.0	97.5	76.4	27.0	98.9	97.7
2	31.2	3.52	501.3	27.1	96.6	84.2	26.2	96.6	64.2	28.4	94.0	82.4	28.5	94.3	84.8	29.3	94.2	98.5
3	31.1	3.41	494.0	28.1	96.5	78.4	26.4	94.7	51.9	29.5	97.4	75.4	29.7	99.1	78.7	31.6	99.7	94.0
4	30.3	3.67	540.6	27.4	98.9	70.2	25.3	98.1	61.7	30.2	97.3	81.9	29.0	101.4	78.0	29.9	102.5	97.2
5	30.2	3.27	506.0	27.0	97.2	72.9	25.1	94.4	55.5	29.1	96.0	76.7	29.0	98.8	78.7	29.8	97.9	94.2
6	31.7	4.10	605.6	28.5	95.9	75.0	26.9	90.7	63.2	29.8	96.3	82.6	29.5	98.8	84.2	32.2	101.7	98.8
7	28.9	3.69	545.6	27.4	98.6	90.3	25.7	97.6	71.2	29.1	100.5	90.9	29.4	100.8	89.9	30.1	103.5	99.9
8	30.6	3.94	537.0	28.2	95.4	88.5	26.9	95.9	77.7	30.6	98.6	93.7	30.1	99.0	97.1	30.8	98.5	105.7
9	32.8	3.54	516.7	28.7	99.7	83.7	28.0	96.9	56.3	29.6	100.6	89.8	29.8	105.1	85.3	30.7	103.4	99.2
Mean value	30.6	3.55	514.2	27.5	97.2	79.7	26.0	95.5	61.3	29.2	97.6	83.0	29.1	99.4	83.7	30.2	100.0	98.4
Standard deviation	±1.4	±0.37	±60.0	±1.0	±1.5	±7.2	±1.4	±2.2	±9.1	±1.2	±2.6	±6.9	±0.9	±3.3	±6.7	±1.5	±3.1	±3.5
Coefficient of variation	4.6	10.4	11.7	3.6	1.5	9.0	5.4	2.3	14.8	4.1	2.7	8.3	3.1	3.3	8.0	5.0	3.1	3.6

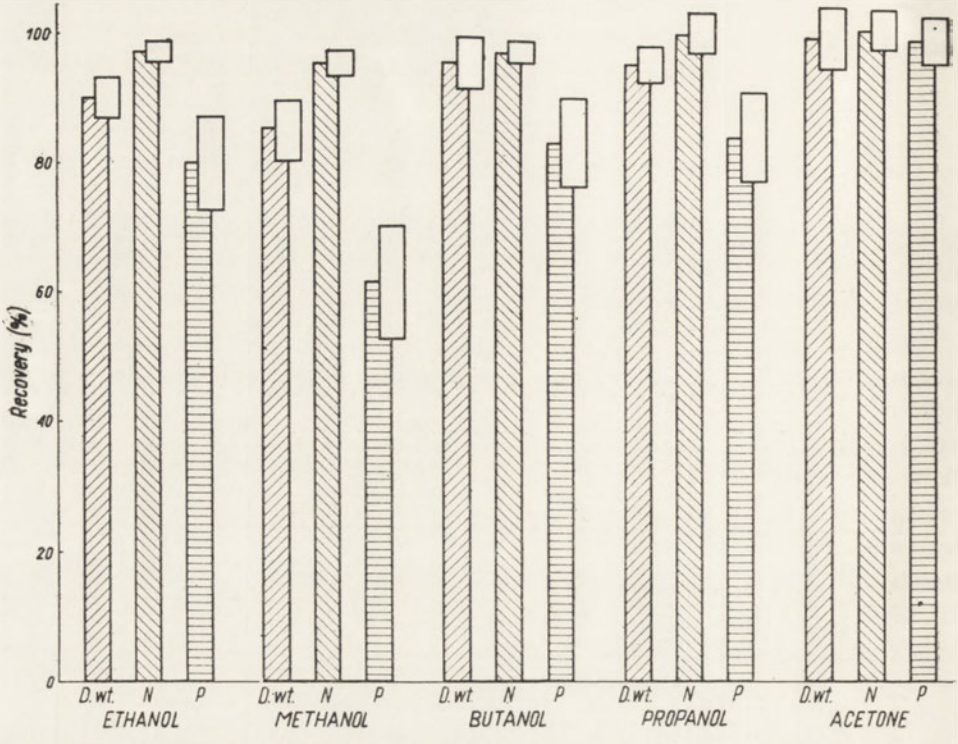

Fig. 1. Recovery of the dry weight, phosphorus and nitrogen in the tissues after freeze-substitution with different media

ethanol or methanol were used. The decrease, however, was statistically significant only in the case of methanol. The observed decrease may be explained as resulting from the elution of phospholipids, and of some compounds containing phosphorus. The losses of nitrogen may be connected with losses of phosphoproteins, e.g. in methanol.

The decrease of nitrogen was found to be rather small; it was most marked with methanol, amounting to 5%. The decrease of phosphorus was much larger and with methanol amounted to 40%. Phosphorus found in the tissues added to that found in the dehydrating media amounted to about 100% of phosphorus in fresh tissue (Table 2). The decrease of nitrogen and phosphorus, according to the data reported in the literature, does not seem to be connected with the elution of nucleic acids and proteins but is probably associated with compounds of low molecular weight.

The smallest losses of nitrogen and phosphorus and no changes in dry weight were observed when acetone was used. Acetone seems to be the best dehydrating medium and is also known as one of the better

fixatives used in the histochemistry of enzymes [1, 14]. Methanol (used in many laboratories because of its high penetrability) and ethanol seem to be less suitable. Under the conditions used, more time was required to complete dehydration when using higher alcohols (butanol and propanol) because of their high viscosity.

Table 2

Recovery of phosphorus in tissues and in dehydrating media after freeze-substitution

Average values in percent of P content in fresh tissue, ± S. D. are given

Substituting medium	Recovery		
	In tissue	In substituting medium	Total
Ethanol	79.7±7.2	19.7±7.2	99.4
Methanol	61.3±9.1	35.0±7.8	96.3
Butanol	83.0±6.9	15.4±8.4	98.4
Propanol	83.7±6.7	17.3±7.1	101.0
Acetone	98.4±3.5	5.1±2.5	103.5

Of the alcohols used in F - S as substitution media, methanol and ethanol proved to be the least suitable. The losses of tissue substances were very high and amounted to 10% of dry weight in ethanol and 15% in methanol. Methanol eluted about 5% of the tissue nitrogen, and 40% of phosphorus. The elution into ethanol was lower, amounting to 3% of nitrogen and 20% of phosphorus. When higher alcohols, propanol and butanol, were used as substitution media the losses were smaller but still amounted to 2% of nitrogen and 16% of phosphorus. It was found that the best medium for F - S was acetone, which gave virtually no losses in dry weight and in nitrogen content, and only 2% of phosphorus content.

SUMMARY

The decrease of dry weight and the content of nitrogen and phosphorus in rat liver during freeze-substitution with 5 dehydrating media (ethanol, methanol, butanol, propanol and acetone) at -79° were determined.

Taking into consideration the elution of nitrogen and phosphorus and the decrease of the dry weight, the authors suggest acetone to be the most satisfactory medium for the freeze-substitution method.

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ELUCJA NIEKTÓRYCH SUBSTANCJI TKANKOWYCH PRZY UTRWALANIU METODĄ "FREEZE-SUBSTITUTION"

Streszczenie

Przebadano ubytek suchej masy oraz azotu i fosforu wątroby szczura w czasie substytucji pięcioma rozpuszczalnikami organicznymi (etanol, metanol, butanol, propanol, aceton) w temperaturze mieszaniny suchego lodu z etanolem. Uzyskane wyniki pozwalają stwierdzić, że aceton jest najlepszym środowiskiem, biorąc pod uwagę jako kryteria wypłukiwalność azotu i fosforu oraz ubytek suchej masy.

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Vol. IX 1962 No. 2

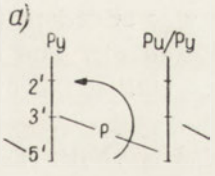
W. SZER and D. SHUGAR

A NOTE ON THE STABILITY OF PYRIMIDINE NUCLEOSIDE CYCLIC PHOSPHATE METHYL ESTERS AND THE MODE OF ACTION OF RIBONUCLEASE

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

It is well known from the work of Todd *et al.* [for review see ref. 1] and Markham & Smith [4] that pancreatic ribonuclease catalyses the hydrolysis of monoesters of pyrimidine nucleoside 3′-phosphates but is inactive against the corresponding dimethyl esters, i.e. triesters of phosphoric acid. The pyrimidine nucleoside 2′ : 3′-cyclic phosphates are likewise substrates of this enzyme.

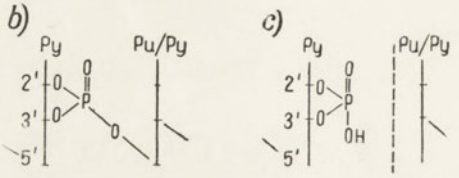
In view of the foregoing, and the earlier dilatometric investigations of Linderstrom-Lang *et al.* [3] and Vandendriessche [9], it is generally accepted that the depolymerizing activity of ribonuclease is a two-stage reaction, of which the initial step is a specific transphosphorylation, i.e. a transfer of the phosphodiester linkage from position 5′ to 2′ of a pyrimidine nucleotide as shown in (a):



The result is the formation of pyrimidine nucleoside cyclic phosphates and/or purine oligonucleotides with a terminal pyrimidine nucleoside cyclic phosphate residue. The second phase of action of the enzyme involves the opening of the cyclic phosphate groups with the formation of nucleoside-3′-phosphates or purine oligonucleotides with a terminal nucleoside-3′-phosphate.

It appeared of interest to us to examine the mechanism of the above-mentioned transphosphorylation reaction and, in particular, to examine

whether the cyclization and formation of an "internal" triester such as in diagram (b):



is actually the first step in the action of ribonuclease, followed by hydrolysis of the 5′ linkage, or whether cyclization results from depolymerization and occurs simultaneously with the transfer of the phosphodiester linkage (c). This proved to be possible through the use of the methyl ester of uridine-2′ : 3′-cyclic phosphate, and the corresponding cytidine derivative.

During the course of a previous investigation [6] on the *N*-methylation of uridine-2′ : 3′-phosphate, *N*-methyluridine-2′ : 3′-methyl phosphate was isolated and found to be resistant to ribonuclease. Since independent evidence showed that *N*-methylation itself was sufficient to block ribonuclease action [6, 7], it appeared worthwhile, in relation to the foregoing, to examine the behaviour of uridine-2′ : 3′-methyl phosphate (I), which was prepared as follows:

To a 25 ml. flask containing 216 mg. (0.66 mM) uridine-2′(3′)-phosphate in 5 ml. ethanol was added 750 mg. (3 mM) *N*,*N*′-dicyclohexylcarbodiimide. The mixture was left for 12 hr. at room temperature, following which paper chromatography demonstrated that cyclization had proceeded quantitatively [cf. 5]. The solution was then carefully titrated with an ethereal solution of diazomethane to the point where spectral analysis of an aliquot indicated that *N*-methylation was beginning to take place. Solvent was then rapidly removed under reduced pressure. About 5 ml. water was added to the residue and the crystals of *N*,*N*′-dicyclohexylurea filtered off. The solution was then taken to dryness and the residue crystallized from ethanol and ether, yield 165 mg. (64% theor.).

Analysis: calculated for $C_{10}H_{13}O_8N_2P$: N - 8.72%; P - 9.68%; observed: N - 8.62%; P - 9.47%.

The product did not form a salt with calcium chloride in anhydrous ethanol and the UV absorption spectrum was identical with that for uridine-2′(3′)-phosphate. In acid it was converted quantitatively to uridine-2′(3′)-methyl phosphate which, as was to be expected, was partially hydrolysed by ribonuclease.

Cytidine-2′ : 3′-methyl phosphate (II) was obtained by an analogous procedure, but the titration in this case was not as critical since the ring nitrogen does not readily methylate.

Table 1

Paper chromatography

Solvents used: *A*, propan-2-ol — ammonia (d = 0.88) — water (7 : 3 : 1, by vol.); *B*, propan-2-ol — water (7 : 3, v/v)

Compound	R_F with solvent	
	A	*B*
Uridine	0.50	0.67
Uridine-2′(3′)-phosphate	0.17	0.36
Uridine-2′:3′-phosphate	0.37	0.43
Uridine-2′(3′)-methyl phosphate	0.45	0.49
Uridine-2′:3′-methyl phosphate (I)	0.65	0.80
Cytidine	0.55	0.65
Cytidine-2′(3′)-phosphate	0.23	0.41
Cytidine-2′:3′-phosphate	0.42	0.50
Cytidine-2′:3′-methyl phosphate (II)	0.60	0.68

Ascending paper chromatography, using Whatman no. 1, was employed for identification of the various compounds. The R_F values of compounds are given in Table 1.

Enzymatic trials

The compounds examined were made up to a concentration of 10 mg./ml. in 0.1 M-acetate buffer at pH 7.8, and crystalline pancreatic ribonuclease (Armour) added to a concentration of 1 mg./ml. Following 2 hr. incubation at 37°, the solutions were examined by paper chromatography. During this period, it was found that both the uridine and cytidine cyclic phosphates underwent complete hydrolysis to the 3′-phosphates, as expected. The corresponding monomethyl esters were, however, unaffected.

It consequently follows that enzymatic hydrolysis of internucleotide linkages does not proceed *via* a cyclic triester intermediate. On the other hand, the mechanism proposed by Witzel [11], according to which enzymatic hydrolysis by ribonuclease is regarded as a special case of acid-catalysed hydrolysis in which the proton donor is the enzyme, is in agreement with the foregoing results. It, in fact, follows from these that one of the prerequisites for the action of ribonuclease is the existence of a potentially dissociable phosphate hydroxyl. It is worth noting that a similar situation prevails for the hydrolytic action of snake venom phosphodiesterase, which is inactive against the dimethyl esters of UMP, CMP and related compounds [8].

Finally, it is worth noting that internal triesters such as in diagram (b) may be formed by the action of dicyclohexylcarbodiimide on dinucleotides such as UpU, which may readily be isolated on an ECTEOLA column [cf. Witzel, 10]. Such "dinucleotides" are resistant to ribonuclease. It should be recalled in this connection that uridine-2′(3′)--methyl phosphate will undergo cyclization in anhydrous medium in the presence of dicyclohexylcarbodiimide, but at a relatively slow rate in comparison to uridine-2′(3′)-phosphate [6].

Hydrolysis of nucleoside triesters

Contrary to expectations [1], both uridine-2′:3′-methyl phosphate (I) and cytidine 2′:3′-methyl phosphate (II) were found to be relatively stable at room temperature, a finding which is pertinent to the interpretation of the mechanism of hydrolysis of nucleoside 3′ (and probably 2′) dimethyl phosphates, previously examined by Todd *et al.* [2]. The course of hydrolysis of such triesters was postulated as proceeding *via* the intermediate I (II) which, however, could not be detected due to its presumed instability. This supposed intermediate is, in fact, sufficiently stable to permit its isolation by paper chromatography even in solvents which are not too acid or alkaline. This might have been anticipated from our earlier results involving the preparation of *N*-methyluridine-2′:3′-methyl phosphate. In agreement with Todd *et al.* [1] this supposed intermediate could not be detected during the course of hydrolysis of nucleoside-2′(3′)-dimethyl phosphates and it is consequently not an intermediate in this reaction.

SUMMARY

The 2′:3′-cyclic methyl phosphates of uridine and cytidine have been synthesized. Both compounds are completely resistant to pancreatic ribonuclease, in accordance with the accepted mode of action of this enzyme as catalysing a specific transphosphorylation. Both compounds are sufficiently stable for purposes of isolation and for paper chromatography. This made it possible to demonstrate that they are not, as hitherto supposed, intermediates in the hydrolysis of pyrimidine (and, presumably, purine) nucleoside 2′(3′)-phosphate triesters.

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UWAGI O TRWAŁOŚCI ESTRÓW METYLOWYCH CYKLICZNYCH NUKLEOTYDÓW PIRYMIDYNOWYCH I O MECHANIZMIE DZIAŁANIA RYBONUKLEAZY

Streszczenie

Otrzymano estry metylowe 2′:3′-cyklicznych fosforanów urydyny i cytydyny. Obydwa związki są całkowicie odporne na działanie rybonukleazy trzustki. Jest to zgodne z przyjętym mechanizmem działania tego enzymu, polegającym na katalizowanej transfosforylacji. Określono dodatkowy warunek specyficznego działania enzymu, a mianowicie konieczność istnienia jednej zdolnej do dysocjacji grupy wodorotlenowej w reszcie fosforanowej. Obydwa otrzymane związki okazały się na tyle trwałe, że nadawały się do wyodrębnienia i chromatografii bibułowej. W ten sposób wykazano, że nie stanowią one nietrwałych związków przejściowych przy hydrolizie pochodnych trójestrowych 2′(3′)-fosforanów nukleozydów pirymidynowych (i prawdopodobnie purynowych), jak przyjmowano dotychczas.

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DANUTA ROŻYNKOWA and S. MAGAS

^{131}I-LABELLED FAT IN THE STUDIES ON FAT ASSIMILATION IN RATS

Department of General and Experimental Pathology, and 2nd Department of Internal Medicine, Medical School, Poznań

Fat labelled with ^{131}I is commonly used for the evaluation of intestinal fat absorption. Recently ^{131}I-labelled triolein was used by several authors [8] in studies on fat tolerance curves in patients with atherosclerosis. Some authors [10], however, suggest that since ^{131}I is not a natural fat component, it cannot be a proper tracer of lipid metabolism.

The present work was carried out to answer to what a degree the orally administered radioiodinated fat can be the tracer of ingested fat. For this purpose (a) the radioactivity in the digestive tract and in the blood was followed, (b) the influence of other fats on the absorption of iodinated fat was studied, and (c) the distribution of radioactivity in some tissues and in their lipid extracts was estimated.

MATERIAL AND METHODS

For the experiments 70 adult male rats weighing 200-300 g. were used. Known amounts of ^{131}I-labelled fat were pipetted into the animals' mouths opened with a special dilator (a plexiglass plate with a large hole). This precaution was made to avoid any losses of the administered fat. During 24 hr. before the experiment the rats were given only a solution containing 5 g. glucose and 0.1 g. KI per 100 ml. Potassium iodide was given to diminish the accumulation of ^{131}I by the thyroid gland.

The amount of fat administered was 0.3 - 0.4 g. of oil per rat, except in the preliminary experiments performed to establish the optimal dose of fat.

Triolein labelled with ^{131}I (product of the Radiochemical Centre, Amersham, England) or ^{131}I-labelled olive oil prepared according to Vetter & Vaell [11] was used. No differences dependent on the kind of

fat given were observed. The radioactivity of the doses administered was 1.3 - 40 μc. The radioiodinated fat was diluted with natural olive oil or with olive oil iodinated with non-radioactive iodine. No free $^{131}I^-$ ions were found in the radioactive fat, as tested by estimating the radioactivity of the aqueous extracts.

At various time-intervals (0.5 - 24 hr.) after the administration of ^{131}I-labelled fat the rats were anaesthesized with ether and the blood samples, fragments of the digestive tract, parenchymal organs and samples of other tissues were taken. The samples were weighed (accuracy 0.01 g.). For the calculation of the total blood radioactivity the blood volume was assumed to be 5% of the rat's body weight [7]. In some of the rats, blood samples (0.3 - 0.4 g.) from the tail vein were taken several times. In the small intestine the radioactivity of its content and of the intestinal wall was determined. For the determination of the lipid-bound radioactivity, the homogenized tissues were extracted with a chloroform - methanol mixture according to Bligh & Dyer [3].

The radioactivity was estimated with the scintillation counter and LE-5 electronic scaler. The standard was prepared from a known amount of ^{131}I-labelled fat diluted with chloroform in a volumetric flask. The radioactivity of known volume of standard was measured under the same geometric conditions.

RESULTS

The usefulness of ^{131}I-labelled fat

Before the investigation of the fate of ingested fat in various organs and tissues two questions were to be considered: what is the rate of absorption of ^{131}I-labelled fat into blood, and whether this absorption differs from that of natural unlabelled fat.

The radioactivity of the intestinal content, intestinal wall and peripheral blood at different time-intervals after the ingestion of labelled fat is illustrated on Fig. 1. Fig. 2 shows the results of blood radioactivity estimation in 5 rats; it can be seen that the maximum appeared mostly after 3 to 4 hr. after fat ingestion.

For the proper evaluation of lipaemia by means of radioactivity it was necessary to establish the part of activity associated with the lipid fraction. The radioactivity of the lipid extract was measured and about 70% of the total activity was found in the lipid fraction (Fig. 3). The same ratio persisted during 24 hr. after fat administration. These data suggest that in this kind of experiments the radioactivity of blood can be the measure of alimentary lipaemia in healthy rats.

To see whether the intestinal absorption of iodinated fat differs from that of a normal fat, experiments were made in which the rats were given ^{131}I-labelled fat alone, or with the addition of various amounts of natural oil or oil iodinated with non-radioactive iodine (Table 1). The

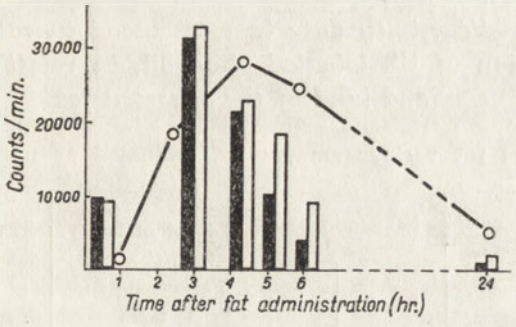

Fig. 1. Radioactivity of the intestinal content, intestinal wall and the total blood of rats after administration of 100 mg. of ^{131}I-labelled triolein (20 μc). The values for blood are averages from samples collected from tail veins of 2 rats. Black columns denote intestinal content, white ones intestinal wall; (O), total blood

percentage of radioactivity administered, found in the total amount of blood was nearly constant and amounted to about 6%; it was almost independent of the amount of fat given and its iodine content. If the

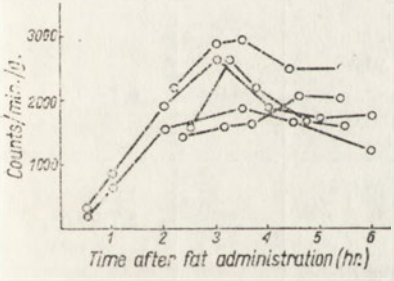

Fig. 2. Radioactivity of the blood collected from tail veins of 5 rats after the administration of 100 mg. of ^{131}I-labelled triolein (20 μc)

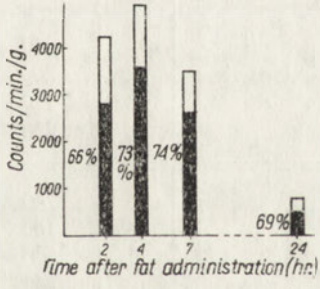

Fig. 3. Radioactivity of blood and blood lipid extract after the administration of 60 mg. of ^{131}I-labelled triolein (40 μc). The black portion of each column denotes lipid activity; the white, non-lipid activity. Average values from 16 analyses are given

absorption rate of the iodinated fat differed from that of the unlabelled one, the radioactivity of the total blood would depend on the kind and amount of the added unlabelled fat. As this was not the case, it seems

that the iodinated oil does represent the course of alimentary lipaemia as well as the normal non-iodinated one.

Table 1

Percentage of radioactivity dose in total blood in rats 6 to 8 hr. after the administration of ^{131}I-labelled olive oil, in relation to the amount and kind of the ingested fat

To the radioactive fat various amounts of iodinated or natural oil were added

Fat added	Rat no.	Fat administered (mg.)	Fat administered (μc)	Dose of fat (mg./100 cm² absorbing surface)	% of radioactivity dose in blood	Average, ±S.D.
Non-iodinated oil	1	18	7.2	4.5	5.0	5.7±1.3
	2	19	6.5	4.7	6.3	
	3	170	8.2	42.5	6.2	
	4	170	6.7	42.5	5.2	
	5	220	7.0	55.0	5.9	
	6	320	7.6	80.0	4.6	
	7	560	6.6	140.0	7.7	
	8	620	7.8	155.0	4.9	
	9	1019	7.5	255.0	3.7	
	10	1150	6.7	287.0	7.9	
Iodinated oil (non-radioactive)	11	60	4.8	15.0	5.5	6.2±0.8
	12	200	4.8	50.0	6.0	
	13	370	4.8	92.0	6.0	
	14	400	6.5	100.0	7.2	
None	15	40	1.3	10.0	5.5	5.7±0.6
	16	100	3.2	25.0	5.5	
	17	220	7.0	55.0	5.8	
	18	400	12.8	100.0	6.6	

The results presented in Table 1 indicate also the relation between the amount of fat ingested and the alimentary lipaemia. The low percentage, about 6% only, of administered labelled fat found in blood agrees with the very rapid exchange of exogenous fat with the tissue lipids [4].

The above preliminary experiments supported in many details the findings of other authors [8, 12] and confirmed the usefulness of ^{131}I-labelled fat in studies on intestinal fat absorption.

The optimal dose of fat

The amount of fat, 6 hr. after the administration of which the radioactivity remaining in the intestine was the lowest, was considered to be the optimal dose. The amount of fat given was calculated per 100 cm^2 of intestinal absorbing surface. The absorbing surface was assumed to be 1.5 cm^2 per 1 g. of body weight [5]. The same rats were used for these experiments and the experiments described in Table 1.

In two series of experiments the same results were obtained (Fig. 4). The optimal amount of fat was found to be 80 - 100 mg. per 100 cm^2 of intestinal absorbing surface, i.e. about 0.24 - 0.45 g. of fat for a rat

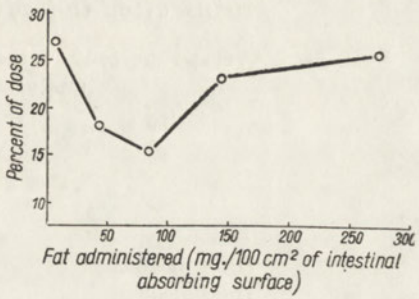

Fig. 4. Percentage of radioactivity remaining in the digestive tract 6 hr. after the administration of varying amounts of oil containing constant amounts of ^{131}I-labelled fat. Average values of duplicate measurements are given

weighing 200 - 300 g. After the administration of this dose the percentage of fat remaining in the intestine after 6 hr. was the lowest. Larger doses caused marked prolongation of the intestinal absorption, and smaller doses (1 drop, i.e. about 20 mg.) remained for a comparatively long time in the stomach, as no food was given to the rats after fat administration. In further experiments the above mentioned optimal dose of fat has been used.

The distribution of radioactivity in tissues

The percentage of radioactivity in various rat tissues at different time-intervals after the administration of radioiodinated fat was estimated. Blood samples, digestive tract, liver, other parenchymal organs and thyroid gland were removed. The rest designated as "remaining tissues" consisted of muscles, adipose tissue, integuments and other soft tissues. The results of estimations are presented in Table 2. Attention should be paid to the rapid and regular increase of radioactivity in the "remaining tissues". The ^{131}I content in the thyroid did not exceed 1% of the amount given. This may be easily explained by the blocking action of the administered potassium iodide.

In order to estimate the distribution of radioactivity in the "remaining tissues" the radioactivity of particular tissues, i.e. integuments (skin, hair and subcutaneous tissue), muscles and adipose tissue was determined. The results calculated per 1 g. of tissue were expressed as percentage of the amount administered. The values found at different time-intervals after the feeding are presented in Fig. 5 and compared with the data concerning the blood and the heart muscle.

Table 2

Distribution of radioactivity in rat tissues and organs after the administration of ^{131}I-labelled fat

Average percent values of the dose administered from analyses of 8 rats are given

Tissue	Time after fat administration (min.)			
	105	150	195	330
Digestive tract	80	66	48	31
Total blood	2.5	4	6.5	5
Liver	2	3	4	4
Other parenchymal organs	1.5	2.5	4	5.5
Thyroid (blocked)	0.5	0.5	0.5	0.5
Remaining tissues	13.5	24	37	54

The course of radioactivity in the heart muscle and depot fat is similar to that in the blood. A marked increase of radioactivity per 1 g. of blood, heart muscle and integuments was observed during the first 3 hr. after the administration of ^{131}I-labelled fat. The respective values for the skeletal muscle and perirenal adipose tissue were much lower. A continuous rise in the radioactivity of integuments was observed. Continuous passage of inorganic ^{131}I into the skin and skeletal muscles may be inferred from the shape of the curves.

The high radioactivity found in the heart muscle agrees with the data of other authors [2, 4]. Special attention should be paid to the very high radioactivity of the integuments. The results obtained may be interpreted either by the mechanism of exogenous fat exchange in the superficial body fats being different from that of the deeper layers, or by the accumulation of $^{131}I^-$ ions, which are formed in the course of catabolism of the administered fat.

The latter possibility was confirmed by the experiments presented

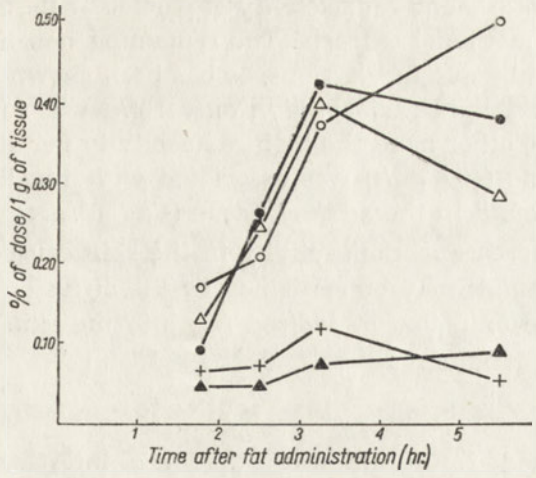

Fig. 5. Percentage of radioactivity dose per 1 g. of rat tissues after the administration of ^{131}I-labelled fat. Average values of samples taken from 8 rats are given. (O), integuments; (●), blood; (△), heart muscle; (▲), skeletal muscle; (+), depot adipose tissue

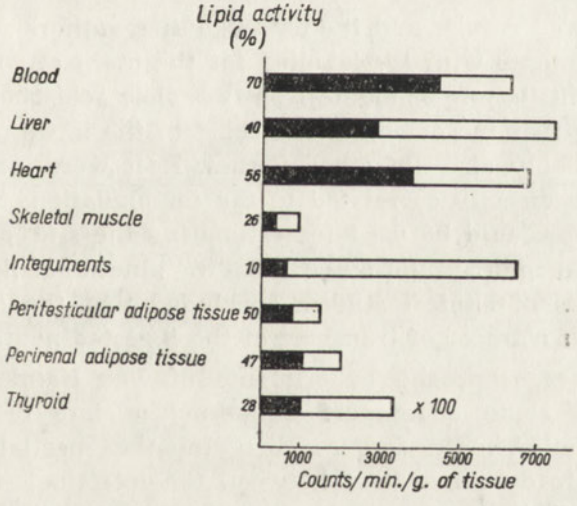

Fig. 6. Lipid and non-lipid radioactivtiy per 1 g. of rat tissues 6 hr. after the administration of ^{131}I-labelled fat. Results are averages of duplicate experiments. Values for thyroid are reduced a 100 times. The black portion of each column denotes lipid activity, the white, non-lipid activity

in Fig. 6. The lipid-bound radioactivity of particular tissues was estimated in chloroform - methanol extracts. The remaining non-lipid radioactivity was supposed to be due to $^{131}I^-$ ions, as has been shown by other authors [6, 9]. The lowest lipid radioactivity, only 10%, was found in the integuments. On the other hand the high radioactivity per 1 g. of blood, liver and heart muscle (40 - 70%) was associated with the lipid fraction and the same was found in the adipose tissue (47 - 50%).

The rapid increase of radioactivity in the skin caused in 90% by the non-lipid compounds may be explained by the diffusion of the $^{131}I^-$ ions liberated by the oxidation of iodized fat, into the skin as iodide space.

DISCUSSION

The presented results indicate that the intestinal absorption of ^{131}I-labelled fat was the same as the absorption of normal fat. It was also shown that the assimilation of the ingested ^{131}I-labelled fat can be followed by direct estimation of radioactivity in intestine and eventually in blood. In other tissues and organs the estimation of lipid extracts should also be made. If the lipid and inorganic iodine fractions are estimated, the amount of inorganic ^{131}I may be considered as the tracer of the amount of catabolized exogenous fat. In this case the accumulation of iodide by the thyroid gland should be previously diminished by the administration of potassium iodide to minimize the formation of iodine-bound protein.

The presented results and the data of other authors seem to suggest that in experiments with ^{131}I-labelled fat the main attention should be paid to the splitting off of iodine from the oleic acid chain. It has been reported [1, 9] that the deiodination occurs in the intestinal wall, in the liver and probably also in other tissues. If it were proved that the deiodination is directly correlated to the fat oxidation, then studies on the deiodination could be used for estimating the site and rate of fat metabolism. George *et al.* [6] considering the kinetics of lipid metabolism and the results of clinical studies concluded that the ^{131}I-labelled fat may be used as a tracer of transport of the ingested neutral fat.

On the other hand, the experiments of Van Handel & Zilversmit [10] indicated some differences between the fate of the ^{14}C- and ^{131}I-labelled fat. The difference lies in the partial liberation of ^{131}I-marker already somewhere between the intestinal lumen and the thoracic duct. This, however, should not eliminate the radioiodinated fat from studies on lipid metabolism, provided that the deiodination process and its relation to the utilization of lipids are better known and taken into account. Moreover, the fact of iodine not being a natural

physiological lipid component may appear advantageous. The ^{131}I, once split off, is never reincorporated into tissue lipids. On the contrary, the isotopes of carbon or hydrogen may be to some extent included again into the lipid metabolic cycle.

SUMMARY

The intestinal absorption and further distribution of ^{131}I-labelled fat in rat tissues were studied. The course of the blood radioactivity, the percentage of the administered dose found in total blood, the blood lipid radioactivity and the optimal dose of fat to be given were determined.

The radioactivity per 1 g. of the skeletal muscles and adipose tissue was much lower than that of blood, heart muscle and integuments. In the lipid extracts of blood, liver, heart muscle and adipose tissue the greater part of radioactivity was found. In the integuments and skeletal muscle the prevalence of non-lipid radioactivity has been demonstrated.

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TŁUSZCZ ZNAKOWANY ^{131}J W BADANIU PRZYSWAJANIA TŁUSZCZU U SZCZURÓW

Streszczenie

Badano wchłanianie jelitowe i dalsze rozmieszczenie w tkankach szczurów tłuszczu znakowanego promieniotwórczym jodem ^{131}J. Określono przebieg radioaktywności krwi, procent dawki odnajdywanej we krwi całkowitej, radioaktywność lipidową krwi oraz optymalną dawkę tłuszczu.

Radioaktywność na gram mięśni szkielowych oraz depozytowej tkanki tłuszczowej była kilkakrotnie niższa od radioaktywności krwi, mięśnia sercowego oraz powłok skórnych. Wyciągi lipidowe krwi, wątroby, mięśnia sercowego oraz tkanki tłuszczowej zawierały znaczną część radioaktywności, natomiast powłoki skórne i mięśnie szkieletowe wykazywały przewagę radioaktywności nielipidowej.

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L. ŻELEWSKI, M. ŻYDOWO and JADWIGA PURZYCKA

THE EXCRETION OF CITRATE IN MALE AND FEMALE RATS AFTER PROLONGED ACIDOSIS OR ALKALOSIS

Department of Biochemistry, Medical School, Gdańsk

The amount of citric acid excreted in the urine depends on the acidification or alkalization of the organism [2]. The aim of the present study was to investigate the influence of prolonged acidification or alkalization on the citrate output in male and female rats. The possibility of sex differences was suggested by the increase of citrate excretion under the influence of oestrogens [8, 4, 6].

METHODS

For experiments 18 white rats kept on a standard laboratory diet were used. The animals were divided into three groups with three males and three females in each group. One group were given 0.05 N-HCl instead of drinking water, the second one 0.1 N-$NaHCO_3$, and the control group had tap water to drink. The acidification and alkalization lasted for six months since the eighth week of life of the animals [10]. Then the animals were placed in metabolic cages, the urine was collected and citric acid [1], calcium and inorganic phosphorus [7] were estimated during seven successive days.

Afterwards the animals were killed by decapitation, the blood was collected into a heparinized vessel and citrate estimated [1]. Liver and kidney were homogenized in a KCl - borate solution (0.1 M-KCl buffered with 0.039 M-borate, pH 7), to get a 5% homogenate. From the kidney homogenate, mitochondria were prepared as described previously [9]. To obtain liver mitochondria the liver was homogenized in 0.25 M-saccharose - 0.002 M-EDTA.

Oxygen consumption has been measured in a Warburg apparatus at 30° for 1 hr. Two ml. of incubation medium contained: 250 μmoles

saccharose, 2 μmoles EDTA, 15 μmoles $MgCl_2$, 24 μmoles tris, pH 7.4, and either 30 μmoles succinate or 45 μmoles citrate. In the central compartment a filter paper strip and 0.2 ml. 10% KOH were placed. To the Warburg flask 2 ml. of incubation medium and 1 ml. of cellular fraction suspension in KCl-borate were added.

Nitrogen content in the homogenates and mitochondria was determined with the Kjeldahl procedure in a Parnas-Wagner apparatus using boric acid to bind ammonia.

Diet and reagents. The standard laboratory diet was suggested by Professor Dr. A. Szczygieł; it contained: 20% rye, 20% wheat, 22% oats, 15% bone powder, 6% yeast, 8% powdered milk, 3% margarine, 3.5% caseine, 1% cod liver oil, 1% NaCl and 0.5% $CaCO_3$.

Tris(hydroxymethyl)aminomethane, was L. Light & Co. Ltd. product. Succinic acid was Eisenach, repacked by Fabryka Odczynników Chemicznych, Gliwice. Other reagents were obtained from Fabryka Odczynników Chemicznych in Gliwice.

RESULTS

The mean daily excretion of citrate, calcium and inorganic phosphorus is summarized in Table 1. In comparison with the control group an increase of citrate excretion in alkalotic rats and a decrease in acidotic ones were found. Both in acidotic and in control groups the citrate excretion was higher in females than in males. There were similar sex differences in calcium excretion both in acidotic and in

Table 1

The excretion of citrate, calcium and inorganic phosphate in the urine of acidotic, alkalotic and control rats

Each group consisted of 3 animals; the urine was collected and investigated on 7 successive days. The average values are given in μmoles per kg. body weight per day, ± S. E.

Compound	Sex	Rats		
		Acidotic	Alkalotic	Control
Citrate	Males	183± 14	790± 37	405± 26
	Females	390± 31	960± 73	690± 41
Calcium	Males	142± 15	190± 25	200± 22
	Females	445± 30	200± 22	470± 40
Inorganic P	Males	2390±155	1930± 84	2800±132
	Females	2860±119	1640±116	2650±132

control animals, but in alkalotic rats no sex differences either in citrate or in inorganic calcium excretion were observed. There was no parallelism between the excretion of citrate and calcium. No differences in inorganic phosphate excretion between the acidotic and control groups were observed; in alkalotic rats the excretion was smaller.

Table 2

Citrate in the blood of acidotic, alkalotic and control rats

Each group consisted of 3 animals. The values are given in μmoles per liter, ± S. E.

Sex	Rats		
	Acidotic	Alkalotic	Control
Males	113±13	125±3.6	125±16
Females	143±13	149±23	140±11

In the blood of acidotic, alkalotic and control rats the same concentration of citrate was found (Table 2). There were no sex differences in the level of citrate in blood.

Table 3

Oxidation of citrate and succinate by the homogenates and mitochondria of the kidney and liver from acidotic, alkalotic and control rats

Each number represents a mean from 3 animals, ± S. E. The values expressed as μl. O_2 per hr. per mg. nitrogen

Tissue preparation	Substrate added	Rats					
		Acidotic		Alkalotic		Control	
		Males	Females	Males	Females	Males	Females
Homogenate							
Liver	None	50±16	56± 6	48± 6	66± 6	40± 2	58± 6
	Citrate	71±13	75± 6	58± 5	79± 5	75± 2	75± 2
Kidney	None	19± 1	25± 6	19± 1	22± 2	19± 0.6	25± 2
	Citrate	66± 9	99±14	59± 3	81± 7	78± 6	83±17
Mitcchondria							
Liver	Citrate	55± 3	49± 4	58± 7	54± 2	55± 7	57± 2
	Succinate	127±16	157±18	148± 4	135± 3	153± 9	144±17
Kidney	Succinate	190±24	269±51	247±45	248±40	217±33	270±45

The experiments presented in Table 3 show the oxygen consumption by the homogenates and mitochondria of liver and kidney with either citrate or succinate as substrates. No significant differences could be found in the respiration of preparations obtained from either acidotic, alkalotic or control rats.

DISCUSSION

Many factors such as the diet, vitamin D, parathyroid hormone, and disturbances of acid - base balance influence the amount of citrate excreted in the urine [5]. The experiments performed so far have been carried out on human subjects or experimental animals acidified or alkalized in various ways for short periods of time. A marked decrease of citrate excretion in acidosis and an increase in alkalosis have been shown by Clarke *et al.* [2] and Crawford *et al.* [3]. In our experiments the same differences persisted also during acidification and alkalization lasting for over 6 months. It was also found that the acidotic females excreted much more citrate than acidotic males. This sex difference was smaller in the control group and almost insignificant in the alkalotic group.

The greater citrate excretion by females than by males was accompanied by increased calcium excretion. The amount of inorganic phosphate in the urine was lower in the alkalotic rats, the excretion of this compound seems, however, to be independent of the sex.

Prolonged acidification and alkalization was without effect on citrate level in the blood in both sexes despite the fact that the differences in the amounts excreted in the urine were very marked.

The acidification or alkalization of animals had no influence on the ability of liver or kidney to oxidize citrate and succinate. Therefore the differences in the excretion of citrate between acidotic and alkalotic rats and between males and females can not be explained by the changes in the oxidative activity.

A higher excretion of citrate in females than in males may be due to the influence of female sex hormones. Shorr *et al.* [8], Edwards *et al.* [4] and Panagopoulos *et al.* [6] demonstrated an increased citrate excretion in women after administration of therapeutical doses of oestradiol benzoate.

SUMMARY

1. In rats prolonged acidosis caused a decrease of citrate excretion in the urine, whereas prolonged alkalosis caused a marked increase.

2. The excretion of citrate was higher in acidotic females than

in males. Smaller sex differences were found in the control rats and none in the alkalotic animals.

3. The concentration of citrate in the blood and the ability of kidney and liver mitochondria and homogenates to oxidize citrate were the same in acidotic, alkalotic and control animals.

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WYDALANIE CYTRYNIANU PRZEZ SZCZURY PODDANE DŁUGOTRWAŁEMU ZAKWASZANIU I ALKALIZOWANIU

Streszczenie

1. Długotrwałe zakwaszenie szczura powoduje spadek, natomiast alkalizacja wzrost wydalania cytrynianu z moczem.

2. W grupie zakwaszonej wydalanie cytrynianu jest większe u samic niż u samców. Mniej wyraźne różnice występują w grupie kontrolnej; brak ich w grupie alkalizowanej.

3. Stężenie cytrynianu we krwi oraz jego utlenianie przez mitochondria i homogenaty wątroby i nerki w badanych grupach nie wykazują istotnych różnic.

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L. ŻELEWSKI and J. UMIASTOWSKI

DECREASE OF CITRATE EXCRETION IN THE URINE OF FEMALE RATS AFTER OESTRADIOL BENZOATE ADMINISTRATION

Department of Biochemistry, Medical School, Gdańsk

The results of earlier studies suggested some connection between oestrogens level in the organism and urine citrate excretion. An increase of citrate excretion in women both in the middle of the menstrual cycle [7] and after therapeutical doses of oestradiol benzoate [5] has been reported. In our previous experiments [9] on rats submitted to prolonged acidosis females excreted more citrate than males. The subject of the present communication is the influence of large doses of oestradiol benzoate on the excretion of citrate in the urine of female rats.

MATERIALS AND METHODS

In each series of experiments eight female, litter-mate, albino rats fed with a standard diet [9] have been used. The animals, aged 3 months and weighing 150 - 220 g. were divided into two equal groups, one of which served as controls.

During the experiments the rats were kept in metabolic cages and the urine was collected into cylinders containing 1 ml. of 1 N-H_2SO_4. Oestradiol benzoate dissolved in arachis oil was twice injected intramuscularly (with one day's pause), each dose containing 10 mg. per kg. body weight.

Citric acid in the urine was estimated according to Beutler *et al.* [1].

Reagents. One of the preparations of crystalline oestradiol benzoate was a gift from Hoechst A. G., Frankfurt am Main; the other one was purchased from Warszawskie Zakłady Farmaceutyczne.

Arachis oil was from Warszawskie Zakłady Farmaceutyczne; its properties as specified by the producer were as follows: specific gravity, 0.911 - 0.915; iodine number, 85 - 99; acid number, less than 2; saponifi-

cation number, 188 - 196; refractive index, 1.468 - 1.472. Other reagents were from Fabryka Odczynników Chemicznych in Gliwice.

RESULTS

The data presented in Table 1 concern eight rats, four of which were given oestradiol benzoate in arachis oil, and the other four, not subjected to injections, served as controls. Estimations of citrate were carried out for three days before the administration of oestradiol and seven days after the first injection. On the same days citrate was estimated in the urine of control rats. In rats treated with oestradiol benzoate a marked decrease of citrate excretion was observed on the third day, the lowest values being found on the fourth and fifth day.

Table 1

Effect of oestradiol benzoate on the citrate excretion in the urine of female rats

Oestradiol benzoate (10 mg. per kg. body weight) was injected on the days marked with an asterisk. The values represent μmoles citric acid per kg. body weight per day. In the lower part of the Table the Student's *t* values are given

Treatment	Rat no.	Day of experiment									
		1	2	3*	4	5*	6	7	8	9	10
		Citrate excretion									
Oestradiol benzoate	1	585	520	598	495	318	266	171	171	171	321
	2	350	308	287	270	213	58	18	11	26	29
	3	450	467	595	500	390	280	218	193	208	266
	4	396	442	412	240	187	125	78	115	222	354
None	5	530	520	542	560	700	495	520	320	332	520
	6	800	700	730	675	865	615	667	270	323	352
	7	323	240	342	574	495	407	307	255	173	385
	8	343	353	350	410	406	292	276	270	219	292

	Day after the first injection						
	1	2	3	4	5	6	7
	Student's *t* value						
Oestradiol benzoate	1.73	5.47	13.0	11.4	12.5	4.89	3.79
None	1.08	4.28	0.48	1.57	1.74	2.69	0.73

The values of Student's function t were calculated from the equation:

$$t = \frac{|\overline{X} - \mu|}{\sigma} \sqrt{n-1}$$

where $\overline{X}$ is the mean of the differences between the urine citrate content before the administration of oestradiol and the content on a particular day after the injection; μ denotes an expected mean of the differences for a population on which oestradiol benzoate has no influence, hence in this case $\mu = 0$; σ is the standard deviation of the differences and n is the number of rats. If the value of t is higher than 3.182, then for 3 degrees of freedom the hypothesis that oestradiol has no influence on the urine citrate excretion may be rejected with the probability of 0.95. If $t \geqslant 5.841$ the corresponding probability is equal to 0.99 or more [6].

The results collected in Table 2 also concern eight rats divided into two groups. After the daily urine citrate excretion had been estimated

Table 2

Effect of oestradiol benzoate on citrate excretion in the urine of female rats

Oestradiol benzoate in arachis oil (10 mg. per kg. body weight), or arachis oil alone, were injected on the days marked with an asterisk. The values represent μmoles citric acid per kg. body weight per day.
In the lower part of the Table the Student's t values are given

Treatment	Rat no.	Day of experiment						
		1	2*	3	4*	5	6	7
		Citrate excretion						
Oestradiol benzoate in arachis oil	1	450	432	400	255	151	193	182
	2	385	348	291	192	130	62	94
	3	365	385	322	244	151	68	78
	4	495	417	450	219	182	229	255
Arachis oil alone	5	805	817	564	564	677	682	630
	6	457	495	292	407	313	510	427
	7	400	276	286	296	455	354	312
	8	203	276	270	193	130	135	108

	Day after the first injection				
	1	2	3	4	5
	Student's t value				
Oestradiol benzoate in arachis oil	2.38	7.05	11.6	11.7	11.2
Arachis oil alone	2.09	2.43	1.73	1.43	2.34

for two days, oestradiol benzoate in arachis oil was injected to one group of rats, and arachis oil alone to the control group. After oestradiol injections the urine citrate excretion decreased similarly as in the former experiments and the calculated values of t allow to reject the hypothesis that oestradiol has no effect on urine citrate excretion, within the same confidence limits.

Table 3

Effect of oestradiol benzoate on the citrate excretion in the urine of female rats

The rats were injected oestradiol benzoate in arachis oil or arachis oil alone, on the second and fourth day of experiment. The values represent μmoles citric acid per kg. body weight per day. In the lower part of the Table the Student's t values are given

Treatment	Rat no.	Day of experiment			
		1	2	6	7
		Citrate excretion			
Oestradiol benzoate in arachis oil	1	436	442	115	73
	2	680	660	680	485
	3	374	333	224	208
	4	441	390	250	161
Arachis oil alone	5	313	367	348	370
	6	640	807	740	510
	7	213	260	208	208
	8	323	405	317	354

	Day after the first injection	
	4	5
	Student's t value	
Oestradiol benzoate in arachis oil	1.90	13.7
Arachis oil alone	1.30	2.84

In the third series of experiments (Table 3) the citrate excretion was estimated for two days before oestradiol benzoate or arachis oil alone have been administered, and on the fourth and fifth day after the first injection. On the fourth day a fall in urine citrate was observed in all the rats but one, and there was a significant decrease in all rats on the fifth day. The values of t for this series of experiments allow to draw the same conclusion as for the former two series.

The results of all three series of experiments have shown that oestradiol benzoate administered to female rats in two single doses of 10 mg. per kg. body weight each, caused a decrease of urine citrate excretion. The comparison of the *t* values for the rats to which no injections were made with these for the rats injected with arachis oil indicates that the arachis oil was without any effect on the urine citrate excretion.

DISCUSSION

The results obtained in rats after administration of large doses of oestradiol benzoate differed from those observed on human subjects. An increase of urine citrate excretion in women was observed by Shorr *et al.* [7] in the middle of menstrual cycle; similar increase was observed in women after administration of therapeutical doses of oestradiol benzoate. Panagopoulos *et al.* [5] explained the increase of citrate excretion as caused by the decrease of acetylation reactions and subsequent increase of citrate synthesis from active acetate and oxaloacetate. The decrease of urine citrate in rats observed in our experiments may be explained by direct or indirect influence of oestrogens on filtration or reabsorption of citrate in the kidney, by their inhibiting influence on the synthesis of citric acid or by increased oxidation of citrate.

We do not know of any experimental data indicating the possible influence of oestrogens on renal filtration or reabsorption. The *in vitro* experiments of Cochran & DuBois [2] provide some evidence against the inhibition of citrate synthesis; in the presence of fumarate and pyruvate, oestradiol was without effect on the citrate formation. On the other hand, increased citrate oxidation in the presence of oestradiol was observed by Jonas & Hollander [4].

The observed effect of large doses of oestradiol may by explained by increased citrate oxidation. Villee & Hagerman [8] reported an oestrogen-sensitive transhydrogenase which catalyses the reaction

$$\text{reduced TPN} + \text{DPN} \rightleftharpoons \text{TPN} + \text{reduced DPN}.$$

Greengard *et al.* [3] reported a regulatory effect of hypophysis on the DPN level. An increased DPN level in liver of hypophysectomized rats was observed; it was enhanced by the intraperitoneal administration of nicotinic acid amide. It can be assumed that large doses of oestrogen increased the DPN concentration through the inhibition of hypophysis trophic hormones. The increase of DPN might shift the reaction catalysed by the oestrogen-sensitive transhydrogenase toward oxidized TPN which is necessary for the oxidation of citric acid.

Such a mechanism might explain the necessity of large doses of oestrogens and also the tardy appearance of the maximum decrease of urine citrate excretion.

SUMMARY

A decrease of urine citrate excretion has been observed in female rats which were given two doses of 10 mg. oestradiol benzoate per kg. body weight twice every other day. The lowest excretion was observed on the fourth and fifth day after the first injection.

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SPADEK WYDALANIA CYTRYNIANU W MOCZU SZCZURÓW PŁCI ŻEŃSKIEJ PO PODANIU BENZOESANU ESTRADIOLU

Streszczenie

1. U szczurów płci żeńskiej, którym podawano dwukrotnie w odstępach dwudniowych benzoesan estradiolu (10 mg/kg wagi) zaobserwowano spadek wydalania cytrynianu z moczem.

2. Najniższe wartości wydalonego cytrynianu zaobserwowano na czwarty lub piąty dzień od podania pierwszej dawki.

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ALICJA GRUDZIŃSKA

UNDETERMINED NINHYDRIN-POSITIVE COMPOUND IN HUMAN SALIVA

Department of Biochemistry, Medical School, Gdańsk

Berry in 1951 [3] stated that in human saliva an amino acid of unknown nature is always present in relatively high concentration. In the previous paper [8] it was demonstrated that in deproteinized salivas an unidentified ninhydrin-positive compound is present, which, however, could not be detected in the urine.

Moor & Gilligan [11] reported that the main amino acid in human saliva is the γ-aminobutyric acid, which together with glycine forms 2/3 of amino acids nitrogen. This view, although not confirmed by other authors, is quoted in reviews by Elliot & Jasper [7] and Rauch [13].

The present communication describes an attempt to characterize the unknown ninhydrin-positive compound and to reinvestigate the occurrence of γ-aminobutyric acid in saliva.

MATERIALS AND METHODS

Mixed, resting saliva of 20 healthy adults of both sexes aged 18 - 35 years was examined. Proteins were removed by adding to 10 ml. of saliva 40 ml. of 96% ethanol, and centrifuged. The supernatant was evaporated on the water bath at 50 - 60°. The dry residue was dissolved in 10% aqueous solution of *iso*propanol acidified with HCl (4 ml. of 6 N-HCl per 1000 ml. of *iso*propanol) so that 1 ml. of the solution was corresponding to 10 ml. of saliva. In the preliminary estimation the saliva after deproteinization was desalted on Dowex 50 column, but as no differences were found on chromatograms, in further experiments the desalting was omitted.

The chromatographic separation of amino acids was carried out on 3 cm. wide strips of Whatman no. 1 filter paper; the volumes applied

were 20, 40 and 60 µl. The ascending chromatography in *n*-butanol - - acetic acid - water (4:1:1, by vol.) after Berry *et al.* [4] was used. This solvent system, which was always used for the first separation of saliva amino acids, is further referred to as the Ist solvent system. The chromatograms were developed three times for 12 hr., sprayed with 0.1% solution of ninhydrin in acetone, and then dried for 5 min. at 60°.

From the chromatograms developed in the Ist system the areas containing the examined *X*-compound were cut out, divided into small strips and eluted with 10% aqueous solution of *iso*propanol. After 24 hr. the fluid was decanted and evaporated at 60°. The dry residue was dissolved in *iso*propanol. The amount of *iso*propanol was the same as that used for chromatography in the Ist solvent system. The solution was rechromatographed in 9 solvent systems (for their composition see Table 1). The rechromatography was also performed by the two- -dimensional technique, as used by Moor & Gilligan [11]. The solvent in the first run was phenol and in the second one collidine, according to Dent [6].

Table 1

Solvent systems in the one-dimensional chromatography used for the rechromatography of the eluate

The composition of solvent systems is given on a v/v basis

Symbol	Composition	References
A	*n*-Butanol - formic acid - water (75:15:10)	Awapara & Sato [2]
B	*n*-Butanol - acetic acid - water (4:1:5)	Slotta & Primosigh [15]
C	*n*-Butanol - acetic acid - water (6:1:2)	Asen *et al.* [1]
D	*n*-Butanol - methanol - water (2:2:1)	
E	96% Ethanol - water (70:30)	
F	*tert.* Butanol - formic acid - water (70:15:15)	
G	Pyridine - isoamyl alcohol - water - dimethylamine (10:10:7:0.3)	Block *et al.* [5]
H	Pyridine - acetic acid - water (50:35:15)	
I	2,6-Lutidine - collidine - water (1:1:1) - 2% dimethylamine	Dent [6]

To identify the chromatographically separated compounds the following tests were made: ninhydrin test; Reindel & Hoppe iodine test [14]; photography in UV light; aniline phthalate test [12]; Bial's orcinol reaction [5 (p. 136)]; reaction with alkaline solution of picric acid [10]; test with phosphomolybdenic acid for choline [9]; test with hydroxylamine hydrochloride for choline esters [16].

The hydrolysis of the eluate of the X-compound was performed at 100° in 1 N-HCl for 1 hr. in 2 N-HCl for 15, 30 and 90 min. and in 6 N-HCl for 12 hr.

Reagents. α-Aminobutyric acid and histidine (Fluka); α-amino*iso*-butyric acid, β-aminobutyric acid, β-amino*iso*butyric acid and γ-amino-butyric acid (Hoffman la Roche); aspartic acid, leucine, and lysine (Schuchard); alanine, glycine, and serine (Merck); glutamic acid and threonine (Chemapol); valine (U.S.S.R.); ninhydrin (B.D.H., London).

RESULTS

On the chromatograms developed in the Ist solvent system four amino acids were located in the vicinity of the X-compound, namely β-aminobutyric acid, β-amino*iso*butyric acid, γ-aminobutyric acid and valine. These four amino acids, the eluate of the X-compound and also α-aminobutyric acid and α-amino*iso*butyric acid were chromatographed in 9 various solvent systems and localized with ninhydrin. The respective R_F values, given in Table 2, show that the X-compound is not identical with any of the compared amino acids, as there is always a system in which the unknown compound has a different velocity than the compared amino acids.

The failure to identify the X-compound with the known and available amino acids suggested its peptide nature. Therefore hydrolysis of the eluate was carried out, but even after the 12 hr. hydrolysis in 6 N-HCl at 100° the R_F value of the ninhydrin-positive spot was not changed.

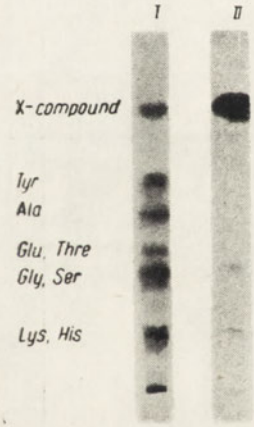

Fig. 1. Chromatogram of saliva amino acids developed in the Ist solvent system; (I), stained with ninhydrin; (II), stained with iodine

The next attempt to identify the X-compound was the iodine test of Reindel & Hoppe [14]. The iodine test is positive with peptides and also with some amino acids. It appeared that in the Ist solvent system the ninhydrin-positive X-compound was located in the same place as the iodine spot (Fig. 1), whereas in other solvent systems significant

Table 2

R_F of the X-compound in saliva developed in 9 solvent systems as compared with R_F of amino acids migrating in its vicinity in the Ist solvent system

Composition of the solvent systems as given in Table 1; (—), negative reaction with ninhydrin (Nin) or iodine (I_2).

Compound	Rechromatography in the solvent system																	
	A		B		C		D		E		F		G		H		I	
	Nin.	I_2	Nin.	I_2	Nin.	I_2	Nin.	I_2	Nin.	I_2	Nin.	I_2	Nin.	I_2	Nin.	I_2	Nin.	I_2
Eluate from the X-compound	0.36	0.37	0.32	0.39	0.28	0.40	0.67	0.57	0.75	0.62	0.70	0.69	0.20	0.41	0.66	0.64	0.35	0.45
β-Aminobutyric acid	(—)	0.36	(—)	0.38	(—)	0.35	(—)	0.56	(—)	0.64	(—)	0.70	(—)	0.26	(—)	0.65	(—)	0.39
β-Aminoisobutyric acid	0.36	0.36	0.38	0.38	0.33	0.33	0.56	0.56	0.64	0.64	0.69	0.69	0.27	0.27	0.64	0.64	0.39	0.39
γ-Aminobutyric acid	0.28	0.28	0.33	0.33	0.30	0.30					0.67	0.67	0.22	0.22	0.72	0.72		
α-Aminobutyric acid	0.43	(—)	0.32	(—)							0.72	(—)	0.32	(—)				
α-Aminoisobutyric acid	0.43	(—)	0.31	(—)	0.34	(—)					0.72	(—)	0.31	(—)				
Valine	0.59	(—)	0.52	(—)	0.45	(—)					0.72	(—)	0.39	(—)				

differences in R_F values of both spots were found. The eluate of the X-compound rechromatographed in the systems B, C, D, E, G, and I gave two spots: the first was ninhydrin-positive and iodine-negative, the second one was ninhydrin-negative and iodine-positive. R_F of both spots are given in Table 2.

It appears that the undetermined X-compound separated from chromatograms of deproteinized saliva consists of at least two separate compounds; one of them reacted with ninhydrin and was not found in urine, the other gave an iodine reaction and was present in all the examined urines.

The chromatograms of the five C_4 amino acids and valine were stained with iodine. Positive reactions were obtained with the two β-amino acids and the γ-amino acid. α-Amino acids and valine were iodine--negative. In the 9 solvent systems used none of the three iodine-positive amino acids had the same R_F as the X iodine-positive spot.

Since none of the two X-compounds could be identified with the standard amino acids used, some tests for other nitrogen compounds were performed. The photography of the chromatograms in the light of a quartz lamp has shown that none of the X-compounds absorbed the ultraviolet light. The Bial's orcinol reaction was negative, and no reduction of aniline phthalate was observed. Tests with phosphomolybdenic acid for choline, and with hydroxylamine hydrochloride for choline esters were negative, as well as the reaction with picric acid for creatinine.

The presented experiments, contrary to the results of Moor & Gilligan [11], did not show the presence of γ-aminobutyric acid in human saliva. To elucidate this discrepancy the chromatographic technique as used by these authors was employed and it was found that the eluate of the X-compound separated in the Ist solvent system, migrated on two-dimensional chromatograms in the same way as γ-aminobutyric acid. However, the paper chromatography in various solvent systems indicated that the ninhydrin-positive spot is not γ-aminobutyric acid.

The observation that γ-aminobutyric acid does not occur in human saliva is in agreement with the generally accepted view that this amino acid is present only in the tissues of the central nervous system, and even in the cerebrospinal fluid it is found only in very small quantities.

SUMMARY

Using paper chromatography in various solvent systems a ninhydrin--positive compound of human saliva, identified by some authors as γ-aminobutyric acid, was found to be another but not yet identified

compound. It was further separated into two different spots, one ninhydrin-positive and one iodine-positive.

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NIEZIDENTYFIKOWANY NINHYDRYNO-POZYTYWNY ZWIĄZEK W ŚLINIE CZŁOWIEKA

Streszczenie

Stwierdzono, że występujący w ślinie człowieka ninhydryno-pozytywny związek nie jest, jak przypuszczali niektórzy autorzy, kwasem γ-aminomasłowym. Przy użyciu różnych układów rozpuszczalników udało się rozdzielić chromatograficznie ten związek na dwa składniki, z których jeden dawał dodatnią reakcję z ninhydryną, a drugi z jodem.

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M. SZYMONA

PURIFICATION AND PROPERTIES OF THE NEW HEXOKINASE UTILIZING INORGANIC POLYPHOSPHATE

Department of Physiological Chemistry, Medical School, Lublin

The presence in *Mycobacterium phlei* of a new enzymatic system phosphorylating glucose with inorganic polyphosphate was first demonstrated by Szymona [18]. The same activity was observed in other *Mycobacteria* and in the virulent strain of *Corynebacterium diphtheriae* [21]. Recently, Dirheimer & Ebel [2] confirmed these findings in both *M. phlei* and *C. xerosis*. Preliminary data on the preparation and properties of the corresponding enzyme were presented earlier [19, 20].

In this paper the results of a more detailed study on the polyphosphate hexokinase have been described.

MATERIALS AND METHODS

Bacterial preparations. M. phlei was grown at 37° on solid Lowenstein media in Roux flasks for about two weeks. The bacilli were collected by centrifugation, washed with distilled water and dried with cold acetone. The resulting acetone powder could be stored for months in the refrigerator without appreciable loss of activity. Cell-free extracts were prepared either by homogenizing acetone-dried cells (10% suspension in tris[1]-HCl buffer, 0.05 M, pH 7.4) in the Potter apparatus for 10 min. or by grinding each gram of acetone powder with 3 g. powdered glass with gradual additions of the same buffer up to about 25 ml. over a period of 15 min. In either case the homogenates, pH 7.4, were centrifuged at 20 000 *g* for 30 min. in the presence of

[1] Abbreviations used are: tris, tris(hydroxymethyl)aminomethane; ortho-P, orthophosphate; poly-P, polyphosphate; TCA, trichloroacetic acid; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5′-monophosphate; G-6-P, glucose-6-phosphate.

deoxyribonuclease added to facilitate the clear-cut sedimentation of cell debris (about 5 mg. per 100 ml. homogenate).

Reagents. Crystalline tripolyphosphate ($Na_5P_3O_{10}.6\,H_2O$) and poly-P in the form of Graham salt, $Na(PO_3)_x$, were kindly donated by Prof. E. Thilo. The Graham salt was found to be contaminated with low molecular phosphates which accounted for about 15% of total P. When analysing 12 μg.P of the Graham salt by paper chromatography, several phosphate spots were detected in addition to the bulk of high molecular poly-P which remained at the start line. The impurities could be eliminated by dialysis or by precipitating high molecular poly-P with $BaCl_2$ at pH 2.5 and treating the washed insoluble with an aqueous suspension of Dowex 50 (H^+ form). The minimum average molecular weight, as determined potentiometrically [25] on such a Dowex 50 treated supernatant was established to correspond to 9 phosphate groups per molecule. Taking into account the possibility of a degradation during the analytical procedure, the obtained value will be among the lowest molecular weights reported for Graham salt in the literature [3, 22]. Stock solutions were prepared by dissolving 100 mg. of the salt in 5 ml. water with an addition of NaOH solution to bring pH to about 7. They could be stored for weeks in the cold without appreciable decomposition. In most experiments dialysed poly-P solutions were employed.

ATP and AMP were used as the sodium salts (C. F. Boehringer & Soehne). The barium salt of ADP (Light) was dissolved in dilute HCl, solid Na_2SO_4 added, and the supernatant after centrifugation neutralized with NaOH. Calcium phosphate gel was prepared according to Keilin & Hartree [9] and used after 8 months of storage at 2 - 4° (14.4 mg. dry matter per 1 ml. suspension). Ammonium sulfate was the Merck reagent. DEAE-cellulose was obtained from the Serva Co. (Entwicklungslabor. Heidelberg), and Dowex 50 from the Dow Chemical Co. Deoxyribonuclease, salt-free, and ribonuclease, protease- and salt-free, were purchased from the Mann Research Laboratories. Protamine sulfate was a reagent of the Nutritional Biochemicals Corporation. Apyrase was purified from potatoes according to Székely [17]. The preparation contained 184 units per ml. (specific activity 123 units per mg. protein). If not otherwise indicated, tris-HCl mixtures served as buffer (Light). Powdered glass (50/120 mesh, J. W. Towers) proved satisfactory for preparing buffered homogenates. Sugar substrates were as follows. D-Glucose (anhydr. puriss., Gliwice); D-glucosamine (Light); D-mannose (Difco Lab.); D-fructose (B.D.H.); glucose-6-phosphate, barium salt (B.D.H.); potassium gluconate (recrystallized from methanol) was obtained from Prof. M. Doudoroff. Glass distilled water was used throughout the study. All other reagents were high grade commercial preparations.

Methods. Protein was determined by the method of Lowry *et al.* [13]. The content of nucleic acid was estimated in the Hilger spectrophotometer. Phosphates were measured by the method of Fiske & Subbarow [4] using 1% metol (methyl *p*-aminophenol sulfate) in 3% $NaHSO_3$ as a reducing agent.

Measurement of hexokinase activity. The phosphorylation was followed by the disappearance of acid-labile phosphate that was not accounted for as ortho-P. If not otherwise stated the incubation mixtures contained in a total volume of 0.48 ml.: tris buffer, pH 8.5, 40 μmoles; glucose, 6 μmoles; $MgCl_2$, 2 μmoles; Graham salt in amount corresponding to 3 μmoles of ortho-P; KCl, 140 μmoles, and the enzyme the addition of which started the reaction. In some cases controls without enzyme or without substrate were included. At zero time and at two equal time intervals of incubation (short enough to indicate a linear course of the reaction) 0.1 ml. aliquots were pipetted into 2 ml. water with subsequent addition of 0.25 ml. of 25% TCA. Usually 1 ml. volumes were taken in duplicate for ortho-P and acid-labile P determinations. Acid-labile P was determined after 10 min. hydrolysis at 100° in 1 N-H_2SO_4. In order to assay the activity of the homogenates in presence of endogenous polyphosphate only, 0.1 ml. aliquots of incubation mixtures were pipetted into centrifuge tubes with 2.9 ml. water followed by an addition of 1 ml. 4 N-H_2SO_4 and heating for 15 min. at 100°. The precipitates were then discarded by centrifugation and the supernatants were used for P determinations. Ortho-P was determined in the same way except that boiling was omitted. In the MGK colorimeter (Medizinische Geräte Fabrik VEB, Berlin, Type AKC) 0.5 μmole phosphate gave a scale reading of 330 with the filter 665.

Chromatography. Descending and ascending chromatograms on Whatman no. 1 filter paper were performed. For the analysis of phosphorus compounds a solvent consisting of 40 ml. *iso*propanol, 10 ml. water, 20 ml. 20% TCA and 0.3 ml. 25% ammonia was used [5]. The spots were detected by means of the Hanes & Isherwood reagent [6] and a UV lamp. In preliminary experiments with homogenates and crude extracts, acetone - pyridine - water (2:1:1, by vol.) proved to be convenient for quick chromatography of deproteinized incubation mixtures. Sugar substrates as well as their reducing esters were located with alkaline silver nitrate spray [23].

In some cases glucose was determined by the method of Somogyi [16] and Nelson [15] after precipitating the proteins and phosphoric esters with barium hydroxide and zinc sulfate [24].

Other experimental details are given in the table or figure legends.

RESULTS

Phosphorylation in homogenates and cell-free extracts

When acetone powder homogenates of the Lowenstein grown bacilli were incubated with glucose, a phosphorylation took place at the expense of endogenous phosphate donor. The volutin granules disappeared and the acid-labile P decreased. The product which was a mixture of both glucose-6- and fructose-6-phosphate accumulated on incubation and could be readily detected by paper chromatography. The overall results were similar to those observed with acetone-dried *C. diphtheriae* [21]. The quantitative data concerning the endogenous activity of acetone-dried *M. phlei* are shown in Table 1. The enzyme eventually responds to an addition of exogenous phosphate donor, be it ATP or Graham salt. The rates of glucose phosphorylation were determined in cell-free extracts. When in an early experiment 0.3 mg. protein of cell-free extract was incubated with 10 μmoles glucose, 2 μmoles $MgCl_2$, 4.7 μmoles acid-labile P of Graham salt (undialysed), 120 μmoles KCl and 30 μmoles buffer, pH 9, in a total volume of 0.73 ml., the reaction proceeded at a rate of about 0.21 μmole/min./mg. protein. It was no longer proportional to time after 40 min., and ceased when about 65% of the Graham salt was consumed for phosphorylation. In the presence of appropriate amounts of extract (about 0.1 mg. protein) and under optimal assay conditions the initial rates may exceed 0.35 μmole/min./mg. protein for Graham salt and about half that value for ATP. The mechanism of the reaction was studied with partially purified preparations. An arbitrary unit of activity was taken as that

Table 1

Endogenous phosphorylation of glucose in acetone-dried homogenates of Mycobacterium phlei

Incubation mixture in a total volume of 0.62 ml. contained 30 μmoles buffer, pH 9, 80 μmoles KCl, 4 mg. acetone powder, and where indicated, 5 μmoles glucose. Incubation time 2 hr. Figures represent mg. P per 100 mg. acetone powder. 15-min. P includes ortho-P, acid-labile P of inorganic poly-P, and P split off from other compounds e.g. nucleic acids, during 15 min. of heating at 100° in 1 N-H_2SO_4

Sample	Without glucose added		With glucose added	
	Ortho-P	15-min. P	Ortho-P	15-min.P
Initial	0.267	1.271	0.278	1.255
Final	0.630	1.180	0.406	0.661

amount of enzyme which causes the phosphorylation of one μmole glucose in 60 min. at 37°.

Phosphatase activities were checked on several occasions and found to be too low to invalidate the assay procedure. When in the standard assay mixture glucose-6-phosphate in concentration of 5 μmoles was substituted for Graham salt and incubated for 30 min. in the presence of 0.4 mg. protein of crude extract, as little as 0.06 μmole ortho-P was liberated. Phosphatase activity toward purified poly-P as measured by the increase of ortho-P in the absence of glucose was negligible as well. Marked hydrolysis was sometimes noticed, however, when incubating ATP or undialysed Graham salt. With the use of crude extract the rate of ATP hydrolysis constituted in some cases nearly 30% of the rate of ATP utilization. This ability to split ortho-P could eventually be removed from a two-fold purified fraction by heating it for several minutes at 70°.

Partial purification

Crude, strongly opalescent, yellow cell-free extract, pH 7.4, containing about 3 - 4 mg. protein per ml. was treated with two third volume of neutral, saturated at 2° ammonium sulfate and the precipitate (fraction 2a) after centrifugation discarded. To the supernatant ammonium sulfate powder was added slowly with stirring to reach 80% saturation and the precipitate (fraction 2b) dialysed against water for several hours. The dialysis residue was adjusted to pH about 6 with M-acetate buffer and a solution of 2% protamine sulfate, pH 5, was added dropwise with stirring until the ratio of the optical densities at 280 mμ to that at 260 mμ, as repeatedly determined in the supernatant fluids, was at least 0.70. Attempts to increase the ratio above 0.75 usually resulted in severe losses of protein. The protamine soluble was then refractionated with ammonium sulfate at pH 5 - 6. Fractions precipitating at 45 - 55% (4b) and 55 - 70% (4c) saturation were collected. In the next step small volumes of the most active fractions (4b) were heated in a water bath at 70° for about 30 sec. with stirring. A coagulation occurred when the temperature inside the tubes reached about 60°, whereupon the solutions were rapidly chilled and spun down. Further purification involved dialysis against water for several hours and adsorption on tri-phosphate calcium gel. To each ml. of the enzyme solution containing about 4 mg. protein, pH 5.6 as adjusted with M-acetate buffer, gel suspension was added (1 mg. dry weight per 1 mg. protein) and after 10 min. centrifuged. The supernatant was discarded and the precipitate was successively washed twice with 1 ml. volumes of

0.05 M-buffer, pH 7.4, and once with 0.1 M-buffer, pH 8.0. The elution of enzyme was subsequently achieved with the use of 0.4 M-buffer, pH 9.5. The eluate was then dialysed against 1.5% KCl in 0.025 M-buffer, pH 7.4, and stored in the frozen state for days without considerable loss of activity. All manipulations except heat treatment were carried out in the cold room at 2 - 4°. Table 2 summarizes the results of one of the fractionations. It is evident that the specific activity has increased about 65 times with respect to the original activity of crude extract. The optical density ratio of the readings taken at 280 mμ and 260 mμ was 0.64 and no absorption in the visible region was found.

Table 2

Purification of the polyphosphate hexokinase

Fraction 0-45% $(NH_4)_2SO_4$ sat. of step 4 exhibited about 10% activity and was rejected. Fraction 55-70% sat. (4c) was eventually preserved for further purification or in some cases pooled with fraction 4b. Details see Methods. Enzyme unit: μmoles glucose phosphorylated in 1 hr. at 37°. Specific activity is defined as units of enzyme per mg. protein

Fraction	Protein (mg.)	Total units	Specific activity	Yield (%)
1. Cell-free extract	375	10125	27	
2. First ammonium sulfate precipitation				
b) 40 - 80% saturated	184	9568	52	94
3. Protamine soluble	136	6800	50	67
4. Second ammonium sulfate precipitation				
b) 45 - 55% saturated	45.5	3412	75	34
5. Heat treated supernatant	16.5	3234	196	32
6. Ca phosphate gel eluate	0.95	1729	1820	17

In an alternative procedure DEAE-cellulose was used. In one of the experiments 10.2 mg. protein of a 4-fold purified enzyme preparation (fraction 2b, heated, dialysed against water) was applied in a volume of 2.3 ml. to the top of a DEAE column (1.5 × 10 cm.) which had been pretreated in the usual way and washed with water. The adsorption of enzyme was complete and no activity appeared in the first 20 ml. water washing. The elution of enzyme was carried out with a continuous KCl gradient as established by placing water in the mixing vessel and running in KCl with 0.05 M-buffer, pH 8. Five-ml. fractions were collected automatically in the cold (flow speed about 20 ml. per hour). The enzyme was present in the range of 13 fractions, respective KCl concentrations rising linearly from 0.13 M to 0.22 M. Its maximal activity indicated a 55-fold purification. The much lower activity toward ATP

appeared to be distributed over the same range of fractions. It was difficult to obtain accurate results, however, because of irregularities in the time course of phosphorylation with some fractions.

Activators and inhibitors

The enzyme requires magnesium ions and neutral salts for maximum activity. Table 3 shows the results of experiments with varying concentrations of magnesium chloride. An apparent K_m value as determined by the double reciprocal plot approximates 2×10^{-3} M.

The effect of KCl was examined at about this concentration of magnesium. It can be seen from Fig. 1 that KCl greatly stimulated the rate of phosphorylation and a maximum was obtained when the final

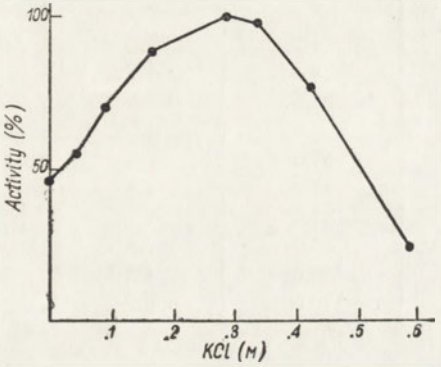

Fig. 1. Effect of KCl on the initial rate of glucose phosphorylation with inorganic poly-P. Reaction mixtures contained: indicated concentrations of KCl; buffer, pH 8.5, 40 μmoles; glucose, 6 μmoles; acid-labile P of Graham salt, 3 μmoles; $MgCl_2$, 1 μmole and 0.04 ml. of enzyme (fraction 4bc, dialysed) in a total volume of 0.48 ml.

concentration of potassium chloride amounted to about 0.29 M. When the quantity of $MgCl_2$ was doubled at this point, an increase of the rate became apparent (Table 4). Further additions of Mg^{2+} and/or inorganic poly-P resulted, however, in lower rates.

Magnesium can be replaced by manganese but not by calcium. The action of KCl can be duplicated by NaCl, Na_2SO_4, $(NH_4)_2SO_4$. Dialysed preparations are largely inactive unless supplemented with magnesium, maximum activity being attained in the presence of neutral salts at high ionic strength.

The enzyme is inhibited by heavy metals such as zinc or mercury. When 7 μg. protein of a gel eluate was incubated at pH 7 (succinate buffer) with $ZnSO_4$ in conc. 10^{-2} M for 20 min., 92% inhibition resulted. Sodium fluoride in conc. 1.6×10^{-2} M caused about 30% inhibition.

Table 3

The effect of magnesium-ion on the polyphosphate hexokinase

Incubation mixtures contained in addition to indicated concentrations of Mg^{2+} and poly-P (dialysed): buffer, pH 8.5, 40 μmoles; glucose, 6 μmoles; and 0.3 mg. protein of dialysed fraction 45-70% $(NH_4)_2SO_4$ sat.(4bc) in a total volume of 0.48 ml. Poly-P expressed as acid-labile P of Graham salt

Poly-P (mM)	$MgCl_2$ (mM)	μmole P/ml. taken up in	
		5 min.	10 min.
6.0		0.25	0.55
6.0	2.1	0.9	1.65
6.0	4.2	1.2	2.2
6.0	8.4	1.3	2.35
7.2	27.0	1.5	2.9
6.8	33.0	1.65	3.3
6.0	57.0	1.7	3.5
3.5	57.0	1.7	2.4
12.0	57.0	1.7	3.5
6.0	83.0	1.8	3.2
6.0	120.0	1.25	2.5
6.0	170.0	0.9	1.5

Table 4

The rate of glucose phosphorylation in presence of varying amounts of KCl, $MgCl_2$, and Graham salt

Conditions of the reaction as described in Table 3

Poly-P (mM)	$MgCl_2$ (mM)	KCl (M)	μg.P/ml./min.
6.0		0.29	1.2
6.0	2.1	0.29	14.6
6.0	4.2	0.29	17.5
6.0	3.8	0.19	15.6
3.0	4.2	0.29	15.8
6.0	1.6	0.29	12.6
6.8	4.2	0.29	16.9
14.0	4.2	0.29	13.0
14.0	8.4	0.29	15.8
14.0	13.0	0.29	13.7

Iodoacetate was ineffective when incubated for 30 min. in conc. 1.4×10^{-2} M at pH 8.

The region of optimum pH is rather broad and extends from 8 to 9 (Table 5).

Table 5

Effect of pH on the rate of phosphorylation of glucose with inorganic polyphosphates

Standard assay conditions except varying buffer (0.1 M). Acetate and carbonate were used at pH 5.6 and 10.0 respectively. Enzyme: 0.1 mg. protein of fraction 2, heated

pH	5.6	7.4	8.0	8.5	9.0	10.0
Relative activity	0.6	0.75	1.0	1.0	1.0	0.2

The most purified enzyme is unstable at alkaline pH values, losing half its activity at pH 9.5 within 24 hr. when stored in solution in the refrigerator. It was found active for several weeks when kept in the frozen state in the presence of KCl at neutral or slightly acidic pH's. Crude extracts, pH 7.4, can be stored in the cold for a few days with relatively small losses of activity.

Kinetics of the reaction

Fig. 2 shows the time course of glucose phosphorylation with the use of purified enzyme and dialysed Graham salt. It is evident from the Figure that about 80% of acid-labile P was taken up when the reaction ceased. At equilibrium an equivalent amount of glucose disappeared as determined with the Somogyi-Nelson reagent after Zn-Ba precipitation. Another experiment of this type involved, besides phosphorus determinations, the use of paper chromatography. To facilitate separation lower amounts of KCl were added to the incubation mixtures. The composition of the latter was as follows. Dialysed Graham salt in amount corresponding to 4.2 μmoles acid-labile P, 4 μmoles $MgCl_2$, 10 μmoles glucose, 80 μmoles of buffer, pH 8.5, 15 μmoles of KCl, and 0.15 ml. of enzyme in a total volume of 0.77 ml. A control was set up in parallel without glucose. Aliquots were analysed for acid-labile P disappearance and ortho-P increase. Virtually no ortho-P formation was found in either case. Again, as in the previous experiment, 81% of Graham salt has been utilized in the sample containing glucose with no change in the control. For chromatographic analyses 50 μl. volumes of the incubation mixtures were applied to Whatman paper and developed as in Methods.

When using the Hanes-Isherwood reagent and the UV test, no difference could be observed between the initial and final samples of the control. The sample incubated with glucose revealed the spot of G-6-P and

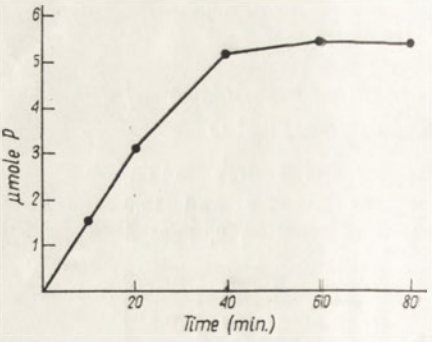

Fig. 2. Phosphorylation of glucose with inorganic polyphosphate. Buffer, pH 8.5, 120 µmoles; glucose, 30 µmoles; acid labile P of Graham salt, 6.7 µmoles; $MgCl_2$, 12.8 µmoles; KCl, 335 µmoles and 0.15 ml. of 1-month-old gel eluate. Total volume 1.51 ml.

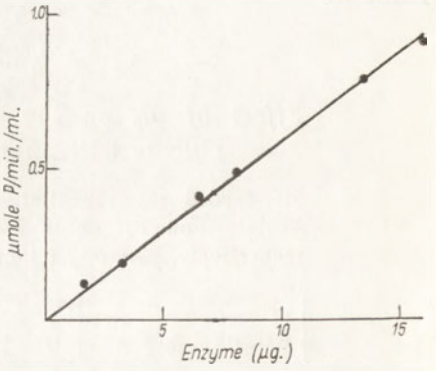

Fig. 3. Phosphorylation of glucose as a function of enzyme concentration. Standard assay conditions. Enzyme: gel eluate

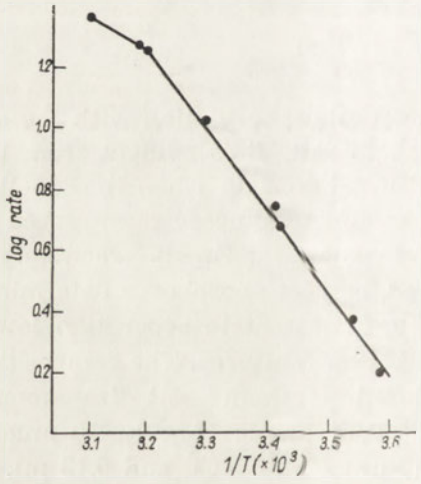

Fig. 4. Arrhenius plot for the effect of temperature. Standard assay conditions. Enzyme: gel eluate (20 µg. protein per ml. incubation mixture)

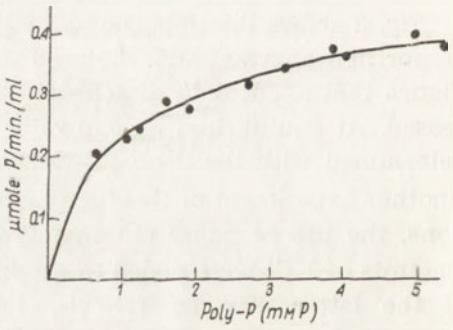

Fig. 5. Effect of poly-P concentration on the initial rate of glucose phosphorylation. Standard assay conditions except varying amounts of poly-P. Enzyme: gel eluate (13 µg. protein per ml. incubation mixture)

a small spot of tripolyphosphate as compared with the authentic compound. There was no Graham salt left after incubation.

The effect of enzyme concentration on the phosphorylation of glucose is represented in Fig. 3. The initial rate is directly proportional to the amount of enzyme over a ten-fold range of dilution.

The effect of temperature is shown in Fig. 4. The energy of activation for Graham salt as calculated from the Arrhenius plot amounts to 12 900 cal.

The dependence of rate on substrate concentration is shown in Fig. 5.

Specificity

All the enzymatic preparations tested catalysed the phosphorylation of both D-glucose and D-glucosamine. No phosphorylation of D-fructose, D-mannose, or D-gluconic acid could be detected. Glucose and glucosamine are probably phosphorylated by the same enzyme, since the rate of phosphorylation is virtually the same in the presence of both these substrates (Table 6). In another experiment in which 5 times lower concentrations of either glucose or glucosamine were used, essentially the same results were obtained.

Table 6

Phosphorylation of glucose and glucosamine in mixture

Figures in μmoles represent acid-labile P which disappeared on incubation with indicated sugar. Reaction tubes contained: 10 μmoles of either sugar, or 10 μmoles of each in mixture, 60 μmoles buffer, pH 8, 120 μmoles KCl, 12 μmoles of P of Graham salt, 6 μmoles of $MgCl_2$ and 0.05 ml. of enzyme (fraction 4bc, heated)

Time (min.)	Glucose (μmoles P)	Glucosamine (μmoles P)	Mixture (μmoles P)
5	1.4	1.45	1.4
10	2.85	2.8	2.9
20	5.8	5.6	5.7

The enzyme preparations were also capable of utilizing ATP and the question arose whether there were two separate enzymes operating. The activity toward ATP has been followed under a variety of conditions. It required magnesium ions and appeared to be stimulated by KCl, but the effect of potassium chloride was distinct at low Mg^{2+} concentrations only. The pH optimum lay in the same range from 8 to 9. Attempts of differential heating were unsuccessful as both activities remained unaffected after 2 min. at 60° or 1 min. at 70°. The heating of fraction

2b at 70° for 10 min. resulted in about 40% loss of each of the two activities. The inhibitory action of NaF appeared to be equal with either substrate. When a 2-fold purified enzyme was dialysed for 24 hr. against water neither activity was abolished. However, the ratio of the two activities as determined occasionally in various preparations was not constant and changed in favour of inorganic polyphosphate (Table 7). In fact, old DEAE eluates were much less active toward ATP. This difference of stability suggests the presence of two different hexokinases, one of which may be active with inorganic poly-P only.

Table 7

Comparison of activity with Graham salt and ATP

Standard assay conditions, except DEAE eluates which were incubated in a total volume of 1 ml. for 15 and 30 min. Same concentrations of acid-labile P of either substrate were used

Enzyme preparation	Ratio of activity Poly-P/ATP
Crude extract	1.0 - 2.0
Fraction 40 - 80% $(NH_4)_2SO_4$ sat.	2.5
Ca phosphate gel eluate	3.3
DEAE eluate, fresh	4.5
DEAE eluate, after 2 months of storage	~15.0

Another kind of evidence for the lack of interrelation between the activities comes from the experiment with purified potato apyrase (Table 8). It is clear that apyrase completely abolished the activity with ATP while that with Graham salt remained intact. At the same time apyrase has proved uncapable of splitting ortho-P from the latter substrate; nor was its activity toward ATP inhibited by the polyphosphate.

There was some activity with ADP. When 5 μmoles of ADP was incubated for 30 min. with 2 μmoles of $MgCl_2$, 6 μmoles of glucose, 120 μmoles of KCl, 30 μmoles of tris buffer, pH 9, and 0.1 ml. of fraction 2b in a total volume of 0.60 ml., 12% of the substrate became used up for phosphorylation and nearly 35% underwent hydrolysis. An addition of 2.5 μmoles of ADP to the incubation mixture containing 4.0 μmoles P of Graham salt, 4 μmoles of $MgCl_2$, 250 μmoles of KCl, 8 μmoles of glucose, 60 μmoles of buffer, pH 8.5, and 0.5 ml. of a DEAE eluate in a total volume of 1.05 ml. did not alter the rate of activity which amounted to 0.9 μmole utilized per 15 min. both in the samples with and without ADP.

Table 8

Effect of potato apyrase on glucose phosphorylation

Incubation mixtures contained in each experiment: buffer, pH 8, 60 μmoles; KCl, 120 μmoles; and where indicated glucose, 10 μmoles; Graham salt in amount corresponding to 9 μmoles of acid-labile P, or ATP, 5.5 μmoles acid-labile P, with $MgCl_2$ 4.5 or 2.5 μmoles resp. Apyrase was added in a volume of 0.05 ml. (9 units). Fraction 2b was used as the source of hexokinase. Total volume 0.65 ml. Incubation time: 15 min.

Additions	Ortho-P increase (μmole)	Acid-labile P utilization (μmole)
Poly-P, apyrase	0	0
ATP, apyrase	5.4	0
ATP, poly-P, apyrase	5.6	0
Poly-P, glucose, hexokinase	0.25	3.5
ATP, glucose, hexokinase	0.4	1.2
Poly-P, glucose, hexokinase, apyrase	0.2	3.45
ATP, glucose, hexokinase, apyrase	5.5	0

No phosphorylation could be stated with either pyro- or tripolyphosphate used as substrates.

A separate experiment was devised to examine the effect of ribonuclease on the poly-P utilization. To 0.2 ml. of fraction 2b, heated, 0.05 ml. of ribonuclease solution was added (25 μg.) and incubated at 37° in a cellophane tube with simultaneous dialysis against 20 ml. of buffer, pH 7.4. After 1 hr. the hexokinase activity of the dialysis residues was determined. In both samples equal activity was found. The same results were obtained when using gel eluate.

DISCUSSION

The experiments both with crude extracts and purified preparations clearly demonstrate the presence of an activity utilizing inorganic polyphosphates for the phosphorylation of glucose. A similar reaction was previously observed in *C. diphtheriae* [21] and a trace of it in *E. coli*. In contrast to this, acetone-dried yeast cells were active with ATP only (M. Szymona, unpublished data).

Since purified gel eluates possess no hydrolytic activity towards poly-P as established both by ortho-P determinations and paper chro-

matography in the presence of glucose as well as in its absence, the phosphorylative reaction dealt with in this work cannot be due to any phosphatase side-reaction. The results are at variance with those of Hoffmann-Ostenhof [8] who observed a slight transphosphorylating activity of a phosphatase preparation from yeast.

According to the literature, inorganic polyphosphates may function as secondary, phosphagen-like energy donors, which are formed and utilized *via* the ADP - ATP system. Specific enzymes have been discovered in yeast [7, 27], *E. coli* [10, 11], *C. diphtheriae* [11] and recently in *C. xerosis* [14]. Some of them were purified to a considerable extent. In the experiments of Kornberg [11], a synthesis of ATP from the polyphosphates could be coupled with glucose phosphorylation, provided hexokinase and ADP were added. Winder & Denneny [26] observed a utilization of poly-P for the phosphorylation of glycerol in cell-free extracts of *Mycobacterium smegmatis*. The reaction was catalysed by glycerol kinase and a polyphosphate-AMP-transpherase. For the phosphorylation to take place, an addition of AMP was required. We looked for the latter enzyme in *M. phlei* and were able to find it in extracts prepared from living cells by disintegration in the Potter apparatus. When as little as 0.3 μmole AMP was incubated for 1 hr. with 0.2 ml. of dialysed, volutin-rich extract, a remarkable disappearance of the metachromatic granules became evident in the microscope. There was no such change without AMP added. The reaction proved to be confined to the narrow range of pH from 6 to 7. However, the enzyme invariably was lacking in the extracts from acetone-dried cells and thus its action in the phosphorylation of glucose must be ruled out. The experiments with apyrase which splits ADP as well as ATP are interpreted to mean that the utilization of polyphosphate in the reaction studied does not involve the adenylic system. As a matter of fact, the phosphorylation with Graham salt can proceed readily in purified, exhaustingly dialysed preparations, without any nucleotide added. Thus, a two-step mechanism which would require first the synthesis of ATP and then its utilization by a common hexokinase is very unlikely, the more so that ATP used as a substrate does not lead to equal or higher specific activities. On the contrary, the ability to utilize ATP has been lower than that to utilize polyphosphate. Although no systematic studies were carried out on the utilization of ATP in the present work, some of the results seem to indicate that the respective activities may be due to similar but separate enzymes. The difference was best revealed when storing DEAE eluates in the cold for several weeks. Under these conditions the activity with ATP decreased faster than that with poly-P, the ratio being much higher in favour of poly-P after 2 months of storage.

All the above findings support the conclusion that the phosphorylation with poly-P is catalysed by a new type of kinase. It seems most simple to assume that long chain polyphosphates are being utilized directly up to the stage of tripolyphosphate according to the equation:

$$(PO_3^-)_n + \text{glucose} \rightarrow (PO_3^-)_{n-1} + \text{G-6-P}$$

On the other hand, some undiffusable nucleic acid was present in the purified enzyme as ascertained spectrophotometrically and one might speculate about its role. However, the experiments showed no effect of ribonuclease on the poly-P utilization.

Whatever the ultimate explanation might be, the enzyme is of interest for several reasons. In contrast to the yeast hexokinase which is uncapable of utilizing poly-P [1] and possibly unlike other ATP requiring kinases, this one has proved to utilize polyphosphates rather than ATP. Experiments have shown that about 80% of acid-labile phosphorus of Graham salt became converted into glucose-6-phosphate, the rest being accounted for as a low molecular residue. However, the molecular weight of the Graham salt used was relatively low and it would not be surprising if higher polymers were utilized with even greater efficiency. In fact, polyphosphates of biological origin may consist of more than 150 phosphate residues [12]. Furthermore, the high rate of activity amounting to 27 μmoles/hr./mg. protein in crude extracts, as well as other characteristics, e.g. the activation energy, allow to qualify the corresponding enzyme among those of biological importance. In view of the non-homogeneity of the Graham salt, no efforts were made to establish the Michaelis constant; nevertheless, a rough estimation of the data, some of which are shown in Fig. 6, gives a K_m of the order of 1×10^{-3} M of acid-labile P expressed as ortho-P, or 9 times less if expressed in moles of the polyphosphate composed of 9 P residues. This figure is rather high but yet attainable in living cells. It must be added, however, that the kinetic data as described for the low molecular Graham salt, may not apply to polyphosphates of much higher condensation grades.

SUMMARY

A new activity capable of utilizing inorganic polyphosphate for the phosphorylation of both glucose and glucosamine has been found in cell-free extracts of *M. phlei*. The corresponding enzyme which can be described as a polyphosphate hexokinase was purified approximately 65-fold by repeated ammonium sulfate fractionation, heat treatment and elution from calcium phosphate gel. The purified preparation requires

magnesium and is most active at high ionic strength in the range of pH 8 - 9. It does not utilize di- or tripolyphosphate but possesses some phosphorylating activity with ATP.

Evidence was presented for the assumption that there exist in *M. phlei* two separate hexokinases, one of which utilizes inorganic polyphosphates without the intermediation of the adenylic system.

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CZĘŚCIOWE OCZYSZCZENIE I WŁASNOŚCI NOWEJ HEKSOKINAZY WYKORZYSTUJĄCEJ NIEORGANICZNY POLIFOSFORAN

Streszczenie

W ekstraktach bezkomórkowych *M. phlei* znaleziono nowy typ kinazy zużytkowującej nieorganiczny polifosforan w postaci soli Grahama do fosforylacji glukozy i glukozaminy. Odnośny enzym, który można określić mianem heksokinazy polifosforanowej, został oczyszczony ok. 65-krotnie przy pomocy siarczanu amonowego, ogrzewania i adsorbcji na żelu fosforanowo-wapniowym, a także na kolumnie z dwuetyloaminoetylocelulozy. Heksokinaza polifosforanowa wymaga jonów magnezu i jest najbardziej aktywna przy dużej sile jonowej w zakresie pH 8 - 9. Oczyszczone preparaty enzymatyczne wykazują wielokrotnie mniejszą aktywność w stosunku do ATP i nie wykorzystują do fosforylacji pyroani trójpolifosforanu.

Przedstawiono dowody przemawiające za występowaniem u *M. phlei* dwóch różnych heksokinaz, z których jedna wykorzystuje bezpośrednio nieorganiczny polifosforan bez współudziału systemu adenilowego.

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ZOFIA SZYMANOWSKA, P. POSZWIŃSKI, K. MURAWSKI and K. ZAKRZEWSKI

HUMAN SERUM ALBUMINS IN STARCH-GEL ELECTROPHORESIS

Department of Biochemistry and the Blood Derivatives Division, Institute of Haematology, Warszawa

The resolution of human serum albumin, obtained after the method of Cohn [3], into several components using starch-gel electrophoresis and a discontinuous system of buffers was recently reported by Saifer, Robin & Ventrice [8]. They claimed to be able to prepare these components in a pure state from starch-gel after electrophoresis and suggested, therefore, that these subfractions represent a series of stable polymeric albumin derivatives which may result from ethanolic fractionation. We have, however, observed that it was also possible to separate the albumin isolated from normal human serum, into 4 - 5 components by means of electrophoresis, without the treatment of protein with alcohol. The results of experiments presented in this paper afford further evidence that the phenomenon of albumin association is reversible. The mechanism of this reversible aggregation of albumins observed in starch-gel electrophoresis remains, however, unclear and is open to further investigations.

METHODS

Human serum albumin used in this study was ethanol isolated Cohn Fraction V [3]. The protein was additionally purified by repeated fractionation with ethanol and dialysis. The final preparation was 99% homogeneous in free-boundary electrophoresis and exhibited no detectable impurities in paper electrophoresis. Another preparation of albumin from human serum used in this study, was obtained by means of starch-gel electrophoresis, as further fully described.

Starch-gel electrophoresis was performed in a vertical apparatus in borate buffer as described by Smithies [10]. Dimensions of the tray were 295 × 120 × 80 mm. The time of electrophoresis was 18 hr. at

16 mA and at room temperature. The starch for electrophoresis was acid-hydrolysed commercial potato starch [9].

Preparation of proteins from starch-gel. After electrophoresis, the gel was sliced into two horizontal halves and a small sample of the gel was stained with naphthalene black to visualize the positions of protein fractions. Appropriate area was then cut out from the unstained gel (taking into account the contraction of the gel during staining), finely cut and placed on a sintered glass (Schott G3) filter. A cellophane bag filled with saline was tied under the funnel and the whole was placed in an electrode vessel containing saline adjusted to pH 8.5 - 9 with 0.1 N-NaOH. The funnel was filled with saline and connected with another electrode vessel through a cotton-wool bridge. The whole arrangement, similar to that described by Moretti, Boussier & Jayle [5], is shown in Fig. 1. A current of 12 mA was applied for 48 hr. After this time, the content of the bag was additionally dialysed for 24 - 48 hr. against distilled water in a refrigerator, filtered through paper and freeze-dried. The powder was then redissolved in a small amount of borate buffer, centrifuged, the undissolved starch was discarded and a clear supernatant was used for electrophoresis.

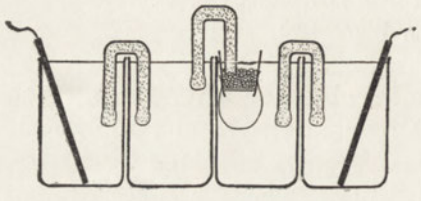

Fig. 1. Preparation of protein from starch-gel by electrodialysis. Details in Methods

RESULTS

The resolution of human serum albumin in starch-gel electrophoresis is shown in Fig. 2. Identical patterns, consisting of four or five distinct components decreasing in broadness and intensity from the fastest to the slowest, were obtained both for ethanol fractionated albumin (Cohn Fraction V) and for albumin prepared from serum by means of starch-gel electrophoresis. In this latter case, the albumin from several serum runs was collected, extracted from the gel by electrodialysis, freeze-dried and subjected to starch-gel re-electrophoresis. This experiment shows clearly that the action of alcohol on protein in the course of preparation, if any, is not a specific factor leading to the formation of protein aggregates demonstrated in starch-gel electrophoresis.

When after electrophoresis, as shown in Fig. 2, the separated albumin components were cut out from the gel, extracted, concentrated and re-

-run, in each case the components were resolved as in the original albumin preparation, provided the amount of protein used for electrophoresis was sufficient. The results of such an experiment are illustrated in Fig. 3 which shows the electrophoresis of albumin and re-electrophoresis of its first (main) and second fractions. The same results were

Fig. 2.

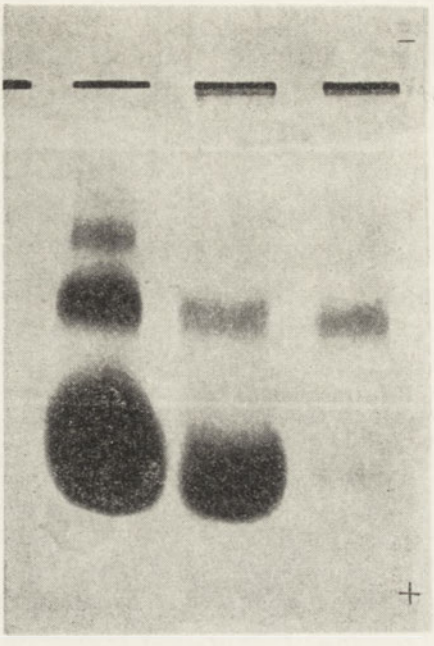

Fig. 3.

Fig. 2. Starch-gel electrophoresis of albumin (Fraction V)

Fig. 3. Starch-gel electrophoresis of albumin (left) and its first and second components (middle and right, respectively)

obtained with the slower components. No difference was found in the patterns obtained for fractions derived from ethanol fractionated albumin and for those derived from electrophoretically isolated protein.

The quantitative distribution of albumin components was determined in one experiment only. In five electrophoretic runs total amount of 91 mg. of albumin (Fraction V) has been applied to electrophoresis. A total of 69 mg. (76%) of protein was recovered. The first fraction amounted to 70%, the second to 17%, and the third to 13% of the whole preparation. Significant error depending on the admixture of soluble starch may be, however, involved in this calculation, as the concentration of albumin was determined only spectrophotometrically from the extinction coefficient at 277.5 mμ [2] while soluble starch exhibits also some absorption at this wavelength.

DISCUSSION

The presented experiments confirm the data of Saifer, Robin & Ventrice [8] who observed that apparently homogeneous human serum albumin may be resolved into several subfractions by means of starch-gel electrophoresis. In addition, we have found that this is true not only for ethanol fractionated albumin but also for albumin fraction isolated from normal human serum using preparative starch-gel electrophoresis. The action of alcohol presumably is not the only factor which might cause the appearance of aggregated albumin in starch-gel electrophoresis. A common step in preparation of both the ethanol fractionated and the electrophoretically isolated albumin is drying of the protein from the frozen state, which might possibly induce the polymerization of protein. The resolution in starch-gel electrophoresis of protein aggregates which are apparently homogeneous in other electrophoretical techniques is now a well-known phenomenon and constitutes a basis for the differentiation of the haptoglobin types [1] and it seems to explain the heterogeneity of myeloma proteins in starch-gel electrophoresis [6]. When whole normal or pathological serum is used instead of the separated albumin fraction there are no signs of heterogeneity of this protein in starch-gel electrophoresis, as has been shown by Poulik & Smithies [7] by means of two-dimensional technique; this suggests the specific influence of preparative procedures on the electrophoretical results. It must, however, be noted that a possibility of a real electrophoretical heterogeneity of albumins seems to exist in view of the recent findings that native sheep and crystalline bovine, serum albumins may be separated into 2 or 3 subfractions by paper electrophoresis in tris buffer [4].

Another result of the experiments presented in this paper is a finding that starch-gel electrophoresis is not only able to reveal the polymerization of albumin molecules but also to resolve the preparatively obtained aggregates into original components. In this case, the original component is always present in quantitative predominance, as can be seen from Fig. 3, but all other subfractions are clearly visible. This may be due to an equilibrium resulting from the reversible association and dissociation of albumin monomers occurring in starch-gel. These results are contrary to those of Saifer, Robin & Ventrice [8], who obtained only the original fraction in repeated electrophoresis. The resolution of electrophoretically isolated fractions into several components in our experiments was, however, possible only when a sufficient amount of protein (from several electrophoretic runs) was isolated and re-run, which was not done by the above cited authors. There is another possibility viz.

that the procedures employed by us (electrodialysis and freeze-drying) might induce dissociation and/or association of protein and might be, too, the cause of discordant results.

In view of the data presented in this paper and earlier by Saifer, Robin & Ventrice [8] it would appear that both the specific resolving power of starch-gel and slight molecular alterations occurring during the preparation of albumin are responsible for the phenomenon of association and dissociation of albumin when subjected to electrophoresis on starch-gel. This fact is of practical importance for analysis of proteins using starch-gel electrophoresis. The protein heterogeneity indicated by this method may be in some instances due to small denaturation changes in the protein molecule and/or to high resolving properties of the gel which may possibly induce phenomena of association and dissociation of protein.

SUMMARY

In one-dimensional starch-gel electrophoresis, borate buffer, pH 8.5, human serum albumin separates into 4 or 5 distinct fractions. The resolution occurs in case of albumin which was ethanol fractionated as well as in case of electrophoretically prepared protein. Electrophoretically obtained albumin fractions are not stable molecular entities as may be judged from reappearance of all subfractions in re-electrophoresis of isolated single component. The possible causes of this phenomenon and its importance for the analysis of proteins by means of starch-gel electrophoresis are discussed.

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ALBUMINY SUROWICY LUDZKIEJ W ELEKTROFOREZIE NA ŻELU SKROBIOWYM

Streszczenie

W jednowymiarowej elektroforezie na żelu skrobiowym (bufor boranowy, pH 8,5) albuminy surowicy ludzkiej rozdzielają się na 4 do 5 odrębnych frakcji. Rozdzielają się zarówno albuminy frakcjonowane etanolem, jak również otrzymane elektroforetycznie. Uzyskane elektroforetycznie frakcje albumin nie stanowią stabilnych gatunków molekularnych, ponieważ wszystkie podfrakcje pojawiają się odwracalnie w re-elektroforezie wyizolowanego pojedynczego składnika. Omówiono przypuszczalne przyczyny tego zjawiska oraz jego znaczenie dla badania białek przy użyciu elektroforezy na żelu skrobiowym.

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RECENZJE KSIĄŻEK

RECENT DEVELOPMENTS IN THE CHEMISTRY OF NATURAL PHENOLIC COMPOUNDS. Proceedings of the Plant Phenolics Group Symposium, edited by W. D. Ollis. Pergamon Pres, 1961, str. VIII + 237.

Książka ta zawiera referaty wygłoszone w kwietniu 1960 r. na Sympozjum „grupy fenoli roślinnych". „Grupa fenoli roślinnych" stanowi luźne powiązanie szeregu badaczy pracujących w tej dziedzinie, zbierających się na dyskusje robocze. Obecne sympozjum było trzecim dorocznym zebraniem. Książka nie stanowi dokładnego odbicia toku obrad, lecz przedstawia specjalne opracowanie do druku referowanych tam zagadnień.

R. W. Rickards omawia biosyntezę związków fenolowych z acetylo-koenzymu A. W. B. Whalley mówi o związkach między strukturą a biogenezą fenoli roślinnych. H. Grisebach przedstawia biosyntezę izoflawonów.

Postępom w dziedzinie poznania struktury naturalnych związków fenolowych, zawierających elementy izoprenowe, poświęcony jest rozdział napisany przez W. D. Ollis'a (redaktora) i I. O. Sutherland'a. C. H. Hassal i A. I. Scott napisali następny rozdział, o roli wolnych rodników w tworzeniu związków przez fenole. R. D. Haworth i E. Haslam dają krótkie ujęcie swych nie ogłoszonych jeszcze badań nad gallotaninami i biosyntezą kwasu galusowego. Chemii tanin poświęcone też są trzy następne rozdziały napisane przez O. T. R. Schmidt'a i W. D. Ollis'a oraz L. Hörhammer'a i H. Wagner'a. Dwa ostatnie rozdziały omawiają głównie strukturę nowych związków, mianowicie A. Dreiding omawia beta-cyjaniny, a W. D. Ollis i O. Sutherland nowe antybiotyki o charakterze fenolowym.

Książka przeznaczona jest przede wszystkim dla chemików-organików, pracujących nad strukturą związków biologicznych, oraz dla biochemików, zainteresowanych barwikami roślinnymi, substancjami garbnikowymi itp. Jednakże i biochemik ogólny, interesujący się naturalnymi związkami fenolowymi, ze względu na rosnące znaczenie chinonów dla zrozumienia procesów oksydoredukcji, znajdzie w niej wiele interesujących informacji o podstawowym znaczeniu. Chciałbym tu zwłaszcza podkreślić rozdział o roli wolnych rodników w mechanizmie sprzęgania fenoli z innymi związkami oraz obficie rozsiane szczegóły dotyczące nowoczesnych metod określania budowy związków.

Józef Heller