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OXIDATION OF METHANOL IN THE ORGAN OF VISION

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The toxic action of methanol differs from that of its higher homologues in that it causes damage to the optic nerve. This fact has long been attributed to the different metabolism of methanol leading to the formation of formaldehyde [11]. This was experimentally confirmed and constitutes the starting point of current concepts of methanol intoxication [1]. Poisoning with methanol has two aspects: the narcotic effect of unchanged methanol and the toxic effect due to the products of oxidation of methanol, chiefly formaldehyde [7]. The toxic effect of the accumulation of formaldehyde can be explained by its marked ability to react with various constituents of the living organism, particularly with the amino group of the amino acids. Its highly specific effect on retina and optic nerve is more difficult to explain. The oxidation of methanol to formaldehyde can be supposed to occur more rapidly in retina and optic nerve than in other tissues.

Two metabolic pathways of methanol in the animal body are known: oxidation by the alcohol dehydrogenase (ADH) [15] and oxidation by catalase [10]. Ethanol and methanol are oxidized by liver ADH, but the latter much more slowly. Crystalline liver ADH, however, oxidizes only ethanol. Theorell & Bonnichsen [15] thought that the failure of the crystalline enzyme to oxidize methanol can be explained by the presence in the non-crystalline preparation of an additional factor essential for the oxidation of methanol.

Oxidation of methanol in the presence of catalase and hydrogen peroxide was demonstrated by Keilin & Hartree [10] and formulated as the reaction: $CH_3OH + H_2O_2 = HCHO + 2H_2O$. Chance [4] showed later that the kinetics of methanol oxidation by catalase agree with the kinetics of methanol disappearance from the blood of experimental animals. He supposed that *in vivo* catalase oxidizes methanol, but probably it does not participate in the oxidation of ethanol, which is oxidized by dehydrogenase. The ability to oxidize methanol was found [9] in most tissues tested, which is in agreement with the wide distribution of catalase, while ADH was found only in the liver. These data show the important role of catalase in the oxidation of methanol.

If the supposition that methanol is oxidized more readily in retina and optic nerve than in other tissues is correct, high concentrations of catalase should be present in both these tissues. So far no data could be found in the literature. Only Feinstein [6], who studied the distribution of catalase in rat tissues, found a particularly low catalase activity in brain as compared with other tissues. He did not, however, investigate the content of catalase in the optic nerve or in retina.

In the presented paper the activity of catalase in the oxidation of methanol in retina and optic nerve was determined. As the activity found was very low, the oxidation of methanol by alcohol dehydrogenase preparation from retina was investigated.

METHODS

Preparation of material. Eyeballs were secured 2 - 4 hr. after slaughter from 2 - 3-month-old calves, 1 - 2-year-old cows, 1 - 2-year-old pigs, and in one case human eyeballs 8 hr. after death. After the incision of the cornea the lens was separated. The eyeball was then opened and the vitreous humor was removed; the retina was next prepared, the main stem of the retinal artery being removed. The isolated parts of the eyeball were rinsed with physiological saline solution and homogenized in saline solution. 7 - 11% homogenates were made for the determinations of catalase and 50% homogenates for the preparation of alcohol dehydrogenase. All procedures during the preparation of the material were carried out at 0°.

For comparative estimations of catalase content the homogenates of the oculomotor nerve of calves, and liver, kidneys and lungs of calves and pigs were made.

Determination of catalase. The activity was estimated by the method of Feinstein [6], in which the number of m-Eq. of sodium perborate decomposed was calculated per mg. of tissue. The values presented are averages of ten determinations, with the exception of the human material with which only two determinations were made.

Determination of ADH activity. The method of Bonnichsen & Brink [3] was used for ADH isolation from retina and liver. Although this method does not yield crystalline ADH, the preparations were catalase-free. Tissue homogenate was mixed for 3 to 4 hr., then left for 12 hr. at 4°. After this time the homogenate was centrifuged and the turbid red supernatant was heated for 15 min. in a water bath at 52°, cooled to 20 - 25° and centrifuged; ammonium sulphate was added to the supernatant (349 g. per liter) and after 30 min. at 4° the mixture was centrifuged. The water-clear supernatant was adjusted to pH 6 by ammonia, and ammonium sulphate (208 g. per liter) was added. After 30 min. at 4° the mixture was centrifuged again and the supernatant discarded. The sediment was dissolved in a small volume of 0.01 M-phosphate buffer, pH 7, and dialyzed for 24 hr. at 4° against 0.01 M-phosphate buffer, pH 7. The enzyme solution prepared in this way did not exhibit any catalase activity.

The activity of the ADH preparation was determined in glycine buffer, pH 9.6, to which appropriate amounts of methanol or ethanol and DPN^+ were added.

The incubation mixture contained: glycine buffer pH 9.6, 2.1 ml.; 0.01 M-ethanol, or methanol, 0.6 ml.; enzyme preparation after dialysis, 0.3 ml.; DPN^+, 0.25 mg. Simultaneously two control samples were prepared: in the first one the enzyme preparation was omitted and in the second, the substrate (ethanol or methanol). Instead of the omitted solution bi-distilled water was added.

ADH activity was measured by the rate of DPNH formation. The extinction at 340 mμ was read in a Hilger spectrophotometer.

The second method used to determine ADH activity was the colorimetric estimation of formaldehyde or acetaldehyde formed from methanol or ethanol, respectively. Formaldehyde was determined with the chromotropic reagent [5], and acetaldehyde with *p*-dihydroxydiphenyl reagent [14]. The incubation was carried out at 37° and stopped after determined intervals by adding 2N-H_2SO_4.

Reagents. Methanol and 96% ethanol were bi-distilled; formaldehyde c.p., sodium perborate c.p., potassium permanganate, ammonium sulphate, *p*-dihydroxydiphenyl were products of Fabryka Odczynników Chemicznych in Gliwice. Acetaldehyde (paraldehyde), ammonium hydroxide, sodium chloride, monobasic potassium phosphate, and dibasic potassium phosphate were products of Merck. Chromotropic acid and TPN were obtained from Light & Co.; DPN from K & K Lab., New York; glycine from B.D.H. Sulphuric acid was the product of Riedel de Haen.

RESULTS

Determination of catalase

The results of determinations of catalase activity in calf, pig and human organs of vision and in other tissues of pigs and calves are presented in Table 1. For comparison, the catalase activity in various rat tissues, as given by Feinstein [6], is also included.

A very low catalase activity was found in the optic nerve and retina of all four investigated species. In the retina of pig catalase activity was more than 300 times lower than in the liver. These results do not support the supposition that the oxidation of methanol to formaldehyde in the retina is due to catalase. If the damage to the optic nerve in methanol intoxication is due to the formation of formaldehyde then the occurence of a dehydrogenase specifically oxidizing methanol in retina might be postulated.

Determination of dehydrogenase

It must be noted that even in the absence of substrate (ethanol or methanol) DPN was slowly reduced by the dialyzed preparation of ADH from the retina. Evidently this preparation contained some unidentified substrate capable of reducing DPN. Attempts to eliminate this factor were unsuccessful, and in the spectro-

Table 1

Catalase activity in different organs of calf, pig, man and rat

The activity determined by the method of Feinstein [6] is expressed as m-Eq. sodium perborate decomposed per mg. of tissue. Average values from 10 experiments are given; in parentheses are the limit values.

Tissue	Calf	Pig	Man*	Rat (according to Feinstein [6])
Vitreous humor	0.0	0.0	0.0	—
Lens	0.0	0.0	0.0	—
Optic nerve	0.015 (0.0087—0.0208)	0.035 (0.034—0.041)	0.0027	—
Retina	0.0028 (0.0015—0.0036)	0.017 (0.015—0.02)	0.0015	— —
Liver	0.654 (0.650—0.663)	0.593 (0.570—0.660)	—	0.810
Kidney	0.0190 (0.0187—0.0193)	0.399 (0.363—0.425)	—	0.331
Lung	0.0312 (0.0236—0.0339)	0.355 (0.345—0.360)	—	0.053
Brain	—	—	—	0.0074

*Average of two determinations.

photometric determinations it was necessary to introduce a second blank (see Methods, control sample II) without the addition of substrate. The determinations of dehydrogenase activity were based on the difference between the extinction at 340 mμ of the sample with the substrate added and the extinction of the

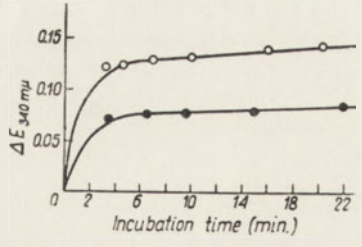

Fig. 1. Reduction of DPN by alcohol dehydrogenase preparation from retina with: (○), methanol and (●), ethanol as substrates

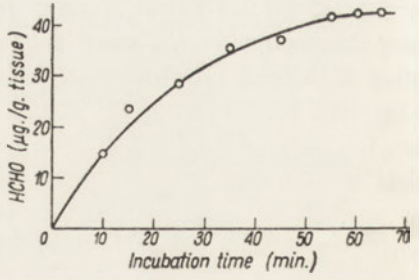

Fig. 2. Formation of formaldehyde from methanol incubated with alcohol dehydrogenase preparation from retina (average values of 5 determinations)

second blank. The E value of the first blank (control sample without the enzyme preparation) was taken as zero point. At 340 mμ no difference was found between the extinction of the enzyme preparation from the retina and that of bi-distilled water; hence the extinction of the first blank could be regarded as equal to the

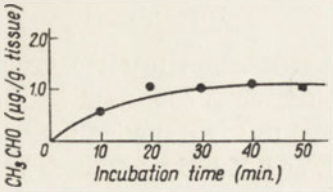

Fig. 3. Formation of acetaldehyde from ethanol incubated with alcohol dehydrogenase preparation from retina (average values of 5 determinations)

extinction of the studied sample at zero time. For technical reasons (mixing of the enzyme preparation with buffer, substrate and DPN, and transfer of the mixture into the cuvettes of the spectrophotometer) from 80 to 100 seconds elapsed before the first measurements could be made. Fig. 1 shows the rate of DPNH formation

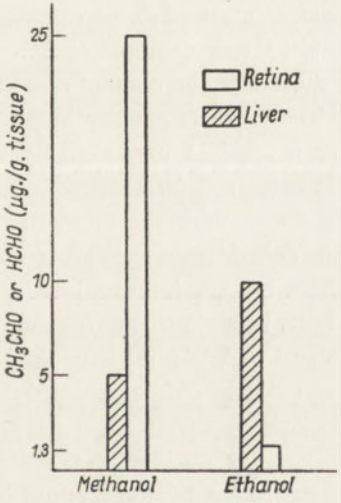

Fig. 4. Activity of alcohol dehydrogenase in pig retina and liver with methanol or ethanol as substrates. Formaldehyde or acetaldehyde in μg. formed after 20 min. incubation per gram of tissue

with methanol and ethanol as substrates. Methanol and ethanol were oxidized by the enzyme preparation from the retina, but the oxidation of methanol was much more rapid. The same results were obtained when the formation of formaldehyde and acetaldehyde was determined (Figs. 2 and 3).

Without DPN no detectable amounts of aldehyde were formed by retina ADH from methanol and ethanol. The enzyme was also inactive when TPN was used instead of DPN.

The comparison of retina and liver ADH activity measured by aldehyde formation is presented in Fig. 4. These data indicate that liver ADH oxidizes ethanol more quickly than methanol, and that methanol is the better substrate for retina ADH.

DISCUSSION

The generally accepted assumption that the toxic effect of methanol is due to the formation of formaldehyde is supported also by the experiments of Potts & Johnson [13]. They showed that in ox retina formaldehyde is 1000 - 3000 times more active than methanol, and 25 - 75 times more active than formate, in inhibiting oxygen uptake and carbon dioxide production. Therefore the presence of an enzyme oxidizing methanol at a concentration higher in retina than in other tissues could be anticipated, or alternately the presence of an enzyme specific for methanol and not found in other tissues. The two known enzymes capable of oxidizing methanol are catalase and alcohol dehydrogenase. In rat liver methanol is oxidized by catalase although ADH is present; this was demonstrated by Mannering & Parks [12] who showed that in liver homogenates 1,2,3-aminotriasole, a specific catalase inhibitor, inhibited the formation of formaldehyde from methanol. The liver, however, contains large amounts of catalase; other rat tissues contain it in much smaller amounts, as was demonstrated by Feinstein [6]. Results obtained in the present work show that the content of catalase in the retina of cattle, pigs and man is very low. The catalase activity in pig's retina is 300 times lower than in the liver. Therefore it appears very unlikely that oxidation of methanol in retina and optic nerve is catalyzed by catalase.

It is therefore necessary to consider the alternative, namely the oxidation of methanol by an appropriate dehydrogenase. The retina contains axerophthol dehydrogenase [16, 2], but there are no informations concerning the specificity of this enzyme, and particularly its behaviour towards the one or two-carbon alcohols. It was only shown by Hubbard & Wald [8] that partially purified and crystalline alcohol dehydrogenases from the liver oxidize axerophthol in the presence of DPN, in contrast to yeast dehydrogenase, which does not possess this ability.

In the present work a partially purified, catalase-free alcohol dehydrogenase preparation was obtained from retina by the method of Bonnichsen & Brink [3]. The preparation oxidized methanol to formaldehyde in the presence of DPN; the reaction was estimated by the formation of DPNH and formaldehyde. The preparation oxidized also ethanol, but more slowly. The alcohol dehydrogenase preparation from pig liver obtained by the same method oxidized methanol much more slowly than ethanol. These results are in agreement with the data of Theorell [15].

In view of the results obtained it may be concluded that (1) in retina the oxidation of methanol is catalyzed by dehydrogenase and not by catalase, and (2) the preparation of retina alcohol dehydrogenase has a different substrate specificity than liver ADH.

According to Theorell [15] the crystalline liver ADH oxidizes only ethanol, the low activity towards methanol found in partially purified preparations being due to some contaminations. If pure ADH from the retina were obtained it could be expected to be an enzyme specifically oxidizing methanol. However, even if crystalline retina dehydrogenase would oxidize ethanol, it would nevertheless be distinct from liver ADH.

Whether ADH from the retina, which exhibits greater specificity towards methanol than towards ethanol, is identical with axerophthol dehydrogenase or is a separate enzyme, remains to be elucidated.

The authors are gratefully indebted to Prof. Dr. B. Skarżyński for valuable suggestions and encouragement. They are also indebted to Dr. T. W. Szczepkowski for methodical advice.

SUMMARY

1. Oxidation of methanol in the retina by catalase and alcohol dehydrogenase (ADH) was studied.

2. The determinations of catalase activity in the retina of three species of animals did not support the supposition that catalase oxidizes methanol in retina.

3. Partially purified preparation of ADH from pig retina oxidized methanol much more rapidly than ethanol. ADH was DPN- but not TPN-dependent.

4. Preparation of liver ADH was more active with ethanol than with methanol as substrate.

5. Presumably in retina methanol is oxidized by an ADH differing from liver ADH.

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UTLENIANIE METANOLU W NARZĄDZIE WZROKU

Streszczenie

1. Badano proces utleniania metanolu w siatkówce świni, biorąc pod uwagę możliwości udziału w tym procesie katalazy lub dehydrogenazy alkoholowej (ADH).

2. Oznaczenia aktywności katalazy w siatkówce trzech gatunków zwierząt wykazały, że możliwość utleniania w siatkówce metanolu przez katalazę jest mało prawdopodobna.

3. Z siatkówki świni uzyskano preparat ADH, który utlenia metanol znacznie szybciej niż etanol. ADH współdziała z DPN nie z TPN.

4. Preparaty ADH z wątroby wykazały znacznie większą aktywność wobec etanolu niż wobec metanolu.

5. Uzyskane wyniki dają podstawę do przypuszczenia, że metanol jest utleniany w siatkówce przez ADH odmienną od wątrobowej.

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BIOSYNTHESIS OF GLUTATHIONE IN HUMAN BLOOD CELLS

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The origin of glutathione[1] in erythrocytes has been widely discussed. It has been supposed that glutathione is formed in the bone marrow during the erythropoiesis and is present in mature cells throughout their life-span [17]. Later Anderson & Mosher [1] showed that shortly after the administration of ^{35}S-labelled cystine into the stomach of rats the isotope appears in glutathione of erythrocytes. Dimant, Landsberg & London [8] demonstrated that the half-life time of glutathione in blood is much shorter than that of haem. Elder & Mortensen [10] demonstrated *in vitro* the incorporation of labelled glycine into glutathione of rat erythrocytes, and in recent years Kasbekar & Sreenivasan [15] studied the synthesis of glutathione in isolated rat erythrocytes. However, no detailed investigations of the biosynthesis of glutathione in human erythrocytes have been made.

Still less is known about the occurence and eventual biosynthesis of glutathione in other cellular elements of blood. The presence of GSH in human leucocytes is certain, but there are contradictory data as to its level [2, 12]. From the work of Morito & Asada [18] the presence of GSH in thrombocytes can only be supposed. In thrombocytes and leucocytes, however, the synthesis of glutathione has not yet been investigated.

In the present study the content of GSH in erythrocytes, leucocytes and thrombocytes isolated from the blood of healthy human subjects was determined, and an attempt was made to elucidate whether all these cells are able to synthesize glutathione.

MATERIAL AND METHODS

Pure suspensions of thrombocytes, leucocytes and erythrocytes. They were obtained by fractionating 80 ml. of fresh human blood, as previously described [16]. Venous blood was mixed (9:1) with 5% aqueous solution of versene (ethylenediaminetetraacetate disodium, product of Light) and centrifuged to obtain plasma

[1] Abbreviations used are: GSH, glutathione; GSSG, oxidized glutathione; Gly, glycine; Cys, L-cysteine; Glu, L-glutamic acid.

with a high content of thrombocytes. The thrombocytes were isolated from the plasma by the method of Campbell *et al.* [6] and suspended in the incubation solution described below. The remaining leucocytes and erythrocytes were separated by centrifugation and sedimentation [4, 16] and suspended in the incubation solution.

Since versene, even in low concentrations may modify the activity of various enzymes [26], the synthesis of GSH by erythrocytes obtained from citrate-blood was also investigated, and no differences were found.

Determination of GSH in blood cells. For the determination of GSH, distilled water and a drop of toluene were added to the centrifuged blood cells, and the mixture was shaken. After cytolysis the proteins were precipitated with 5% sulphosalicylic or metaphosphoric acid. In the supernatant GSH was determined by means of the nitroprusside, alloxan or glyoxalase methods.

The nitroprusside method was employed in the modification of Grunert & Philips [11], the measurement of extinction being read at 520 mμ and the amount of GSH calculated from a standard curve obtained with a preparation of glutathione (Eastman Co.).

Determination of GSH by the alloxan method was made according to Patterson & Lazarow [19]. Extinction of the GSH-alloxan complex was measured at 305 mμ with an "Uvispec" (Hilger) spectrophotometer, the amount of GSH being calculated from a standard curve. Both methods are simple and convenient, but not very specific, and the results can be too high due to other SH-compounds. Therefore, the more specific glyoxalase method was also used with the titrimetric modification as proposed by Schroeder & Woodward [21]. A purified preparation of yeast glyoxalase and an aqueous solution of methylglyoxal were obtained according to Umbreit *et al.* [27]. At the used concentrations of glyoxalase and methylglyoxal the results were proportional to the amount of GSH in the range of 5 - 100 μg. GSH per ml.

To determine the oxidized glutathione it was reduced by the method of Dohan & Woodward [9], employing a mercury cathode and sulphosalicylic acid. The surface of the cathode was 10 cm^2, the volume of the sample 2 ml., and the current approximately 30 mA. After 10 min. electrolytic reduction, 1 ml. of the solution on the cathode was withdrawn and GSH was determined. The difference between the content of GSH in the sample before and after the reduction represented the amount of GSSG.

The amount of the determined GSH was calculated per one blood cell. The number of cells in the incubated samples was determined with an haemocytometer. Since the suspensions of thrombocytes and leucocytes were not entirely homogeneous it was necessary to make a correction. It was calculated that this correction was always less than 5% of the GSH determined and therefore could be neglected. The amount of GSH was also calculated per gram of packed cells, as this could be compared with the amount of glutathione in other tissues given by other authors. The results were analysed statistically, with calculation of standard de-

viation and the mean error of the arithmetical means. In cases in which the number of determinations was less than six, statistical analysis was carried out by the simplified method of Rokosz [20]. After the verification of their statistical significance, the results formed the basis for conclusions concerning the relation between the ability to synthesize glutathione and GSH content in the cells.

GSH synthesis. In most of the experiments the blood cells were suspended in a solution (called "typical medium") prepared by mixing 3 parts of 0.154 M-NaCl, 4 parts 0.154 M-KCl, 1 part 0.154 M-$MgSO_4$, and 2 parts of Sörensen's phosphate buffer, pH 7.3. When glucose, as a source of energy for the cells, was added, its concentration was 250 mg.% (27.7 μmoles/2 ml. of the sample). Solutions of glycine (Fabryka Odczynników Chemicznych, Gliwice), L-cysteine (Fluka) and L-glutamic acid (Light) were prepared so that the final concentration of each in the incubation mixture was 10^{-3} M. Usually 20 μmoles of each amino acid dissolved in the incubation solution was added, and the final volume of the sample was 2 ml. For aerobic tests the incubation was carried out in open test tubes immersed in a water bath at 37°, and for anaerobic tests the samples were placed in the Warburg vessels through which nitrogen was passed.

The biosynthesis of GSH was assayed by the increase of GSH or by the incorporation of labelled glycine into GSH molecule.

In the first case the procedure was as follows. Glucose and amino acid(s) were added to 1 ml. of a suspension of blood cells, and the mixture was incubated for 2 hr. Then the blood cells were centrifuged and washed twice with physiological saline in order to remove cysteine. The sedimented blood cells were then cytolysed, and after protein precipitation the content of GSH in the supernatant was determined. Blood cells incubated without the amino acids, with three essential amino acids but without glucose, or with glucose and cysteine but without glutamic acid and glycine, served as controls. The difference in GSH content was taken as the amount synthesized.

When the radioisotope method was used the procedure was as follows. To 1 ml. of blood cells suspension, glucose and a solution containing 20 μmoles of cysteine and 20 μmoles of glutamic acid were added. Next, 0.5 ml. of a solution of labelled glycine was added, obtained by adding an appropriate amount of [2 - ^{14}C] glycine (produced in U.S.S.R.) to 20 μmoles of glycine. The specific activity of glycine in the experiments ranged from 6000 to 12000 counts/min./μmole. After 2 hr. incubation the blood cells were cytolysed, and protein precipitated by adding 2 ml. 10% trichloroacetic acid (TCA). The mixture was filtered and the sediment washed on the filter with 2 ml. TCA. To the filtrate 6 mg. GSH was added as carrier, and GSH was precipitated with $CdCl_2$ and then Cu_2O, according to the procedure of Waelsch & Rittenberg [28]. The crystalline sediment of glutathione mercaptide after washing with water and absolute alcohol was transferred quantitatively to a bakelite planchet and dried under an infrared lamp. The radioactivity was determined by measuring the number of impulses with a scanning

Geiger-Müller counter with an electronic computer, taking into account the self-absorption of beta radiation by the preparation. In some experiments the mercaptide, after its activity had been measured, was rinsed quantitatively out of the planchet, and copper was precipitated by passing H_2S according to the method of Dimant *et al.* [8]. This method permitted to determine the amount of GSH in the sample and its loss during the isolation and purification of mercaptide; moreover, it allowed measurements of activity of GSH in infinitely thin layers to be made. With this procedure it was possible to determine the necessary corrections due to the loss of glutathione during isolation of mercaptide and self-absorption of the preparation, and hence to obtain the true activity of GSH. The specific activity of glycine being known, the amount of GSH into which glycine was incorporated could be calculated from Bloch's equation [5].

RESULTS

Content of glutathione in erythrocytes, thrombocytes, and leucocytes

The results of determinations of GSH in blood cells are summarized in Table 1. It can be seen that erythrocytes contain the greatest part of GSH found in blood. Since, according to Tempka [25], 1 mm^3 of human blood in health contains 5×10^6 erythrocytes, 3×10^5 thrombocytes, and 6×10^3 leucocytes, it can be calculated that 98% of GSH is to be found in erythrocytes, only 0.83% in thrombocytes, and 1.17% in leucocytes (plasma did not contain GSH [14]).

In Table 2 the content of GSH in packed cell sediments of erythrocytes, thrombocytes, and leucocytes is shown. Although these results give only approximate values they throw some light on the GSH content in the cellular elements of blood. The content of the oxidized form of glutathione is presented in Table 3.

Table 1

The content of glutathione (GSH) in cellular elements of human blood

GSH was estimated by the nitroprusside [11], alloxan [19] and glyoxalase [21] methods.

Cells	No. of determinations	Method	GSH/one cell	
			($\mu g. \times 10^{-8} \pm$ S.D.	($\mu mole \times 10^{-10}$)
Erythrocytes	7	Nitroprusside	5.99 ± 0.48	
	6	Alloxan	5.87 ± 0.46	
	6	Glyoxalase	5.84 ± 0.52	
		Average	5.90 ± 0.84	1.92
Thrombocytes	3	Nitroprusside	0.86	
	2	Glyoxalase	0.84	
		Average	0.85 ± 0.086	0.277
Leucocytes	3	Nitroprusside	64.4	
	2	Glyoxalase	55.6	
		Average	60.0 ± 12.2	19.5

Table 2

The content of glutathione in erythrocytes, thrombocytes and leucocytes per gram of packed cells

Values calculated from the data in Table 1.

Cells	GSH/g. of cell mass		
	(mg.)	(μmoles)	Relative content
Erythrocytes	0.489	1.59	1.00
Thrombocytes	0.467	1.52	0.95
Leucocytes	2.73	8.89	5.59

Table 3

The content of reduced and oxidized glutathione (GSSG) in human blood cells

The reduction of GSSG was made according to Dohan & Woodward [9].

Cells	Method	GSH in the sample		GSSG/GSH	GSSG in % of the total glutathione
		Before reduction (μg.)	After reduction (μg.)		
Erythrocytes	Nitroprusside	28	32	0.14	12.5
		61	78	0.28	21.8
	Glyoxalase	45	53	0.18	15.0
		54	58	0.075	7.0
Thrombocytes	Glyoxalase	15	15	0	0
		11	10	0	0
Leucocytes	Glyoxalase	19	21	0.10	9.5
		14	15	0.07	6.6

Determinations of the increase of GSH

In the first experiments the nitroprusside method was used. The controls consisted of incubated cell suspensions, to which glycine, cysteine and glutamic acid were added after the incubation. The data presented in Table 4 seem to suggest that an intense synthesis of glutathione occurred in all human blood cells. However, further experiments showed that the observed increase was non-specific, being caused chiefly by the retention of cysteine in the cells. Cysteine was absorbed by blood cells during the incubation, and the washing of cells with 0.9% NaCl removed only free cysteine. For the absorption and retention of cysteine in the cells the presence of glucose was essential (Table 5).

When the alloxan method was used the error resulting from retention of cysteine in incubated blood cells was somewhat diminished because of the greater specificity of the method. In the glyoxalase method, on the other hand, the results could be lower, probably because of the inhibition of the enzyme's activity by cysteine.

Table 4

The increase of extinction in nitroprusside method after the incubation of blood cells suspensions

Typical medium with glucose added. Conditions of the determinations as described under Methods. To the control samples the 3 amino acids were added after the incubation.

Cells	$E_{520\ m\mu}$				Increase of E
	Control		Incubation with 3 amino acids		
	Air	N_2	Air	N_2	
Erythrocytes	0.14	—	0.38	—	0.22
	—	0.14	—	0.50	0.36
Thrombocytes	0.04	—	0.14	—	0.10
	—	0.04	—	0.13	0.09
Leucocytes	0.05	—	0.10	—	0.05
	—	0.05	—	0.11	0.06

Table 5

The absorption of cysteine by blood cells during the incubation

Typical medium with glucose added. To the control samples the 3 amino acids were added after the incubation. The numbers express the extinction of samples in nitroprusside method

Cells	$E_{520\ m\mu}$		
	Control	Incubation with 3 amino acids	Incubation with cysteine alone
Thrombocytes	0.04	0.12	0.13
Erythrocytes	0.13	0.30	0.35
Erythrocytes, without glucose	0.12	0.10	0.09

Table 6

The biosynthesis of glutathione in erythrocytes estimated by nitroprusside, alloxan and glyoxalase methods

Typical medium with glucose added. The results, μg. GSH per sample, are uncertain due to the presence of cysteine

Sample	Method of GSH determination		
	Nitroprusside	Alloxan	Glyoxalase
Control (without substrates)	31	30	32
With cysteine	58	32	30
With 3 amino acids	52	34	34

It was for this reason, probably, that the glyoxalase method gave erratic and uncertain results. In Table 6 the results obtained in one experiment by the three methods of GSH determination are presented, and in Table 7 the results of trials to assay

Table 7

The biosynthesis of glutathione in blood cells estimated by the glyoxalase method

Typical medium with glucose added. The samples with substrates were incubated with the 3 amino acids, to controls amino acids were added after the incubation.

Cells	Sample	GSH in the sample (μg.)
Erythrocytes	Control	25
	With substrates	31
Thrombocytes	Control	9
	With substrates	8
Erythrocytes	Control	28
	With substrates	26
	With substrates, glucose being omitted	17
Thrombocytes	Control	8
	With substrates	11

the biosynthesis of GSH by the glyoxalase method. From these experiments it seems evident that the determination of GSH increase is not a reliable method for the study of GSH biosynthesis in the blood cells.

Determination of GSH biosynthesis with radioisotope

[2-^{14}C]Glycine was used to investigate the biosynthesis of GSH in relation to the amount of erythrocytes and to the time of incubation (Table 8). As substrates three amino acids or glycine alone were used. From the data in Table 8 it can be seen that within the same blood sample the synthesis was proportional to the number of cells and the time of incubation; between different blood samples, however, marked differences were observed due probably to individual variations.

It was found that erythrocytes are able to incorporate glycine into the GSH molecule without cysteine or glutamic acid being added to the incubation mixture. To elucidate whether GSH glycine was exchanged for [^{14}C]glycine, or whether a complete synthesis of the tripeptide occurred, the incorporation of glycine was studied in different incubation media in the presence of glycine alone or of the three amino acids. From the results summarized in Table 9 it can be seen that in erythrocytes the biosynthesis of glutathione in the presence of glutamic acid, cysteine and glycine was the highest in media containing phosphate, K^{1+}, Mg^{2+} ions (typical medium), and glucose; on the other hand, the incorporation of labelled gly-

cine without the addition of the remaining two amino acids was the highest in incubation media containing NaCl and sodium phosphate, but no glucose. In these conditions the addition of glutamic acid and cysteine or glucose decreased the rate of the reaction. Inhibition of glycine incorporation into erythrocyte-GSH by glucose was most distinct in a medium containing phosphate, K^{1+} and Mg^{2+} ions. The incorporation of glycine into erythrocyte-GSH was completely inhibited by heating the sample for 2 min. in a boiling water bath. In the range of pH 6 - 8 the rate of the reaction changed very little. The results presented in Table 10 show that the rate of the reaction is proportional to the concentration of GSH in the sample. In this experiment it was necessary to haemolyse the erythrocytes because glutathione did not pass through their cell membranes.

Determinations of GSH with the nitroprusside and alloxan methods after the incubation of erythrocytes with glycine only did not show any appreciable increase of glutathione.

Table 8

The biosynthesis of glutathione in erythrocytes estimated by the isotope method

Typical incubation medium with glucose added. Specific activity of glycine in the experiments 1 and 2 was 11300 counts/min./μmole and in the experiments 3 and 4, 5900 counts/min./μmole. The amount of GSH into which glycine was incorporated was calculated from the activity of the isolated mercaptide.

Expt. no.	No. of erythrocytes in the sample ($\times 10^8$)	Incubation (hr.)	Mercaptide - GSH (counts/min./sample $\pm$ S.D.)	GSH formed (μmoles $\times 10^{-3}$)
	Incubation with glycine			
1	30	0	In background limits	0
	30	0.5	21.1±2	3.75
	30	1.0	55.2±3	9.77
	30	2.0	97.3±5	17.23
2	66	2.0	284.0±9	50.28
	33	2.0	131.5±7	23.29
	Incubation with glutamic acid, cysteine and glycine			
3	46	1.0	101.4±5	34.4
	46	2.0	228.8±9	77.9
4	21	2.0	53.8±3	18.3
	42	2.0	120.1±6	40.7

All these data allow to assume that the observed incorporation of labelled glycine into erythrocyte-GSH in the absence of cysteine and glutamic acid was due to the exchange of this amino acid for GSH glycine.

In Table 11 the effect of cysteine and glutamic acid on the rate of incorporation of glycine into GSH in erythrocytes suspended in typical incubation media with 250 mg.% glucose is shown. The results indicate that in the presence of all

Table 9

Influence of medium composition on the erythrocyte-glutathione biosynthesis

Composition of the media: Typical medium, phosphate, Cl^-, Na^{1+}, and Mg^{2+} (see Methods); NaCl-tris, 3 parts of 0.9% NaCl and 1 part of 0.2 M-tris; NaCl-phosphate, 3 parts of 0.9% NaCl and 1 part of sodium phosphate; pH of each medium was 7.3. Specific activity of glycine was 9940 counts/min./μmole. Incubation 2 hr. in open test tubes. The amount of GSH formed was calculated from the activity of mercaptide.

No. of erythrocytes in sample	Incubation medium	Addition		GSH formed (μmoles × $\times 10^{-3}$)
		Amino acid	Glucose (μmoles)	
39×10^8	NaCl-tris	Gly	—	13.5
		Gly, Glu, Cys	—	21.1
		Gly	27.7	12.2
		Gly, Glu, Cys	27.7	24.4
	Typical medium	Gly	—	51.6
		Gly, Glu, Cys	—	20.2
		Gly	27.7	27.1
		Gly, Glu, Cys	27.7	42.5
44×10^8	NaCl-tris	Gly	—	15.4
		Gly	27.7	9.5
	NaCl-phosphate	Gly	—	82.8
		Gly	27.7	46.4
	Typical medium	Gly	—	75.4
		Gly	27.7	35.4
	NaCl-tris	Gly, Glu, Cys	27.7	24.0
	NaCl-phosphate	Gly, Glu, Cys	27.7	58.2
	Typical medium	Gly, Glu, Cys	27.7	68.5
		Gly, Glu, Cys	—	42.4
		Gly, Glu, Cys	5.5	58.2
		Gly	5.5	38.8

Table 10

Influence of the amount of glutathione on the rate of glycine incorporation

Typical incubation medium with glucose added. Erythrocytes were haemolysed by shaking with 0.1 ml. toluene. GSH dissolved in 0.9% NaCl was added to two samples before the incubation.

Sample	GSH content in sample before incubation (μg.)	Mercaptide (counts/min./sample)	Activity (counts/min./100 μg. GSH)
Erythrocytes	120	216±10	180.0
Haemolysate	120	64± 3	53.3
Haemolysate	220	102± 5	46.4
Haemolysate	420	195±10	46.5

Table 11

Influence of cysteine and glutamic acid on the incorporation of glycine into erythrocyte-glutathione

Typical incubation medium with glucose added. To compare the results the activity of mercaptide isolated from a sample incubated with the 3 amino acids was taken as 100%.

No. of erythrocytes in sample	Addition	Mercaptide (counts/min./sample)	Relative activity (%)
32×10^8	Gly	37.2±2	46.4
	Gly, Glu	33.1±2	41.3
	Gly, Cys	44.8±2	55.9
	Gly, Glu, Cys	80.1±4	100.0

three amino acids the activity of mercaptide is about twice as great as in the presence of glycine alone.

In further considerations the amount of GSH (calculated from the activity of mercaptide) formed during incubation with the three amino acids will be called "total biosynthesis", and the amount of GSH found after incubation with glycine alone, the "exchange"; the difference between these two values will be designated as "biosynthesis *de novo*". In Table 12 the comparison of the labelled GSH formed in erythrocytes by exchange, to the total biosynthesis is presented. The results are averages of experiments made with samples of erythrocytes obtained from the blood of 8 healthy persons.

Table 12

Total glutathione biosynthesis and glycine exchange in erythrocytes

The amount of GSH synthesized was calculated from the activity of mercaptide into which glycine was incorporated during 2 hr. incubation in typical medium with glucose added, under aerobic conditions. The results of 8 experiments are given, in which both exchange and total biosynthesis were determined in erythrocytes incubated with glycine or with three amino acids, respectively.

	GSH formed/one erythrocyte (μmole$\times10^{-12}$)	
	Range	Average
Exchange	4.7 - 8.6	6.94
Total biosynthesis	8.7 - 22.0	13.06

The biosynthesis of GSH in human thrombocytes and leucocytes is presented in Table 13. After 2 hr. incubation with the three amino acids the average total biosynthesis calculated per one thrombocyte was 7×10^{-12} μmoles GSH, and 76×10^{-12} μmoles per one leucocyte.

Table 13

Biosynthesis of glutathione in thrombocytes and leucocytes

Typical medium with glucose added. Specific activity of glycine, 8750 counts/min./μmole. Incubation, 2 hr. in open test tubes. Average values obtained with blood cells from three persons.

Expt. no.	No. of cells ($\times 10^8$)	Amino acids added	Mercaptide (counts/min./sample)	GSH formed in sample (μmoles $\times 10^{-3}$)	GSH formed /one cell (μmoles $\times 10^{-12}$)
			Thrombocytes		
1	17	Gly, Glu, Cys	57.7±3	13.2	7.76
2	16	Gly, Glu, Cys	31.0±1.5	7.1	4.43
3	18	Gly	8.7±1.7	2.0	1.11
3	18	Gly, Glu, Cys	70.4±3.5	16.1	8.98
			Leucocytes		
1	0.80	Gly, Glu, Cys	23.6±2	5.4	67.5
2	0.82	Gly	7.8±1.6	1.9	23.1
2	0.82	Gly, Glu, Cys	27.6±3	6.3	76.8
3	0.62	Gly, Glu, Cys	22.8±2	5.2	83.8

The exchange of glycine and the total biosynthesis of GSH per one blood cell can be compared with the previously established content of GSH in the cells (Table 14). The rate of GSH turnover in various blood cells incubated with an excess of substrates under the conditions used can be deduced from these results. If the process of exchange of glycine and biosynthesis *de novo* are considered together, and if it is assumed for the sake of simplification that the course of these reactions in time is rectilinear, it will be found that for the synthesis of an amount of GSH

Table 14

Comparison of the content of glutathione and its biosynthesis in blood cells

The values of GSH-content are averages from Table 1. The exchange values and total biosynthesis of GSH are taken from Tables 12 and 13.

Cells	GSH per one blood cell			GSH	
	Content (μmoles $\times 10^{-12}$)	Exchange (μmoles $\times 10^{-12}$)	Total biosynthesis (μmoles $\times 10^{-12}$)	Formed in % of the content	Exchange/total biosynthesis
Erythrocytes	192.0	6.94	—	3.6	—
	192.0	—	13.06	6.8	0.53
Thrombocytes	27.7	1.11	—	4.0	—
	27.7	—	7.06	25.5	0.15
Leucocytes	1950.0	23.1	—	1.2	—
	1950.0	—	76.0	3.9	0.33

corresponding to the amount of GSH per cell, erythrocytes need approx. 29.4 hr., thrombocytes only 7.8 hr., and leucocytes 51.3 hr.

Calculation of the obtained values of total biosynthesis of GSH in relation to the wet weight of packed cells permits a comparison of this process in cellular elements of blood considered as separate homogeneous tissues (Table 15).

Table 15

Total biosynthesis of glutathione per 1 gram of wet weight of packed blood cells

The values are averages calculated from Table 14.

Cells	Total biosynthesis of GSH (μmole/1 g. of cell mass/2 hr.)
Erythrocytes	0.108
Thrombocytes	0.387
Leucocytes	0.346

In Table 16 the ability of blood cells to incorporate glycine into GSH during incubation in aerobic and anaerobic conditions is compared. The suspensions of different blood cells used in the experiment were obtained from the same blood sample.

Table 16

Biosynthesis of glutathione in blood cells in aerobic and anaerobic conditions

Typical incubation medium with glucose added. Specific activity of glycine 8750 counts/min./μmole. Under "GSH formed" total biosynthesis values are given, except for erythrocytes incubated with glycine only, where the values represent the exchange.

Cells	No. of cells/ sample ($\times 10^8$)	Additions	Gas phase	Mercaptide (counts/min. /sample)	GSH	
					Formed/ sample (μmole $\times 10^{-3}$)	Formed in air/formed in N_2
Erythrocytes	30	Gly	Air	75.5±3	17.23	
		Gly	N_2	106.7±5	24.4	0.747
		Gly, Glu, Cys	Air	135.3±6	30.9	
		Gly, Glu, Cys	N_2	185.9±9	42.4	0.728
Thrombocytes	18	Gly, Glu, Cys	Air	70.4±3.5	16.1	
		Gly, Glu, Cys	N_2	56.6±3	12.9	1.24
Leucocytes	0.63	Gly, Glu, Cys	Air	22.8±2	5.2	
		Gly, Glu, Cys	N_2	35.8±3	8.2	0.634

DISCUSSION

If the amount of GSH is calculated in relation to the wet weight of packed cells, its content appears to be approximately the same in thrombocytes and in erythrocytes, while in leucocytes it is almost six times greater (Table 2). The ratio of leucocyte-GSH to erythrocyte-GSH according to the results of Hardin *et al.* [12] is approximately 6.48, and according to the present work it is 5.59. It appears that in the animal organism the leucocytes are the "tissue" most rich in GSH content [14].

It was found that in erythrocytes the oxidized form of glutathione constitutes about 14% of its total amount and the GSSG/GSH ratio is 0.164. This figure agrees with the results of other authors who employed similar methods [3]. Thrombocytes contain only reduced glutathione; in leucocytes the presence of small amounts of GSSG may be possible.

The attempt to determine the biosynthesis of glutathione in erythrocytes by the nitroprusside, alloxan and glyoxalase methods permitted only the conclusion that probably all the cellular elements of human blood produce small amounts of GSH during the incubation with appropriate substrates. In the case of erythrocytes the determinations made with the alloxan and glyoxalase methods (Tables 6 and 7) indicated that the amount of GSH formed during 2 hr. incubation of cells does not exceed 10% of their original GSH content.

The experiments with labelled glycine (Tables 8 - 16) indicate that the turnover of glutathione occurred not only in erythrocytes but also in thrombocytes and leucocytes. The formation of labelled erythrocyte-GSH may be associated with two reactions: a peptide synthesis *de novo* or an exchange of the amino acid. Snoke & Bloch [22, 23] demonstrated the biological reaction of glycine exchange in GSH. The enzyme isolated by them from yeast catalysed both the reaction of condensation of glutamylcysteine with glycine and the exchange of this amino acid in the tripeptide molecule. On the other hand, the GSH-synthetase obtained from pigeon liver [24] did not mediate the exchange reaction. Dimant *et al.* [8] showed that erythrocytes from human or duck blood suspended in isotonic saccharose solution without the addition of ions, glucose, cysteine and glutamic acid can incorporate glycine into the GSH molecule. The presented results support this observation indicating that human erythrocytes may contain an enzyme catalysing the exchange of glycine in glutathione.

The observed inhibition of glycine exchange by glucose is not yet clear. The inhibition depended on the ionic composition of the medium (Table 9) and may be associated with intracellular phosphorylations. This phenomenon seems to be similar to the Crabtree effect [7, 13] which consists in an inhibition by glucose of oxygen consumption in certain cell cultures.

However, the process of incorporation of glycine into erythrocyte-GSH is not due to the exchange reaction only. The increase of activity of the mercaptide after

the addition of cysteine or glutamic acid to the incubation mixture (Table 11) can be explained only as synthesis of the whole GSH molecule. This biosynthesis *de novo* is enhanced by phosphate, K^{1+} and Mg^{2+} ions, and glucose (Table 9). The determined values of total biosynthesis do not, of course, correspond to the increase of GSH in the cell, but represent the turnover of glutathione. The data in Tables 14 and 15 indicate a particularly intense turnover of GSH in thrombocytes.

The reaction of glycine incorporation into GSH of erythrocytes and leucocytes was enhanced by anaerobic conditions. This agrees with the results of other workers [10, 15] who also observed higher values of GSH biosynthesis in the erythrocytes of rats, incubated under anaerobic conditions.

In contrast to erythrocytes and leucocytes, in thrombocytes the biosynthesis of GSH was diminished in the absence of oxygen (Table 16). The same results were obtained by radioisotope method as by nitroprusside one (Table 4). In this respect, however, the thrombocytes, when compared to other tissues, are not an exception; Bloch [5] demonstrated that in pigeon-liver homogenates the rate of glycine incorporation into GSH was five times greater in aerobic than in anaerobic conditions.

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SUMMARY

The content of glutathione was determined by three methods in erythrocytes, thrombocytes and leucocytes isolated from the blood of healthy human subjects. Erythrocytes and thrombocytes contained approximately 1.5 μmole of glutathione per 1 gram of packed cells, and leucocytes over five times more.

After the incubation of the blood cell suspensions with cysteine, glutamic acid and [2-^{14}C]glycine the labelled glutathione was found in erythrocytes, thrombocytes and leucocytes. The experimental data indicated that two reactions occurred: an exchange of glycine and a synthesis of glutathione *de novo*. The turnover of glutathione was particularly intense in the thrombocytes.

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BIOSYNTEZA GLUTATIONU W ELEMENTACH MORFOTYCZNYCH KRWI LUDZKIEJ

Streszczenie

Zawartość glutationu w erytrocytach, płytkach i leukocytach wyosobnionych z krwi zdrowych ludzi oznaczono trzema metodami. Erytrocyty i płytki zawierają w przybliżeniu 1,5 μmola glutationu na 1 g odwirowanych krwinek, a leukocyty przeszło pięciokrotnie więcej.

Po inkubacji zawiesin krwinkowych z cysteiną, kwasem glutaminowym i [2-^{14}C] glicyną stwierdzono obecność znakowanego glutationu w erytrocytach, płytkach i leukocytach. Dane doświadczalne wskazują na występowanie dwóch reakcji: wymiany glicyny glutationu i syntezy glutationu *de novo*. Odnowa glutationu jest szczególnie szybka w płytkach krwi.

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INFLUENCE OF MALEATE AND N-ETHYLMALEIMIDE ON THE SYNTHESIS OF AMINO ACIDS FROM α-KETOGLUTARATE AND AMMONIA IN THE LIVER AND THE KIDNEY OF RATS

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It is generally accepted [15, 8] that the assimilation of inorganic nitrogen in the animal tissues occurs chiefly by the reductive amination of α-ketoglutarate to glutamate. The synthesis of other amino acids is supposed to take place as a result of transamination between glutamate and the corresponding ketoacids. α-Ketoglutarate for the synthesis of glutamic acid is formed in the tricarboxylic acid cycle, nitrogen is supplied by the tissue ammonia, and hydrogens by the oxidative decarboxylation of ketoglutarate.

According to Krebs *et al.* [16] for the synthesis of one molecule of glutamate two molecules of ketoglutarate are used. One of them undergoes oxidative decarboxylation and provides hydrogens for the reductive amination of the other molecule of α-ketoglutarate. The synthesis of glutamate, therefore, diverts a considerable amount of ketoglutarate from the tricarboxylic acid cycle. It may result in a deficiency of oxaloacetate necessary for the biosynthesis of citrate. This may seriously affect the metabolism of the whole organism and the physiological functioning of the tissue in which the increased glutamate synthesis takes place. In the liver the excessive amination of ketoglutarate may affect the metabolism, leading to the accumulation of acetoacetate [26, 8].

The oxidative decarboxylation of α-ketoglutarate provides hydrogens for various syntheses. Therefore, there may be a competition between the other biosynthetic processes and the amino acid synthesis for hydrogen atoms. The oxidation of α-ketoglutarate gives rise to four molecules of ATP[1]. It may be assumed that the shift of ketoglutarate from Krebs' cycle to amino acid synthesis is hardly to occur in such organs as kidneys, the physiological function of which is to maintain

[1]Abbreviations used: ATP, adenosine triphosphate; NAD, $NADH_2$ oxidized and reduced nicotinamide adenine dinucleotide; GSH, glutathione; CoA, coenzyme A; BAL, 2,3-dimercaptopropanol; tris, tris(hydroxymethyl)aminomethane; NEM, N-ethylmaleimide.

the proper composition of the organism; and if such a shift does occur it is connected with some disturbance of the physiological function of the organ. In connection with this it should be emphasized that the determination of the optimal conditions of the amino acid synthesis *in vitro* is more difficult for the kidney than for the liver [7].

In the present work the amino acid synthesis from α-ketoglutarate and ammonia in cyclophorase preparations of rat liver and kidney was compared. Since both the oxidative decarboxylation and the reductive amination of ketoglutarate are sensitive to SH inhibitors [24, 13, 23], and in rats intoxicated with maleate a transitory increase of α-ketoacids level in blood and urine was observed [10, 3], the effect of maleate and N-ethylmaleimide on glutamate synthesis was also investigated.

METHODS

Preparation of cyclophorase. White Wistar rats were used. After the animals had been killed the liver and kidneys were removed, cut into small pieces and chilled immediately in an ice-cold tris buffer used for homogenization. The tissue was blotted, weighed and homogenized in 6 volumes of the same buffer in an all-glass homogenizer, and cyclophorase was prepared according to Bartley *et al.* [4]. The homogenate was centrifuged at 0° for 15 min. at 2000 *g*, the sedimented cellular granules were washed twice with the volume of buffer equal to that used for homogenization, and centrifuged as before. The sediment consisting of washed nuclei and mitochondria was suspended in 4 volumes of buffer; 1 ml. of this suspension corresponded to about 200 mg. of fresh tissue.

Homogenization medium. 0.05 M-tris-HCl buffer, pH 7.2 containing 10^{-1} M-KCl, 10^{-3} M-$MgSO_4$ and 10^{-3} M-EDTA (disodium salt) was used.

Incubation. The synthesis of amino acids in the liver was investigated in a system containing 75 μmoles of tris pH 7.2, 100 μmoles KCl, 1.5 μmoles $MgSO_4$, 1.5 μmoles EDTA, 60 μmoles α-ketoglutarate and 60 μmoles NH_4Cl. In most experiments with the kidney 5 μmoles of ATP and 50 μmoles of nicotinamide were added.

Before the solutions were mixed they were neutralized to pH 7.2 by NaOH or, in the experiments with the kidney, by KOH. The reaction was initiated by the addition of cyclophorase in the amount corresponding to 200 mg. of fresh tissue. The final pH was 7.2, and the total volume 3 ml. The incubation was carried out in atmospheric air at 38° for 60 min. with continuous shaking.

Analytical methods. The amino acid synthesis was estimated by the decrease of the added α-ketoglutarate, as well as by the increase of the amino-nitrogen. Therefore, when the incubation was finished, two samples were taken. One of them was added to 5 vol. of 10% trichloroacetic acid and the ketoacids were determined in the filtrate by the Friedmann & Haugen method [11]. The second sample was added to 4 vol. of absolute ethanol, the filtrate was alkalized and ammonia

removed by airing, and then the total amino-nitrogen was determined by the Yemm & Cocking method [27].

Reagents. S-cysteinylosuccinate and S-glutathionylosuccinate were obtained by the procedure of Morgan & Friedmann [22]. Both preparations were crystallized twice from water, and S-cysteinylosuccinate additionally from methanol. Other reagents were: α-ketoglutaric acid, c.p. (Politechnika Śląska, Gliwice); NH_4Cl, A.R. (Fabryka Odczynników Chemicznych, Gliwice); maleic acid, A.R. (USSR); NEM, BAL and ATP-disodium salt (Light); glutathione, $MgSO_4$ and EDTA (Merck); tris and cysteine (Fluka).

Controls. It was found that 1 ml. of liver or kidney cyclophorase preparation did not contain detectable amounts of ketoglutarate. There was no decrease of ketoglutarate resulting from the non-enzymatic reaction with ammonium chloride. The control test for the formation of amino-nitrogen was a system which contained all the reagents except ammonium chloride. In this way the increase of amino-nitrogen was caused by the synthesis of amino acid exclusively from the exogenous ammonia.

RESULTS

The removal of ketoglutarate and the amino acid synthesis in the liver and kidney

The efficiency of the oxidative decarboxylation is a major factor influencing the extent of amino acid synthesis. The data in Table 1 show that liver cyclophorase in the absence of exogenous ammonia utilized about 40% of the added α-ketoglutarate. It could be expected according to Krebs' hypothesis that in the presence of exogenous ammonia the disappearance of ketoglutarate will be doubled as a result of dismutation. The oxidative decarboxylation of 120 μmoles of ketoglutarate (40% of the substrate added) provides hydrogens for the reductive amination of the same amount of substrate. On the whole 80% of the added α-ketoglutarate should be used. However, in the presence of an excess of ammonium chloride the removal of ketoglutarate increased by about 150 μmoles per g. of tissue (over 90% of the amount added). It corresponded to the amino acid synthesis which increased by about 150 μmoles of amino-nitrogen per 1 g. of tissue. The ratio of α-ketoglutarate removal to the increase of amino-nitrogen is about 1.8. This indicates that in the liver the presence of ammonia shifted the ketoglutarate metabolism towards the amino acid synthesis. Under these conditions this synthesis occurred with a maximum yield, all hydrogens liberated at the oxidative decarboxylation being utilized for the reductive amination.

In a medium containing no exogenous ammonia the removal of ketoglutarate by kidney cyclophorase (Table 1) was about 30% of the substrate added. It was therefore about 1/4 less than in the liver. In contrast to the liver, the addition of ammonia to the kidney was nearly completely without any effect on ketoglutarate disappearance. Under the same conditions in which liver cyclophorase utilized

Table 1

α-Ketoglutarate utilization and amino acid synthesis in rat liver and kidney

The composition of the incubation mixture and other details under methods. Results are expressed in μmoles/g. of tissue/hr.

Cyclophorase preparation	Experiment no.	α-Ketoglutarate removal					$N\text{-}NH_2$ formed (μmoles)	α-Ketoglutarate removed/ $N\text{-}NH_2$ formed
		Without NH_4^+		With NH_4^+ added		Δ (μmoles)		
		(μmoles)	(% of amount added)	(μmoles)	(% of amount added)			
Liver	1	101.0	34	263.5	88	162.5	153.5	1.7
	2	139.0	46	288.5	96	149.5	155.0	1.9
	3	128.5	43	283.0	94	154.5	150.5	1.9
	4	113.5	38	256.0	85	142.5	139.5	1.8
	average	120.5	40	273.0	91	152.5	149.5	1.8
Kidney	1	100.0	33	115.0	38	15.0	15.0	7.7
	2	101.0	34	117.0	39	16.0	13.5	8.7
	3	85.0	28	81.0	27	-4.0	7.5	10.8
	4	95.5	32	105.0	35	9.5	10.0	10.5
	average	95.5	32	104.5	35	9.0	11.5	9.0

90% of ketoglutarate and formed about 150 μmoles of amino acid, the kidney cyclophorase used up only 35% of ketoglutarate and synthesized only 11.5 μmoles of amino acid. In kidney the ketoglutarate disappearance was nearly 3 times lower and the amino acid synthesis over 12 times lower than in liver. The ratio of the ketoglutarate disappearance to the increase of the amino-nitrogen was 9, indicating that for 9 molecules of ketoglutarate which were utilized only one underwent a reductive amination.

Influence of ATP and nicotinamide

Krebs *et al.* [16] have shown that the addition of ATP to liver homogenate increased the synthesis of glutamate. Also the data of Braunstein *et al.* [6, 7] and Kretowicz [19] indicate the increased amino acid synthesis in the presence of ATP. As can be seen from the data in Table 2 the addition of ATP 1.7×10^{-3} M to the kidney cyclophorase stimulated the amino acid synthesis resulting in the removal of over 90% of ketoglutarate added and in the formation of about 50 μmoles of amino-nitrogen per g. of tissue. The amino acid synthesis was five times greater than without ATP but 2/3 lower than in the liver, in spite of the fact that the ketoglutarate removal in both cases was almost complete.

In the kidney the presence of ATP was necessary for a good yield of the synthesis. It seems that the higher synthesis cannot be explained only as a result of increased α-ketoglutarate utilization, which increased only three times whereas the amino-nitrogen formation five times. The ratio of the ketoglutarate disappearance to

the increase of amino-nitrogen was about 5. It means that for 5 molecules of ketoglutarate which were utilized 4 underwent an oxidative decarboxylation and only one a reductive amination, and that the hydrogens liberated from 3 molecules of ketoglutarate were transferred to another acceptor. This incomplete yield of amino acid synthesis in the kidney as compared with the liver can be explained by a lower activity of the glutamate dehydrogenase or by a transfer of hydrogens to another acceptor.

Table 2

The influence of ATP and nicotinamide on the amino acid synthesis from α-ketoglutarate and ammonia in rat kidney

The composition of the incubation mixture and other details under Methods. Results are expressed in μmoles/g. of tissue/hr.

Additions	Experiment no.	α-Ketoglutarate removal		$N\text{-}NH_2$ formed (μmoles)	α-Ketoglutarate removed/ $N\text{-}NH_2$ formed
		(μmoles)	(% of amount added)		
None	Average data from Table 1	104.5	35	11.5	9.0
Nicotinamide, 50 μmoles	1	116.0	39	21.0	5.5
	2	113.5	38	14.0	8.1
	3	102.0	34	15.0	6.8
	4	78.0	26	11.5	6.8
	average	102.5	34	15.5	6.6
ATP, 5 μmoles	1	267.5	89	51.5	5.2
	2	269.5	90	45.0	6.0
	3	251.0	86	48.5	5.3
	4	286.0	95	57.5	5.0
	average	270.0	90	50.5	5.3
ATP, 5 μmoles, and nicotinamide, 50 μmoles	1	291.5	97	58.5	5.0
	2	294.0	98	58.0	5.1
	3	280.5	94	50.0	5.6
	4	297.0	99	63.5	4.7
	average	290.0	97	57.5	5.0

Both the ketoglutarate oxidation and the reductive amination are NAD-dependent. Nicotinamide is a factor preventing the breakdown of the dinucleotide by NAD-ase. It was found (Table 2) that nicotinamide added to the kidney cyclophorase at the concentration of 1.7×10^{-2} M was without any effect on α-ketoglutarate removal, but the amino acid synthesis increased by about 30% forming about 15.5 μmoles of amino-nitrogen per g. of tissue. The ratio of ketoglutarate utilisation to the increase of amino-nitrogen was lowered to about 6.

When ATP and nicotinamide were added simultaneously the kidney cyclophorase utilized nearly 100% of ketoglutarate. Also the synthesis of the amino acid gave the highest yield under the conditions employed, and was expressed by the increase of 57 μmoles of amino-nitrogen per g. of tissue. The ratio of ketoglutarate removal to the amino acid formation was about 5. This was the lowest value obtained for the kidney. The effect of nicotinamide is not yet clear. As the rate of amino acid synthesis was the highest in the presence of ATP and nicotinamide all further experiments with kidney cyclophorase were carried out in media containing ATP and nicotinamide.

The influence of maleate

The enzymatic system of α-ketoglutarate decarboxylation contains lipoic acid and CoA. Recently Massey [21] demonstrated that the protein SH groups are involved in the hydrogen transfer from lipoic acid onto flavine. Glutamate dehydro-

Table 3

The influence of maleate on the amino acid synthesis from α-ketoglutarate and ammonia in rat liver and kidney

Maleate was added to the incubation mixture in the amounts: 0.3 - 30.0 μmoles for the kidney and 30 - 310 μmoles for the liver. In experiments with the kidney 5 μmoles ATP and 50 μmoles nicotinamide were added. Other conditions as described in Methods. Results are expressed in μmoles/g. of tissue/hr.

Cyclophorase preparation	Maleate (M)	α-Ketoglutarate		$N\text{-}NH_2$		α-Ketoglutarate removed/$N\text{-}NH_2$ formed
		Removal (μmoles)	Inhibition (%)	Formation (μmoles)	Inhibition (%)	
Liver	None	292.0	—	161.5	—	1.8
	1×10^{-2}	287.5	2	160.0	—	1.8
	2×10^{-2}	258.5	12	135.0	16	1.9
	3×10^{-2}	181.0	38	95.5	41	1.9
	4×10^{-2}	129.0	56	55.5	66	2.3
	5×10^{-2}	92.0	68	36.5	80	2.5
	6×10^{-2}	64.0	78	23.0	86	2.8
	7×10^{-2}	39.5	86	16.5	90	2.4
	8×10^{-2}	28.0	90	5.0	97	5.6
Kidney	None	292.0	—	58.5	—	5.0
	1×10^{-4}	270.0	7	67.0	—	4.0
	3×10^{-4}	220.0	25	45.5	22	4.8
	6×10^{-4}	114.5	61	24.0	59	4.8
	1×10^{-3}	67.0	77	14.0	76	4.8
	3×10^{-3}	39.0	87	4.5	92	9.0
	5×10^{-3}	30.5	90	1.0	98	—
	1×10^{-2}	20.0	93	0.0	100	—

genase according to Hellerman *et al.* [13] contains three SH groups and at least two of them are active. The inhibition of glutamate synthesis by SH-reagents may concern different stages of oxidative decarboxylation of ketoglutarate, as well as the reductive amination.

Data shown in Table 3 indicate that a very high concentration of maleate was necessary to inhibit the ketoglutarate disappearance and amino acid synthesis in liver. A 50% inhibition was observed at $3-4\times10^{-2}$ M concentration and nearly complete inhibition of the reactions took place at concentrations of maleate approaching 0.1 M. Maleate affected at about the same degree the ketoglutarate disappearance and the formation of amino-nitrogen.

In the kidney the amino acid synthesis and ketoglutarate disappearance were inhibited by maleate concentrations nearly 100 times lower than in the liver. So, 25% of inhibition occurred already at the maleate concentrations of 3×10^{-4}, that is about 70 times lower than in the liver. Maleate 10^{-3} M produced about 75% inhibition in kidney, while in liver it was necessary to use concentrations over 50 times higher. This indicates that amino acid synthesis in kidney is much more sensitive to maleate than in liver. Similar selective action of maleate on the kidney was found in the experiments *in vivo*. It was shown by the decrease of the SH groups in the kidney of maleate-treated rats [25] and by the multiple tubular defects of the kidney [2].

The influence of N-ethylmaleimide (NEM)

N-Ethylmaleimide is a much stronger inhibitor than maleate. Because of its high reactivity with SH groups it has *in vivo* a strong toxic action even in very small doses, acting probably by blocking the oxidative metabolism. It does not produce, however, multiple tubular defects (Angielski *et al.*, unpublished data).

Data presented in Table 4 show that NEM inhibited the amino acid synthesis at concentrations much lower than those of maleate. Moreover, no differences were observed in its action on the liver and the kidney. At concentrations of 5×10^{-5} M NEM produced 25% inhibition of ketoglutarate disappearance and of amino-nitrogen increase. At 10^{-4} M concentration there was a 75% inhibition, and at the concentration $2-3\times10^{-4}$ M a total inhibition of amino acid synthesis in the kidney as well as in the liver. These concentrations were lower than the effective concentrations of maleate, for the kidney over 10 times and for the liver 500 times. As in the case of maleate there was a parallelism between the inhibition of ketoglutarate utilization and the inhibition of amino-nitrogen formation. These data may indicate that maleate as well as NEM inhibited the oxidative decarboxylation of α-ketoglutarate and not directly the amino acid synthesis. It is known that 10^{-3} M-maleate inhibits the oxidative decarboxylation, but is without any effect on the activity of succinic, malic or *iso*citric dehydrogenase [14, 12, 20]. At the same time it is not likely that the inhibition by the same concentrations of the inhibitor should

Table 4

The influence of N-ethylmaleimide (NEM) on the amino acid synthesis from α-ketoglutarate and ammonia in rat liver and kidney

NEM was added to the incubation mixture in the amounts of 0.03 - 0.9 μmole. In experiments with kidney 5 μmoles ATP and 50 μmoles nicotinamide were added. Other conditions as described in Methods. Results are expressed in μmoles/g. of tissue/hr.

Cyclophorase preparation	NEM (M)	α-Ketoglutarate		$N\text{-}NH_2$		α-Ketoglutarate removed/$N\text{-}NH_2$ formed
		Removal (μmoles)	Inhibition (%)	Formation (μmoles)	Inhibition (%)	
Liver	None	274.0	—	148.0	—	1.0
	1×10^{-5}	271.5	0	162.5	—	1.7
	3×10^{-5}	263.0	4	145.0	2	1.8
	5×10^{-5}	207.5	26	104.0	30	2.0
	7×10^{-5}	137.0	50	71.0	52	1.9
	1×10^{-4}	76.5	72	32.5	78	2.3
	1.5×10^{-4}	63.0	77	19.5	87	3.2
	2×10^{-4}	46.5	83	13.5	93	3.4
	3×10^{-4}	27.5	90	1.0	100	—
Kidney	None	290.0	—	57.0	—	5.1
	1×10^{-5}	294.0	0	70.5	—	4.2
	3×10^{-5}	284.0	2	54.5	4	5.2
	5×10^{-5}	235.0	19	44.0	23	5.3
	7×10^{-5}	148.0	49	30.0	47	4.9
	1×10^{-4}	63.5	78	12.5	78	5.1
	1.5×10^{-4}	29.0	90	2.5	96	12.0
	2×10^{-4}	11.5	96	0.5	99	—
	3×10^{-4}	11.5	96	0.0	100	—

concern the two different reactions to the same degree both in the kidney and in the liver.

From the data presented in Tables 3 and 4 it can be seen that very low concentrations of maleate and especially of NEM, which had no inhibitory effect on the α-ketoglutarate disappearance, stimulated the amino acid synthesis. This strange phenomenon which indicated the increased activity of glutamate dehydrogenase in the presence of low maleate concentrations agreed with the results of Hellerman *et al.* [13]. They have found a similar stimulating effect using very low concentrations of mercuric compounds.

Influence of small-molecular sulfhydryl compounds on the inhibition of the amino acid synthesis produced by maleate and NEM

Dixon & Webb [9] consider maleate as belonging to the group of alkylating inhibitors. The inhibition produced by such compounds is, in general, irreversible. Table 5 presents the results of the experiments on the effect of GSH, cysteine and

Table 5

The influence of sulfhydryl compounds on the maleate inhibition of amino acid synthesis in kidney

Cyclophorase preparation was preincubated with maleate for 15 min.; then substrates and neutral solutions of sulfhydryl compounds were added. Other conditions as described in Table 3 and in Methods

Maleate (M)	Addition (M)	Inhibition of	
		α-Ketoglutarate removal (%)	$N\text{-}NH_2$ formation (%)
6×10^{-4}	None	49	57
	Cysteine, 6×10^{-4}	64	50
1×10^{-3}	None	73	72
	GSH, 3×10^{-3}	69	76
	GSH, 5×10^{-3}	77	85
	Cysteine, 3×10^{-3}	83	83
	BAL, 3×10^{-3}	74	80
	BAL, 5×10^{-3}	58	61

Table 6

The amino acid synthesis from α-ketoglutarate and ammonia in the presence of S-cysteinylossuccinate (CSA) and S-glutathionylosuccinate (GSA) in rat liver and kidney

Maleate, CSA and GSA were added in neutralized solutions. Other conditions as described in Table 3 and in Methods. Results are expressed in μmoles/g. of tissue/hr.

Experiment no.	Kidney			Liver		
	Addition (μmoles)	α-Ketoglutarate removed (μmoles)	$N\text{-}NH_2$ formed (μmoles)	Addition (μmoles)	α-Ketoglutarate removed (μmoles)	$N\text{-}NH_2$ formed (μmoles)
1	None	296.0	57.5	None	271.0	128.5
	Maleate, 15	20.5	0.0	Maleate, 180	26.5	4.5
	CSA, 15	265.0	60.0*	CSA, 180	240.5	120.0*
	GSA, 15	295.0	—	GSA, 180	223.5	—
2	None	290.0	55.0	None	278.5	170.0
	Maleate, 15	26.5	0.5	Maleate, 180	46.5	15.0
	CSA, 15	296.5	53.5*	CSA, 180	296.0	148.5*
	GSA, 15	256.0	—	GSA, 180	251.0	—
3	None	262.5	49.5			
	Maleate, 15	33.0	1.0			
	GSA, 15	248.0	—			

* Corrected by subtracting the theoretical values of $N\text{-}NH_3$ content in CSA.

BAL on the maleate inhibition of amino acid synthesis in the kidney. The amounts of GSH, cysteine and BAL 3 and even 5 times greater than those of maleate were without significant effect. Also in the experiments in which no preincubation of cyclophorase with maleate was carried out, but cyclophorase was added simultaneously with the sulfhydryl compounds, the decrease of the inhibition was not noticed. These data show the irreversible character of the inhibition produced by maleate. It may by possible, however, that the addition product of maleate with small-molecular SH groups had an inhibiting effect. It appeared (Table 6) that S-cysteinylosuccinate as well as S-glutathionylosuccinate at concentrations corresponding to the inhibitory concentrations of maleate had no effect on the amino acid synthesis. It can be also concluded that there was a higher reactivity of the maleate with the SH groups of the oxidative decarboxylation system of the kidney than with the SH groups of GSH, cysteine or BAL.

Table 7

The influence of sulfhydryl compounds on the inhibition of amino acid synthesis caused by N-ethylmaleimide (NEM)

The cyclophorase preparation was preincubated with NEM for 10 min. Other conditions as described in Tables 3 and 5 (for the kidney) and in Methods.

Cyclophorase preparation	NEM (M)	Addition (M)	Inhibition of	
			α-Ketoglutarate removal (%)	$N\text{-}NH_2$ formation (%)
Kidney	1×10^{-4}	None	79	80
		GSH, 3×10^{-4}	46	45
		Cysteine, 5×10^{-4}	42	35
	1.5×10^{-4}	None	96	100
		GSH, 3×10^{-4}	56	55
		Cysteine, 1.5×10^{-4}	60	61
		BAL, 1.5×10^{-4}	21	20
Liver	1×10^{-4}	None	75	86
		GSH, 1×10^{-4}	47	31
		GSH, 3×10^{-4}	22	20
		BAL, 1×10^{-4}	30	38
	1.5×10^{-4}	None	88	92
		GSH, 1.5×10^{-4}	55	47
		BAL, 1.5×10^{-4}	23	40

The inhibition of amino acid synthesis produced by NEM was lowered 30 - 50% both in the kidney and the liver by the addition of sulfhydryl compounds (Table 7). BAL had a stronger effect than the monothiol compounds and overcame the inhibition of NEM by about 70%. These results indicate the reversibility of the inhibition produced by NEM. They also suggest that the reactivity of NEM with

GSH, cysteine and especially with BAL was higher than with the SH groups of the oxidative decarboxylation systems of the kidney and liver. The difference in the effects of maleate and NEM on the amino acid synthesis manifested itself not only by their concentrations necessary to inhibit the reaction, but also by their reactivity with SH groups of the enzymatic system. That might favour the supposition that maleate and NEM act on different SH groups involved in the oxidative decarboxylation of α-ketoglutarate. Of course, it is possible that any of these inhibitors may also affect other active groups of the enzymes.

DISCUSSION

The results presented in this paper show marked differences in the amino acid synthesis from α-ketoglutarate and ammonia in rat liver and kidneys. They concern first of all the yield of amino acid synthesis, and the ratio of ketoglutarate disappearance to the increase of amino-nitrogen.

When comparing the yields of amino acid synthesis in various organs one should take into consideration the specific function of the organ studied as well as the participation of the reaction investigated in the general metabolic pattern. The liver is an organ of biosynthesis and the equilibrium of the reaction α-ketoglutarate $+ NH_3 + NADH_2 \rightleftharpoons$ glutamate $+$ NAD is shifted towards the glutamate synthesis [5]. So in the presence of an excess of ammonia the amino acid synthesis by the liver cyclophorase occurred with the maximum yield. All hydrogens from the oxidative decarboxylation were utilized for the reductive amination. This is shown by the ratio of the ketoglutarate disappearance to the increase of amino-nitrogen, which is nearly 2.

The low amino acid synthesis in the kidney as compared with the liver seems to be the result of the low reductive amination. Only a part of the hydrogens from the oxidative decarboxylation became utilized for the reductive amination of ketoglutarate. The kidney is an organ which constantly carries out an osmotic work. The source of the energy for the active transport in tubule-cells is the oxidative metabolism. The withdrawal of ketoglutarate from the tricarboxylic acid cycle as a result of amination to glutamate would impair the normal functioning of kidneys. According to Krebs [17] the turnover rate of the tricarboxylic acid intermediates is in the kidney twice as great as in the liver. It seems therefore likely that in kidney the rate of hydrogens transfer on to the Keilin-Warburg system considerably exceeds the rate of the reductive amination. Then the small yield of the amino acid synthesis could be explained by the lower activity of the glutamate dehydrogenase [18] and by the faster removal of the hydrogens to other acceptors.

It was shown that maleate inhibits the synthesis of amino acids in liver and kidney. In the liver 75% of the inhibition occurred at the maleate concentrations about 5×10^{-2} M. In the kidney the similar inhibition was obtained at concentrations 50 - 100 times lower. The parallelism of the inhibition in the ketoglutarate removal and amino-nitrogen increase permits to assume that the effect of maleate

on the amino acid synthesis depends really on the inhibition of the oxidative decarboxylation of ketoglutarate.

The increased excretion of ketoacids in maleate intoxication demonstrated previously [3] may indicate that there is an *in vivo* inhibition of the oxidative decarboxylation system. It can be supposed that the selective action of maleate on the kidney corresponds, at least partly, to the specific inhibition of the oxidative decarboxylation. It is not yet clear to what degree the oxidative decarboxylation becomes inhibited *in vivo* in maleate-treated rats, and what is the importance of this inhibition for the whole picture of the intoxication. The *in vivo* administration of BAL in amounts equimolar with maleate prevents the appearance of aminoaciduria [1] whereas *in vitro* even 5-fold excess is without significant effect on the inhibition produced by maleate. It may appear that *in vivo* the inhibition of the oxidative decarboxylation in the kidney is of no great importance for the syndrome of multiple tubular defects.

The present results provide evidence for a selective action of maleate on kidney. It seems also that NEM, in contrast to maleate, affects the kidney and the liver to a similar degree. The concentrations of maleate and NEM necessary to inhibit the amino acid synthesis are different, as well as their reactivity with SH groups of the enzymatic system. These data may suggest that maleate and NEM act on different SH groups of the oxidative decarboxylation system of α-ketoglutarate. The combination of maleate with the SH groups in liver and kidney seems to be irreversible.

SUMMARY

1. The amino acid synthesis in rat liver and kidney was compared. Liver-cyclophorase utilized about 273 μmoles of α-ketoglutarate (91% of the amount added) and synthesized about 150 μmoles of amino-nitrogen per gram of tissue and hour. The rate of ketoglutarate disappearance and amino-nitrogen increase was about 1.8.

2. The presence of ATP (1.7×10^{-3} M) was necessary for obtaining a good yield in kidney-cyclophorase. Then the disappearance of ketoglutarate amounted to 90%; the increase of amino-nitrogen was about 50 μmoles/g. of tissue/hr. The ratio of these reactions was about 5.3.

3. Maleate (5×10^{-2} M) inhibited by 75% the ketoglutarate disappearance and the formation of amino-nitrogen in liver. In kidney a similar effect was obtained at the maleate concentrations 50 - 100 times lower.

4. N-ethylmaleimide (NEM) in much lower concentration (10^{-4} M) exhibited the same 75% inhibition both in kidney and in liver.

5. The same degrees of inhibition of ketoglutarate disappearance and amino-nitrogen formation were observed with maleate as with NEM .This suggests that the inhibition concerns only the first reaction in our system, that is the oxidative decarboxylation of α-ketoglutarate.

6. The compounds produced by maleate with cysteine (S-cysteinylosuccinate) and with glutathione (S-glutathionylosuccinate) were without any effect on the synthesis of amino acids.

7. The inhibition produced by NEM was partly reversed by sulfhydryl compounds (BAL, glutathione, cysteine), whereas the inhibition produced by maleate was irreversible.

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WPŁYW MALEINIANU I N-ETYLOMALEIMIDU NA SYNTEZĘ AMINOKWASÓW Z α-KETOGLUTARANU I AMONIAKU W NERCE I WĄTROBIE SZCZURA

Streszczenie

1. Porównano wydajność syntezy aminokwasów w wątrobie i w nerce szczurów. Cykloforaza wątroby zużywa około 273 μmole α-ketoglutaranu (91% dodanego) i syntetyzuje około 149,5 μmoli azotu aminowego na gram tkanki i godzinę. Stosunek zużycia ketoglutaranu do przyrostu azotu aminowego wynosi około 1,8.

2. Koniecznym warunkiem wydajnej syntezy aminokwasów przez cykloforazę nerki jest obecność ATP ($1{,}7 \times 10^{-3}$ M). Zużycie ketoglutaranu wynosi wówczas 90%; przyrost azotu aminowego około 50 μmoli/g tkanki/godz. Stosunek tych reakcji wynosi około 5,3.

3. Maleinian (5×10^{-2} M) hamuje w 75% zużycie ketoglutaranu i przyrost azotu aminowego w wątrobie. W nerce podobne działanie uzyskuje się przy stężeniach maleinianu 50 - 100 razy mniejszych.

4. N-etylomaleimid (NEM) hamuje syntezę aminokwasów w stężeniach znacznie mniejszych (10^{-4} M) zarówno w nerce, jak i w wątrobie.

5. Stwierdzono równoległość w hamowaniu zużycia ketoglutaranu i przyrostu azotu aminowego przez maleinian i NEM. Na tej podstawie wysunięto przypuszczenie, że zahamowaniu ulega tylko pierwszy etap t.j. oksydatywna dekarboksylacja α-ketoglutaranu.

6. Połączenia maleinianu z cysteiną (S-cysteinylobursztynian) i z glutationem (S-glutationylobursztynian) są bez wpływu na syntezę aminokwasów.

7. Zahamowanie powodowane przez N-etylomaleimid daje się częściowo odwrócić działaniem związków sulfhydrylowych (BAL, glutation, cysteina), natomiast hamowanie powodowane przez maleinian jest nieodwracalne.

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STUDIES ON THE LEBEDEW PROCESS*

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A. von Lebedew prepared a cell-free solution of the fermentation enzyme system by macerating dry yeast with water and separating the supernatant (Lebedew juice) [1]. This procedure can be thought to be a simple extraction of soluble enzymes. Our experiments, however, indicate that it depends on a number of biochemical reactions.

When dried brewer's yeast washed free from readily soluble protein and cofactors was incubated with yeast "Kochsaft" alcohol dehydrogenase (ADH) [1] [2] and fructose diphosphate aldolase [3] appeared in the supernatant. The Kochsaft could be replaced by a mixture of DPN, ATP, and orthophosphate, and in the case of aldolase by a thermostable yeast component. When yeast was incubated with water these enzymes did not appear. Their appearance during incubation with factors was inhibited by iodoacetate.

The effects brought about by cofactors on washed yeast cells we called "enzyme development". Since the cofactors are present in dry yeast we believe that the development of ADH and aldolase are parts of the Lebedew process. In this process both the heat-stable and the heat-labile components of dry yeast react together during maceration; what we did was to separate them first in order to reconstruct the system.

In the present paper we describe experiments in which enzyme activities of different yeast preparations are compared with fermentation rates. We showed that in the first washing from dry cells at least one enzyme, other than aldolase and ADH, is missing and that this enzyme can be released by the developing agents. So long as this enzyme (or these enzymes) is absent the supernatant is not able to ferment glucose. As soon as it is "developed" glucose fermentation can be performed.

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[1] The following abbreviations are used: ADH, alcohol dehydrogenase; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; FDP, fructose diphosphate.

METHODS

Aldolase activity was measured by estimation of the alkali-labile triose phosphate formed out of 11.7 μmoles of FDP within 30 min. [3]; final volume 5.5 ml., pH 7, 38°. The data are μg. of alkali-labile P produced by 1.0 mg. of dry yeast or an enzyme solution corresponding to this amount.

ADH was estimated spectrophotometrically with ethanol and DPN [2]. The data are $\Delta E \times 10^3$ per minute per 0.1 mg. of dry yeast.

Fermentation was measured manometrically. The Warburg vessels contained: 0.167 m-moles of glucose; 4.7 μmoles of FDP; 6.7×10^{-2} M-NaK-phosphate, pH 6.4; 2.1×10^{-3} M-magnesium sulphate; dry yeast or an aqueous yeast preparation. Final volume 1.0 ml.; incubation at 28°; air as gas phase. Fermentation reached its maximum rate usually after 90 min. It was measured per 10 min. or 1 hr.

RESULTS AND DISCUSSION

The fermentation rates, aldolase activity and ADH activity of untreated dry yeast suspension and of macerated yeast suspension were compared. The maceration of dry yeast was performed with three volumes of water, at 37° during 2 hr. The results given in Table 1 show that fermentation rate and enzyme activities

Table 1

Comparison between untreated and macerated dry yeast

Untreated dry yeast suspension, and juice of macerated for 2 hr. dry yeast were used. For details see Methods.

Fermentation (10 mg. yeast) (μl. CO_2/10 min.)		Aldolase (1 mg. yeast) (μg. alkali-labile P/30 min.)		Alcohol dehydrogenase (0.1 mg. yeast) (ΔE/min. $\times 10^3$)	
Untreated	Macerated	Untreated	Macerated	Untreated	Macerated
22	51	28	105	9	22

are more than doubled by maceration. Since fermentation is brought about with cooperation of aldolase and ADH, it could be thought that one of these enzymes might be the rate-controlling factor. This is, however, not the case. For we can obtain a yeast extract similar to Lebedew juice which contains considerable amounts of aldolase and ADH, but which is not able at all to ferment glucose: this is a quickly prepared supernatant of a water suspension of dry yeast. Here certainly the enzyme system is not complete. It has to be assumed that the missing enzyme(s) could be solubilized only by prolonged maceration. If this is true, it may be expected that the juice will become progressively more and more potent in fermenting ability. It is also possible that the increase in fermentation rate will be greater than the increase in the activities of the two enzymes. The experiments presented in

Table 2 confirmed these assumptions. Between the maceration times 0.5 and 4 hr. the activities of aldolase and ADH were increased 4 and 5 times, respectively, whereas the increase in fermentation was about 45-fold: $(16+12)\times\frac{10}{40+20}:211 = 1:45$. It can be concluded that the rate of fermentation is controlled by the enzyme which was absent in the supernatant before the Lebedew process took place.

Table 2

Fermentation of supernatants after different times of yeast maceration; comparison with aldolase and alcohol dehydrogenase activity

	Time of maceration (hr.)	Yeast (mg.)	Activity	
Fermentation	0.5	40	(μl. CO_2/hr.)	16
		20		12
	4.0	10		211
Aldolase	0.5	1	(μl. alkali-labile P/30 min.)	29
	4.0	1		107
Alcohol dehydrogenase	0.5	0.1	(ΔE/min. $\times 10^3$)	8
	4.0	0.1		42

We wanted to demonstrate that this very enzyme which is released by maceration of unwashed dry yeast can also be "developed" by incubating washed cells with the pertinent factors yielding a supernatant able to ferment glucose. As we have seen with ADH, this incubation can be carried out with yeast Kochsaft. However, we prefer to describe an assay with phosphate and an active fraction M prepared from Kochsaft, as development factors.

Baker's yeast was put into one tenth of its weight of hot water in portions, so that the temperature remained above 80°. To the cooled filtrate 0.3 vol. of methanol was added and the formed precipitate was discarded; then fraction M was precipitated with 2.3 vol. of methanol, washed with methanol and dried *in vacuo*. Yield: 0.35% in weight of fresh yeast.

Fraction M contained 1.4% inorganic P and 0.55% acid-labile P (N-HCl, 100°, 7 min.). Since different amounts of fraction M (0 to 25 mg.) meant at the same time different amounts of orthophosphates 13.3 μmoles of phosphate was added to each incubation mixture to minimize the difference in respect to P.

Assays were carried out as follows: 30 mg. of dry yeast was suspended in water containing 15% of glycerol and left for 15 hr. Cell rests were separated by centrifuging and incubated for 7 hr. at 37° with fraction M dissolved in 0.1 ml. of phosphate solution. Then the mixture was diluted with 0.5 ml. of water which contained

so much of fraction M that the amount of the latter became equal in all samples. In this way in all fermentation tests the content in thermostable substances was the same. Fermentation was determined with 0.4 ml. of the supernatant of incubation samples and with the components mentioned before (see Methods).

Table 3

Enzyme development measured as relationship between the fermentation rate and the amount of fraction M

No. of assays	Fraction M (mg.)	µl. CO_2/hr.	µl. CO_2/hr./mg. fraction M
12	5	78	16
14	10	183	18
11	16	270	17
12	25	355	14

CO_2 liberated between 120 and 180 min. was measured. The data were calculated by subtracting 140 µl. CO_2 per hour, which represent the control values given by phosphate in the absence of fraction M. (Average of 7 assays). Results (Table 3) showed that the fermentation of the supernatant was proportional to the amount of fraction M present in the incubation mixture. Apparently, fraction M is able to develop the enzyme (or the enzymes) which complements the juice of macerated cells and controls the rate of fermentation, acting stoichiometrically.

The proportionality stated above is noticeable only when we subtract the phosphate control values.

We are inclined to regard these control values as the effect of extracting the preformed enzyme by phosphate. In the case of ADH we were able to prove such an explanation by comparing samples containing phosphates with the samples to which iodoacetate was added as inhibitor of the development. In the experiments with fraction M, however, which are based on the whole fermentation system the inhibitor technique could not be applied.

We did some preliminary studies in order to identify the enzyme missing before maceration. We observed that the early supernatant of dried yeast which can not yet ferment glucose is already able to ferment fructose diphosphate in the presence of glucose as phosphate acceptor. It seems possible, therefore, that phosphofructokinase is involved.

SUMMARY

The supernatant of a fresh suspension of dry yeast in water contains considerable amounts of aldolase and alcohol dehydrogenase, but is not able to ferment glucose.

The maceration according to Lebedew can be imitated by incubation of washed dry yeast with a thermostable yeast fraction. Similar systems had been used for preparing supernatants with aldolase and alcohol dehydrogenase activities. In these processes called enzyme development ATP and DPN were also effective.

The Lebedew process, therefore, is interpreted as a series of chemical reactions rather than a simple extraction.

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BADANIA NAD PROCESEM LEBEDEWA

Streszczenie

Supernatant świeżej zawiesiny wodnej suchych drożdży zawiera znaczne ilości aldolazy i dehydrogenazy alkoholowej, nie fermentuje jednak glukozy.

Maceracją drożdży według Lebedewa można zastąpić przez inkubację przemytych suchych drożdży z termostabilną frakcją drożdży. Po inkubacji supernatant posiada oprócz aktywności aldolazy i dehydrogenazy alkoholowej również zdolności fermentowania glukozy. Proces zachodzący podczas inkubacji nazwano „rozwijaniem enzymu".

Proces Lebedewa można więc uważać za szereg reakcji chemicznych a nie za zwykłą ekstrakcję.

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ENDOGENOUS RESPIRATION OF *MYCOBACTERIUM PHLEI* AT VARIOUS TEMPERATURES

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This paper is dealing with respiration of "resting" bacteria at various temperatures. It is a part of our investigations on the effect of temperature on the metabolism, started some years ago on insects [1, 2]. The respiration of the bacterial cells grown on culture medium supplies the energy needed for maintenance, growth and reproduction; however in washed bacteria suspended in inorganic buffer solution the growth and reproduction processes are almost negligible. Therefore in this latter case the rate of oxygen uptake corresponds almost exclusively to the energy required for maintenance, and the endogenous respiration might be considered as "basal metabolism".

As the object of our studies *Mycobacterium phlei* was used. High resistance of *Mycobacteria* to starvation [3, 6] allows to expect negligible loss of living cells in short time experiments. To avoid thermal injury, temperatures employed were limited to the range in which *Mycobacterium phlei* can be grown.

MATERIAL AND METHODS

Mycobacterium phlei (a stock-strain from the Institute of Tuberculosis, Warszawa) was grown at 25° or 35° on the surface of the synthetic liquid medium, containing glucose [5]. Bacteria from the 2 - 9th subcultures were harvested from the liquid medium on the 7 - 9th day of growth.

The experiments were performed with non-starved as well as with starved bacteria. In the first case, the bacterial mass was collected from 100 ml. culture medium, washed twice with 100 ml. portions of buffer solution and centrifuged at 2000 *g* for 10 min.; 3 g. of wet bacterial mass was then suspended in 50 ml. buffer solution. Starved bacteria were obtained by substituting buffer solution for the culture medium. The buffer solution was introduced underneath the grown up pellicle and underlayed culture was incubated simultaneously with the non-

starved control, derived from the same subculture. Thereafter the bacterial suspension was prepared, as described above.

For washing, underlayering and suspending of bacteria M/15 phosphate - 0.9% NaCl solution, pH 7, was used.

The oxygen uptake was measured by the manometric technique [7] in air. The flask contained: 0.2 ml. of 20% KOH (in the center well), 1.5 ml. of bacterial suspension (appr. 1 mg. bacterial N), and 1.5 ml. of NaCl - phosphate, pH 7. Equilibration of temperature was attained after 30 min. shaking with opened stopcocks. The rate of oxygen uptake was compared at two different temperatures in two portions of the same suspension, simultaneously in two apparatuses.

The bacterial nitrogen was determined in 1.5 ml. of bacterial suspension by the Kjeldahl's micro-method.

RESULTS AND DISCUSSION

The endogenous respiration of washed bacterial cells was examined at the growth temperature and at the temperatures lower or higher by 10°. The observed values are compared in Table 1. The increase of temperature by 10° over the growth temperature increased twice the endogenous oxygen uptake, the decrease by 10°, reduced it to a half; the value of Q_{10} being 2 in both cases.

Table 1

Endogenous respiration of Mycobacterium phlei *grown at 25° and 35°*

Washed cells suspended in 0.9% NaCl-M/15 phosphate buffer, pH 7. The Warburg flask contained 0.81 mg. N (culture grown at 25°) or 0.78 mg. N (culture grown at 35°); final volume being 3 ml. Results are expressed in μl. O_2/mg. N.

Time (hr.)	Growth at 25°			Growth at 35°		
	Respiration at		Q_{10}	Respiration at		Q_{10}
	25°	35°		25°	35°	
0 - 1	112	170	1.6	71	121	1.7
1 - 2	72	137	1.9	52	116	2.2
2 - 3	60	132	2.2	48	101	2.1
3 - 4	54	117	2.2	44	88	2.0
4 - 5	53	108	2.0	39	86	2.2
5 - 6	58	104	2.2	37	74	2.0

The endogenous respiration decreased with time, especially within the first 2 hours. This decrease, expressed in percent of the oxygen uptake during the first hour, is less dependent on temperature than the absolute values are. The average values of relative decrease calculated from the two series of experiments, carried out at 25° and 35°, are given in Table 2.

Table 2

Endogenous respiration of Mycobacterium phlei *calculated in percent of the value in the first hour*

Conditions are given in Table 1, which shows one of performed experiments. Mean values from 8 experiments carried out at 25°, and 8 experiments at 35° ± S. D. of the mean. The calculated t was compared to $t_{0.01} \pm 2.98$.

Time (hr.)	Respiration at 25°	Respiration at 35°	t
2	76±3.9	83±3.7	1.30
3	72±5.9	77±3.4	0.73
4	64±5.4	67±3.9	0.45
5	63±5.1	65±3.7	
6	60±5.0	60±3.5	

The decrease of endogenous respiration of suspensions of washed mycobacterium cells [3], as of many other bacteria, was reported, but its cause was not discussed. The attempt was made therefore to elucidate this phenomenon.

Table 3

Endogenous respiration of 24-hr.-starved cultures of Mycobacterium phlei, *grown at 25° and 35°*

Washed cells suspended in 0.9% NaCl-M/15 phosphate buffer, pH 7. The Warburg flask contained 0.69 mg. N (culture grown at 25°) or 0.96 mg. N (culture grown at 35°); final volume being 3 ml. Results are expressed in μl. O_2/mg. N.

Time (hr.)	Growth at 25°			Growth at 35°		
	Respiration at		Q_{10}	Respiration at		Q_{10}
	25°	35°		25°	35°	
0 - 1	132	290	2.2	68	145	2.1
1 - 2	100	177	1.8	62	132	2.1
2 - 3	77	165	2.1	55	123	2.2
3 - 4	64	143	2.2	54	120	2.0
4 - 5	67	136	2.0	55	112	2.1
5 - 6	62	143	2.3	50	104	2.0

The experiments carried out with bacteria starved for 1, 4 or 8 days showed that the decrease was not due to exhausting of endogenous substrates. The response of bacteria starved for 1 or 4 days to the change of temperature was the same as that of non-starved controls. The one day starvation did not influence the rate of endogenous respiration (Table 1 and 3). This is consistent with the well known resistance *of Mycobacteria* to starvation and indicates that during the short-time experiments with non-starved, washed cells the loss of living cells can be neglected.

The 4-day-starvation lowers the rate of endogenous respiration by 30 - 40% as related to the non-starved controls of the same age (Table 4). Similarly the 8-day-starvation decreases the rate of endogenous respiration by about 40% (Table 5). It should be emphasized, however, that bacteria starved during 8 days were harvested on the 14th day of growth, because the pellicle can not be underlayed

Table 4

The effect of 4-day-starvation on the endogenous respiration of Mycobacterium phlei

Cultures harvested on 10th day of growth at 35°. Washed cells suspended in 0.9% NaCl-M/15 phosphate buffer, pH 7. The Warburg flask contained: 0.76 mg. N (control culture) or 1.27 mg. N (starved culture); final volume being 3 ml. Results are expressed in $\mu l.O_2$/mg. N

Time (hr.)	Non-starved (control) Respiration at 35°	4-day-starved culture			Decrease of respiration at 35°, due to starvation (%)
		Respiration at		Q_{10}	
		35°	25°		
0 - 1	163	109	48	2.3	33
1 - 2	131	84	45	1.9	36
2 - 3	114	70	40	1.8	39
3 - 4	117	73	36	2.0	38
4 - 5	114	67	35	1.9	41
Sum	639	403	204	2.0	37

Table 5

The effect of 8-day-starvation on the endogenous respiration of Mycobacterium phlei

Cultures harvested on 14th day of growth at 35°. Washed cells suspended in 0.9% NaCl-M/15 phosphate buffer, pH 7. The Warburg flask contained 1.05 mg. N (control culture) or 1.17 mg. N (starved culture); final volume being 3 ml. Results are expressed in $\mu l.O_2$/mg. N

Time (hr.)	Non-starved (control) Respiration at 35°	8-day-starved culture			Decrease of respiration at 35°, due to starvation %
		Respiration at		Q_{10}	
		35°	25°		
0 - 1	60	41	23	1.8	32
1 - 2	61	34	22	1.5	44
2 - 3	48	25	24	1.0	48
3 - 4	38	27	17	1.6	29
4 - 5	42	19	18	1.1	55
Sum	249	146	104	1.4	42

with buffer earlier than on the 6th day of growth. It can be seen from Table 6 that the 60 - 65% decrease of endogenous respiration takes place between the 10th and the 14th day of growth; this holds both for the starved and non-starved cultures. Hence, the cultures grown for 10 days or longer may be considered as "old" ones.

Table 6

The effect of age on the endogenous respiration of Mycobacterium phlei *at 35°*

Cultures grown at 35°. Non-starved, control cultures were harvested on 7th 10th and 14th day of growth. Cultures starved for 4 and 8 days were harvested on 10th and 14th day of growth resp. Washed cells suspended in 0.9% NaCl-M/15 phosphate buffer, pH 7. The Warburg flask contained: Control cultures, 7 days old, 1.15 mg. N; 10 days old, 0.76 mg. N; 14 days old, 1.05 mg. N. Starved cultures, 10 days old, 1.27 mg. N; 14 days old, 1.17 mg. N. The final volume was 3 ml. Results are expressed in $\mu l.O_2$/mg. N.

Time (hr.)	Non-starved cultures (control), age			Starved cultures, age		% decrease of respiration between 10—14th day of growth	
				10 days	14 days		
	7 days	10 days	14 days	Starved		Control	Starved
				4 days	8 days		
0 - 1	154	163	60	109	41	37	38
1 - 2	136	131	61	84	34	46	40
2 - 3	115	114	48	70	25	42	36
3 - 4	114	117	38	73	27	32	37
4 - 5	107	114	42	67	19	36	28

The effect of transferring the surface cultures to the Warburg apparatus i.e. to the conditions of the aerated deep-culture was considered as one of possible causes of the rapid changes in endogenous respiration during the first two hours of experiment. In Table 7 the respiration of two 7 days old cultures derived from the same subculture is compared. The first culture was grown for the whole time on the surface while the second one was after 5 days of surface growth transformed to an aerated deep-culture by stirring with an electromagnetic agitator. The changes in the endogenous respiration with time were the same in both cultures.

It was proved, however, that endogenous respiration of bacterial suspension kept temporarily without aeration was higher under full aerobic conditions than that of continuously aerated cultures (Table 8). The longer the bacterial suspension was kept without aeration the higher was the increase of respiration when the aeration started. This is true for a pause in aeration not exceeding 3 hours. This effect is gradually vanishing during several hours.

The procedure used excluded the aeration for about 1 hr. i.e. from the moment of pellicle harvesting, to the beginning of shaking in Warburg apparatus, including

Table 7

Endogenous respiration of surface culture and deep-culture of Mycobacterium phlei *at 35° and 25°*

Both cultures derived from the same subculture, were grown at 35° and harvested on 7th day of growth. Washed cells suspended in 0.9% NaCl-M/15 phosphate buffer, pH 7. The Warburg flask contained: 1.48 mg. N (surface culture) and 1.24 mg. N (deep culture); final volume being 3 ml. Results are expressed in $\mu l.O_2$/mg. N.

Time (hr.)	Respiration at 25°		Respiration at 35°	
	Surface culture	Deep-culture	Surface culture	Deep-culture
0 - 1	116	116	244	236
1 - 2	78	91	179	170
2 - 3	68	79	137	126
3 - 4	64	71	123	120
4 - 5	61	62	114	117

Table 8

The effect of the pause in aeration on the respiration rate of Mycobacterium phlei, *after the restoration of aeration*

Culture was grown at 35°, harvested on 7th day of growth. Washed cells suspended in 0.9% NaCl-M/15 phosphate buffer, pH 7. The Warburg flask contained 1.15 mg. N; the final volume being 3 ml. Respiration was examined at 35°. Results are expressed in $\mu l.O_2$/mg. N.

Time (hr.)	Continuously aerated (control)	Start of aeration at hr. of experiment			
		1st	2nd	3rd	4th
0 - 1	153	39	40	42	44
1 - 2	136	190	45	44	46
2 - 3	116	130	203	53	45
3 - 4	114	127	145	205	43
4 - 5	107	115	127	135	212
Sum	626	601	560	479	390

washing, centrifugation and suspending of bacteria. During this period the rate of oxygen uptake is limited by the velocity of oxygen diffusion to the liquid and consequently the cells are during this time insufficiently supplied with oxygen. Therefore, when shaking begins the rate of oxygen uptake is abnormally high and gradually drops thereform. This rapid decrease of initial respiration during the first hours of experiment should be considered as a mere effect of experimental conditions. In the later hours of experiment a small, regular decrease by few percent is observed (Table 2). This phenomenon may be due to the decrease of bac-

terial mass, caused by dissimilation of endogenous substrates. The starving *Mycobacteria* dissimilate mainly lipid materials [6]. The respiratory quotient of *Mycobacterium phlei* dissimilating endogenous lipids amounts 0.75 [4]. In our experiments the loss of endogenous carbon calculated from the oxygen uptake per mg. of bacterial N between 3 - 6 hr. amounts to 2 - 3%. This loss of bacterial mass can account for the decrease of respiration observed in later hours of experiment.

Hence, two causes at least are responsible for the decrease of respiration rate in short-time experiments, namely: the effect of the pause in aeration and the decrease in the bacterial mass, owing to catabolic processes. The first cause is responsible for the marked drop occurring within first 2 - 3 hr., the second one for the relatively small decrease for the whole time of experiments. Allowing for these causes and for the extent of their effects it could be concluded that the endogenous oxygen-uptake of washed cells, when referred to the mass of living bacteria, is constant at a given temperature. Q_{10} was found to be 2 within the temperature range studied.

It should be concluded that non-aerated suspensions of *Mycobacteria* are in the state of an "oxygen debt". They "repay this debt" when aeration is restored. This observation is interesting since *Mycobacteria* belongs to the group of obligate aerobes. So it could be believed that the difference between the obligate and facultative aerobes is probably not as clear-cut as commonly accepted.

SUMMARY

The endogenous oxygen uptake of washed cells suspensions of *Mycobacterium phlei* was examined at various temperatures. The rate of oxygen uptake was found to be constant at a given temperature. The changes observed in the course of short-time experiments are due to the pause in aeration, included in the general procedure and to the loss of bacterial mass owing to catabolic processes. Q_{10} value between 25° and 35° amounted to 2. It was found that *Mycobacteria* can run into an oxygen debt.

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ODDYCHANIE ENDOGENNE *MYCOBACTERIUM PHLEI* W RÓŻNYCH TEMPERATURACH

Streszczenie

Badano w różnych temperaturach zużycie endogenne tlenu przez zawiesiny przemytych komórek *Mycobacterium phlei*. Stwierdzono, że intensywność zużywania tlenu jest stała w danej temperaturze. Zmiany zaobserwowane w czasie krótkotrwałych doświadczeń są wywołane dwiema przyczynami: przerwą w przewietrzaniu, związaną ze sporządzaniem zawiesin bakteryjnych, oraz ubytkiem masy bakteryjnej powstającym wskutek procesów katabolicznych. W zakresie temperatur 25° - 35° wartość Q_{10} wynosi 2. Stwierdzono, że *Mycobacteriae* mogą zaciągać dług tlenowy.

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THE TYROSINE TRANSAMINATION AND TYROSINE CONTENT IN *CELERIO EUPHORBIAE**

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The present paper deals with the determination of tyrosine transamination in *C. euphorbiae*. The metabolism of tyrosine in this moth was of considerable interest because of the role in melanogenesis and the reducing properties of this amino acid. In previous studies [7] the tyrosine content was determined by the method of Folin & Ciocalteu [6] and found in whole pupae to be 350 - 440 mg.%. Since this method is not specific enough, in the present study the Ceriotti & Spandrio [3] procedure was used.

MATERIALS AND METHODS

The isolated tissues of diapauzing at 4° pupae, and of the moths of *C. euphorbiae* were homogenized in a glass homogenizer with 9 vol. of 0.01 M-phosphate buffer, pH 7.4 or 8.0, at 4°. The homogenates were then centrifuged at 6 000 r.p.m. for 15 min. at 4°. With the fat-body homogenate the thin lipid layer was removed from the surface and discarded. The extracts thus obtained (3 - 5 ml.) were dialyzed for 48 hr. at 4° against 2 liter of 0.01 M-phosphate buffer used for homogenization. The pupal haemolymph was dialyzed directly.

For comparison, measurements were carried out on rat liver and pigeon liver, heart, and breast muscle. The isolated liver and heart were first perfused with 0.25 M-saccharose in 0.1% sodium citrate.

Reagents [1]: L-tyrosine (Zakłady Farmaceutyczne, Warszawa), α-ketoglutaric acid (Nutr. Biochem. Corp.), *p*-hydroxyphenylpyruvic acid (Sigma), diethyldithiocarbamate (B.D.H.), pyridoxal phosphate (Fluka, Switzerland), 3,4-dihydroxy-

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[1] The following abbreviations are used: T-KG transaminase, tyrosine:α-ketoglutarate aminotransferase; TCA, trichloroacetic acid; DDC, diethyldithiocarbamate; *p*HPP, *p*-hydroxyphenylpyruvic acid; DOPA, 3,4-dihydroxyphenylalanine; PalP, pyridoxal phosphate.

phenylalanine (Biuro Odczynników Chem., Gliwice), pyruvic acid (Xenon, Łódź), α-nitroso-β-naphthol, trichloroacetic acid and sulfanilic acid (Fabryka Odczynników Chem., Gliwice), diazonium salt of sulfanilic acid prepared from sulfanilic acid.

Determination of tyrosine. The isolated tissues were homogenized in 7% TCA (1:50), then the tyrosine content in the deproteinized extracts was determined after Ceriotti & Spandrio [3] and by the Folin & Ciocalteu method [6]. The measurements were made in Bausch & Lomb Spectronic 20 at 510 mμ and 520 mμ, respectively. Tyrosine in standard solution was also dissolved in 7% TCA.

T-KG transaminase. The activity was determined: (1) by the Briggs method as modified by Canellakis & Cohen [2], and (2) by the tautomerase method of Lin, Pitt, Civen & Knox [9].

(1) In the first method 0.7 ml. of tissue extract in 0.01 M-phosphate buffer, pH 7.4, was preincubated with 0.2 μmole PalP, 16 μmoles tyrosine and 20 μmoles DDC for 15 min. at 37°. Then, 32 μmoles of α-ketoglutarate and 0.6 ml. of 0.01 M-phosphate buffer, pH 7.4, were added, final volume being 3.5 ml., and the mixture was incubated for 2 hr. at 37° with continuous shaking. The reaction was stopped with 0.1 ml. of TCA solution (100 g. TCA dissolved in 100 ml. of water), the protein was centrifuged off and the formed *p*HPP was determined colorimetrically. 1 ml. of the reagent containing 0.5 ml. of 1% KH_2PO_4 and 0.5 ml. of 3% ammonium molybdenate in 5 N-HCl was added to 2.5 ml. of the supernatant and the colour was developed for 3 hr. at room temperature. The measurements were made in a Unicam spectrophotometer at 850 mμ. Canellakis & Cohen recommended incubation in nitrogen but our preliminary experiments showed that in insect tissues where the melanogenesis is the main interfering factor, this procedure is not satisfactory. Accordingly DDC was used for inhibition of phenolase and it was added both to the sample and control.

The reaction products were also identified by means of paper chromatography; 0.2 ml. of the deproteinized incubation mixture was applied on Whatman no. 3 filter paper and two chromatograms were developed simultaneously in butanol - acetic acid - water (4:1:5, by vol.). On one chromatogram glutamic acid was visualized with 0.2% ninhydrin in 96% ethanol, while on the second one a spot of *p*HPP was located with 0.5% diazonium salt of sulfanilic acid in 10% Na_2CO_3 [12].

(2) In the tautomerase method the procedure was as follows: 1 - 2 ml. of the tissue extract in 0.01 M-phosphate buffer, pH 8.0, 12 μmoles of tyrosine, 20 μmoles of DDC and 0.1 μmoles of PalP were preincubated for 20 min. at 37°; then, 80 μmoles of α-ketoglutarate was added, total volume being 3.5 ml. After 2 hr. incubation the mixture was deproteinized by the addition of 1 ml. of 20% metaphosphoric acid. The protein was centrifuged off and 0.5 ml. of the supernatant was introduced to 3 ml. of 1 M-sodium borate in 2 M-sodium arsenate and left for 15 min. at room temperature. The absorption of the enol tautomer of *p*HPP formed under these conditions was read at 310 mμ against control containing keto tautomer. This blank

sample was obtained by adding 0.5 ml. of the supernatant to 3 ml. of 2 M-sodium arsenate.

DOPA:α-ketoglutarate transaminase. In the incubation mixture used in the tautomerase method 12 μmoles of DOPA was substituted for tyrosine. The sample was incubated for 4 hr. at 37°, deproteinized by the addition of 1 ml. of 20% metaphosphoric acid and centrifuged, then 0.5 ml. of the supernatant was applied on Whatman no. 1 filter paper and chromatographed as above.

DOPA:pyruvate transaminase. For the determination of this enzymic activity 80 μmoles of pyruvate was added instead of α-ketoglutarate to the mixture as in the previous transaminase assay. After 4 hr. incubation at 37° the reaction was stopped by the addition of 2 vol. of 96% ethanol (if metaphosphoric acid was used for deproteinization a "tailing" was observed on chromatograms in the region of alanine). After the protein was centrifuged off, ethanol was removed from the supernatant in a hot air current. Then 0.5 ml. of the residue was chromatographed on Whatman no. 1 filter paper in propanol - water (7:3, v/v). The spots were located with 0.2% ninhydrin in acetone.

p*HPP oxidase.* This enzyme was estimated as follows: 0.7 ml. of the fat-body extract in 0.01 M-phosphate buffer, pH 7.4, was incubated with 15 μmoles of *p*HPP, total volume being 3.5 ml. After incubation for 2 hr. at 37° with continuous shaking, the reaction was stopped by the addition of 0.1 ml. of TCA solution, and *p*HPP content was determined in deproteinized sample according to Briggs [2].

The occurence of *p*HPP oxidase was also examined by the tautomerase method as follows: 1 ml. of the pupal fat-body extract in 0.01 M-phosphate buffer, pH 8.0, was incubated with 0.5 or 15 μmoles of *p*HPP. After 2 hr. the incubation mixture was deproteinized with 1 ml. of 20% metaphosphoric acid and *p*HPP was determined.

Determination of protein. The spectrophotometric method was employed based on extinction measurements at 260 and 280 mμ and calculated according to the Warburg & Christian equation [13]. Unicam spectrophotometer of SP 500 type was used. The protein content was also determined by the Mejbaum-Katzenellenbogen tannin method [11] and the results were concordant with those obtained by the spectrophotometric method.

RESULTS AND DISCUSSION

The results of tyrosine determination by the Ceriotti & Spandrio method are several times lower than those obtained by the Folin & Ciocalteu method (Table 1). These differences indicate the presence of some substances which in the Folin test are chromogenic like tyrosine.

In the whole pupae diapauzing at 4° the tyrosine content was 111 mg.% and besides large amounts of the Folin-chromogen were found. The latter expressed in the tyrosine equivalents amounted to 209 mg.%. The concentration of tyrosine was almost the same in all the studied tissues but the chromogen content varied.

Table 1

The comparison of the tyrosine content determined by the Ceriotti & Spandrio [3] and by the Folin & Ciocalteu [6] methods

The results are expressed in mg./100 g. of fresh tissue.

Deproteinized tissues		Ceriotti & Spandrio method	Folin & Ciocalteu method
C. euphorbiae pupa	Haemolymph	106.0	100.0
	Fat-body	118.2	446.0
	Muscles	57.1	238.3
	Whole pupa	111.5	320.5
C. euphorbiae moth	Thorax muscles	20.1	166.3
	The remaining tissues	100.4	670.7
Pigeon	Heart	4.7	40.3
	Liver	12.3	92.4
	Muscles	3.7	27.4

The muscles contained 3 times as much of Folin-chromogen as of tyrosine, fat-body 3 times as much, whereas in haemolymph it was absent. In the moth muscles the chromogen content was about 7 times higher than that of tyrosine.

By microbiological method, Duchâteau & Florkin [4] found that in *C. euphorbiae* pupae diapauzing at 5° the tyrosine content in haemolymph is 78 mg.%. The values obtained by the Ceriotti method are of the same order (Table 2).

The high content of tyrosine seems to be specific for insects as compared with the other organisms [10]. In pigeon for instance (Table 2) this value in various tissues was from 4 to 12 mg.%. A non-tyrosine chromogen is also present in pigeon tissues in the amount about 7 times higher than that of tyrosine (Table 1). The high level of tyrosine in insects may be responsible for commonly known aminoacidaemia in this class [8], the phenomenon regarded by Florkin as a characteristic feature of the insect biochemistry [5].

The compounds other than tyrosine that give colour reaction in the Folin test and can occur in the tissues are first of all: DOPA and uric acid. In the Folin & Ciocalteu method 1 mg. of these compounds corresponds to 2.4 and 0.5 mg. of tyrosine, respectively.

To assay the transamination activity it was necessary to ensure at first that *p*HPP produced in the reaction is not further metabolized under the conditions of experiment. No decrease, however, of the added *p*HPP was found by both methods employed on 2 hr. incubation with the insect preparations. Although *p*HPP oxidase activity was lacking DDC was used to inhibit tyrosinase present in the insect tissues.

Table 2

The content of free tyrosine in Celerio euphorbiae *pupae diapauzing at 4° and in pigeon*

Tyrosine was determined after Ceriotti & Spandrio [3]. The results in pupae and pigeon are mean from 14 and 5 determinations, respectively, ±S. D. of the mean; range is given in parentheses.

Deproteinized tissues		Tyrosine mg./100 g. of fresh tissue
C. euphorbiae pupa	Haemolymph	117.89±2.00 (105.90 - 136.61)
	Fat-body	122.48±2.73 (104.68 - 138.80)
	Muscles	70.87±2.58 (53.57 - 86.57)
Pigeon	Heart	4.66±0.83 (4.44 - 5.00)
	Liver	12.25±1.04 (9.63 - 14.76)
	Breast muscle	3.71±0.99 (3.04 - 5.43)

Table 3

The activity of tyrosine:α-ketoglutarate aminotransferase in C. euphorbiae *tissues and in rat liver*

p-Hydroxyphenylpyruvic acid was determined by the Briggs method as modified by Canellakis & Cohen [2]. The amount of protein in incubated samples: haemolymph, 10 mg.; fat-body, 7 mg.; pupal muscles, 2 mg.; moth muscles, 6 mg.; rat liver, 15 mg. The results expressed in μmoles of *p*HPP formed/120 min./100 mg. of protein.

Experiment no.	Pupal tissues			Moth muscles	Rat liver
	Haemo-lymph	Fat-body	Muscles		
1	1.86	1.38	1.85	1.44	4.68
2	1.96	1.29	1.82	1.28	—
3	1.96	1.34	—	—	—
Average	1.93	1.34	1.84	1.36	4.68

Both products of transamination between tyrosine and α-ketoglutarate i.e. *p*HPP and glutamate were ascertained chromatographically. The values of T-KG transaminase activity determined by the method of Canellakis & Cohen in dialyzed extracts from haemolymph, fat-body and muscles of pupae, flight muscles of the moth, and rat liver are given in Table 3, while the results obtained by the tautomerase method in the pupal tissues and pigeon heart are presented in Table 4.

Table 4

The activity of tyrosine:α-ketoglutarate aminotransferase in C. euphorbiae *pupae tissues and pigeon heart muscle*

p-Hydroxyphenylpyruvic acid was determined by the tautomerase method [9]. The amount of protein in incubated samples: haemolymph 17 - 38 mg.; fat-body, 20 mg.; pigeon heart, 3 mg. The results expressed in μmoles of *p*HPP formed/120 min./100 mg. of protein

Experiment no.	Pupal tissues		Pigeon heart
	Haemolymph	Fat-body	
1	1.25	1.83	3.37
2	1.33	1.83	3.06
3	1.25	1.81	3.75
Average	1.28	1.82	3.39

As can be seen the enzymatic activity varies from 1.3 to 1.9 μmoles of *p*HPP produced per 120 min. per 100 mg. of protein with both methods, differing in their pH, and acceptor to donator ratio in the incubation media.

In the determination of T-KG transaminase activity by the Canellakis & Cohen method the increased amounts of PalP had no effect on the transaminase activity. On the contrary, the extinction values in the tautomerase method increased when further amounts of PalP were added suggesting the activation of the enzyme.

Table 5

The effect of pyridoxal phosphate on the optical density in the Briggs method as modified by Canellakis & Cohen [2] and in the tautomerase method [9]

The composition of samples as described in Methods, except the amounts of pyridoxal phosphate which are given in the Table. Complete system contained the extract of pigeon heart (3 mg. of protein). The absorption of pyridoxal phosphate in sodium borate - sodium arsenate mixture without any other additions was read against sodium arsenate.

Pyridoxal phosphate (μmoles/3.5 ml.)	$E_{850\ m\mu}$ (Canellakis & Cohen method)		$E_{310\ m\mu}$ (tautomerase method)		
	Complete system	Without protein	Complete system	Without protein	Pyridoxal phosphate alone
0.1	0.024	0	0.08	0.01	0
1.8	0.024	0	0.65	0.63	0.63
2.4	0.024	0	0.68	0.66	—
3.0	0.024	0	0.72	0.71	—

In order to explain this discrepancy the experiments were performed with the increasing amounts of PalP under conditions employed in both methods. The experiments were made with the enzymatic extract and without the enzymic protein. It appeared that PalP, like *p*HPP forms with borate complexes absorbing at 310 mμ (Table 5).

It was concluded therefore that in the tautomerase method the amounts of PalP added should not exceed 0.1 μmole per 3.5 ml. i.e. the amount recommended by Lin *et al.* [9]. With greater amounts of PalP used, the results obtained by the tautomerase method are not reliable.

The activity of T-KG transaminase in the insect tissues is low, it is half that in pigeon and rat, and is much lower than the activity of aspartate:α-ketoglutarate aminotransferase. The activity of the latter enzyme in the dialyzed muscles of diapauzing pupae was found [1] to be in average 40 μmoles of oxaloacetate per min. per 100 mg. of protein. The corresponding value of T-KG transaminase calculated per min. is 0.015 μmoles i.e. about 2500 times lower.

Because of the low activity of T-KG transaminase it seems doubtful whether this transamination is of some importance in tyrosine metabolism in insects. A more active transaminase may however exist for some other α-ketoacid as specific acceptor.

Tyrosine could be supposed to undergo transamination after its prior conversion to DOPA. Therefore determination of DOPA transaminase was made but this path of metabolism was also found to be quantitatively negligible. Formation of alanine was proved chromatographically when pyruvate was used as an acceptor of amino groups but the reaction was too weak to be measured quantitatively. When α-ketoglutarate was the acceptor the glutamate spot was not revealed at all.

SUMMARY

1. In *C. euphorbiae* pupae the average tyrosine content estimated by the Ceriotti & Spandrio method in haemolymph and fat-body is 120 mg.% and in the muscles 70 mg.%. The tissues contain some substances other than tyrosine reacting as chromogens in the Folin & Ciocalteu method.

2. The activity of tyrosine:α-ketoglutarate aminotransferase in *C. euphorbiae* tissues is very low as compared with that of aspartate:α-ketoglutarate aminotransferase.

3. A slight activity of 3,4-dihydroxyphenylalanine:pyruvate aminotransferase was proved chromatographically.

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TRANSAMINACJA TYROZYNY I ZAWARTOŚĆ TYROZYNY U *CELERIO EUPHORBIAE*

Streszczenie

1. U poczwarki *C. euphorbiae* średnia zawartość tyrozyny oznaczana metodą Ceriotti i Spandrio [3] wynosi w hemolimfie i ciele tłuszczowym 120 mg.%, w mięśniach 70 mg%. Oznaczenia metodą Folina i Ciocalteu [6] dają wartości kilkakrotnie wyższe, ponieważ tkanki zawierają pewne związki, które podobnie jak tyrozyna są chromogenami dla odczynnika Folina.

2. W tkankach *C. euphorbiae* aktywność aminotransferazy tyrozyna:α-ketoglutaran jest niewielka w porównaniu z czynnością aminotransferazy kwas asparaginowy: α-ketoglutaran.

3. Stwierdzono bardzo słabą aktywność aminotransferazy 3,4-dwuhydroksyfenyloalanina: pyrogronian.

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THE SYNTHESIS OF PYRIMIDINE DERIVATIVES IN PLANT MATERIAL USING [6-^{14}C]OROTIC ACID

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In earlier studies [10, 4, 5] we have suggested that in plant material uracil and uridine may be intermediate products of the conversion of OA [1] to UMP. According to literature, however, neither uracil nor uridine seem to be the intermediates of this process in animal organs and microorganisms [9, 6, 3]. Therefore, further studies on the mechanism of utilization of OA in plant material on its path to pyrimidine nucleotides seemed to be indicated.

MATERIAL AND METHODS

Excised 6-day-old wheat blades (variety Dańkowska 40) were fed with [6-^{14}C]OA or with a mixture containing [6-^{14}C]OA and inactive uracil, uridine or UMP, respectively. The emerging various radioactive, acid-soluble substances were isolated and quantitatively determined. The experimental procedure was essentially the same as previously described [10, 5].

In the first experiment (Table 1), six 2 g. samples of blades were placed separately in small vessels containing 1 ml. of a 3.2 mM-solution of [6-^{14}C]OA each, with a specific activity of 7000 counts/sec./μmole. Incubation was stopped after 5, 30, 60, 120, and 240 min., respectively. Sample no. 6 was transferred after 4 hr. of feeding with [6-^{14}C]OA into 1 ml. of distilled water for a period of further 4 hr. incubation.

In another experiment (Table 2) 2 g. samples of wheat blades were incubated in a mixture containing in 1 ml. 32 μmoles of inactive uracil, uridine or UMP, respectively, and 3.2 μmoles of [6-^{14}C]OA. Changes in the rates of radioactivity of the metabolic products of OA were investigated against a sample fed with [6-^{14}C]OA only. Incubations were stopped after 2 hr.

Acid-soluble pyrimidine substances were then isolated from the plant material, identified and quantitatively determined. The procedure included extraction with

[1] The following abbreviations are used: OA, orotic acid; UMP, uridine-5′-phosphate; CMP, cytidine-5′-phosphate; OMP, orotidine-5′-phosphate.

Table 1

Amounts and specific activities of pyrimidine derivatives in wheat blades fed with [6-^{14}C]orotic acid

Amounts expressed in μmoles/2 g. of fresh weight, and specific activity in counts/sec./μmole.

No.	Time of feeding (min.)	Orotic acid					Uracil		Uridine		UMP		CMP	
		Absorbed		Recovered		Metabolized								
		μmoles	counts/sec./μmole	μmoles	counts/sec./μmole	μmoles	μmoles	counts/sec./μmole	μmoles	counts/sec./μmole	μmoles	counts/sec./μmole	μmoles	counts/sec./μmole
1	5	0.06	7000	0.02	6600	0.04	0.02	106	0.27	208	0.09	91	0.05	0
2	30	0.33	7000	0.12	6920	0.21	0.02	478	0.30	1130	0.09	460	0.06	0
3	60	0.46	7000	0.14	7000	0.32	0.02	870	0.50	1490	0.08	907	0.05	42
4	120	0.88	7000	0.20	7000	0.68	0.05	1320	0.62	2510	0.15	1210	0.06	80
5	240	2.06	7000	0.94	6970	1.12	0.07	2050	0.65	4290	0.20	1850	0.08	177
6	240+240 in water	2.06	7000	0.04	6750	2.02	0.03	1490	0.57	2950	0.14	1950	0.08	184

cold 0.6 N-$HClO_4$, adsorption on activated charcoal, column and paper chromatography, spectrophotometric and chemical determinations and finally measurements of radioactivity. All details were already described [5] and only the modifications applied will be mentioned here: (1) An elution of the substances adsorbed on activated charcoal with a mixture of warm acetone - 0.1 N-NH_3 (4:1, v/v) in addition to the previously employed ethanol elution was introduced. Thanks to the OA tracer with high specific activity small quantities of substances spectrophotometrically no longer detectable could still be found in the acetone eluate after exhaustive elution with ethanol. (2) Additional paper chromatography was employed for the purification of OA isolated from plant material, using *n*-butanol - ethanol - water (1:1:2, by vol.), R_F of OA was 0.37. This procedure, following the previously employed column and paper chromatography, increased the specific activity of the isolated OA by 5 - 10%, thus improving the purity of the isolated compound.

The [6-^{14}C]OA was a commercial sample from The Radiochemical Centre, Amersham, England.

RESULTS

Results presented in Table 1 show that under the experimental conditions described, excised wheat blades absorbed rapidly [6-^{14}C]OA from the medium at a rate roughly proportional to the time of incubation. The specific activity of OA recovered from the plant material remained essentially unchanged.

After 5 min. of incubation with [6-^{14}C]OA it was possible to find in the plant material already three radioactive products of its metabolism. These were: uracil, uridine and UMP. Of all three, uridine has shown the highest radioactivity, the activity of uracil and UMP were about half of that of uridine, and no marked differences between the two could be detected. After longer periods of incubation the quantities of the detected compounds as well as their specific activities steadily increased. The specific activity of uridine was always about twice the activity of the two remaining pyrimidine derivatives. CMP showed radioactivity only after 1 hr. of incubation and even after 4 hr. its specific activity amounted to no more than 10% of that of UMP. On interrupting the flow of the tracer and further incubation in water (Table 1, no. 6) a rapid decline of specific activity in uracil and uridine could be observed, whereas the level of UMP and CMP activity remained unchanged.

As can be seen from Table 2 the addition to the medium of uracil, uridine or UMP, respectively, in no way affected the intake and the intensity of OA metabolism in the plant. As was to be expected the addition of inactive uracil to the medium caused a very distinct decrease of the specific activity of uracil isolated from plant material (Table 2, no. 2). However, this was not the case with the specific activities of uridine, UMP and CMP, which on the contrary were considerably higher than in the plants fed with [6-^{14}C]OA only. At the same time a fall

Table 2

Amounts and specific activities of pyrimidine derivatives in wheat blades fed with 3.2 μmoles of [6-^{14}C]orotic acid or with 3.2 μmoles of [6-^{14}C]orotic acid and 32 μmoles of uracil, uridine and UMP, respectively

Amounts expressed in μmoles/2 g. of fresh weight and specific activity in counts/sec./μmole

No.	Incubation	Orotic acid					Uracil		Uridine		UMP		CMP	
		Absorbed		Recovered		Metabolized								
		μmoles	counts/sec./μmole	μmoles	counts/sec./μmole	μmoles	μmoles	counts/sec./μmole	μmoles	counts/sec./μmole	μmoles	counts/sec./μmole	μmoles	counts/sec./μmole
1	[6-^{14}C]OA	1.35	7000	0.47	6960	0.88	0.08	1660	0.63	4100	0.10	1740	0.08	170
2	[6-^{14}C]OA+uracil	1.22	7000	0.32	6930	0.90	1.18	630	0.30	6830	0.06	3930	0.13	228
3	[6-^{14}C]OA+uridine	1.24	7000	0.39	6950	0.85	0.15	1480	2.17	1360	0.12	960	0.12	204
4	[6-^{14}C]OA+UMP	1.29	7000	0.35	6910	0.94	0.40	1300	0.38	5180	0.08	3243	0.12	530

in the amounts of uridine and UMP and a distinct increase in the quantity of CMP could be observed.

Similarly the addition of uridine to the medium caused an increase in its contents in plant material accompanied by a corresponding fall of its specific activity (Table 2, no. 3). At the same time a sharp decrease of the specific activity of UMP could be observed. On the other hand, the amount and the specific activity of CMP were both higher than in the sample fed with [6-^{14}C]OA only.

Contrary to what was anticipated, the introduction of UMP into the medium did not cause any increase of its amounts in plant material, but the quantities of uracil increased fivefold (Table 2, no. 4). At the same time very marked increase in specific activities of UMP and CMP could be observed. Generally the changes observed on addition of UMP to the medium were suprisingly similar to the ones obtained on introduction of uracil.

DISCUSSION

The presented data suggest that the plant contains an enzymatic system which can very efficiently catalyze the metabolism of OA. After 8 hr. of incubation with 2 g. of wheat blades 2.02 μmoles OA changed into other substances (Table 1). This figure is close to the one obtained previously [4], when the concentration of OA in the medium was 3 times higher. This observation would prove that the rate of OA metabolism in the tissue does not depend on its concentration and would also confirm our previous suggestion [10, 5] that the plant may temporarily store an excess of OA as a precursor of pyrimidine nucleotides.

The system engaged in the metabolism of OA does not seem to belong to the type of adaptive enzymes, because OA was metabolized immediately and the intensity of metabolism did not increase in time. This would indirectly prove that OA is a normal plant metabolite. Endogenic concentration of OA in plant tissue is very small indeed, as it cannot be reliably detected spectrophotometrically, and practically no dilution effects could be observed when labelled OA was introduced (see specific activity of OA in Tables 1 and 2). Similarly Hurlbert & Potter could not detect dilution effects of exogenic [6-^{14}C]OA in animal organisms [8].

Uracil, uridine, UMP and CMP were isolated and identified from plants fed with radioactive OA. The synthesis of these compounds in blades incubated for 8 hr. with inactive OA has been previously reported [4]. The suggestion has then been made that uracil and uridine may be intermediate products in the metabolism of OA to UMP in the plant. This suggestion did not, however, exclude the possibility of uridine and uracil synthesis as degradation products from UMP, recently reported in animal organs by Hurlbert & Kammen [7].

The data presented in this paper do not claim to clarify the sequence of metabolic products formed from OA, yet they prove that degradation of UMP cannot be the only source of emergence of uridine and uracil in plants. On the contrary in the case of uridine it would appear that this substance is an intermediary me-

tabolite in the biosynthesis of UMP from OA. This interpretation was arrived at on the basis of the following data: (1) After 5 min. of incubation with radioactive OA the specific activity of uridine in plants was over twice as high as that of UMP (Table 1, no. 1). (2) On extention of incubation time the specific activity of uridine was always higher than that of UMP (Table 1, no. 1 - 5). (3) On interruption of [6-^{14}C]OA flow and further incubation in water, the specific activity of uridine fell considerably, while the activity of UMP remained unchanged (Table 1, no. 6). (4) With the decrease of specific activity of uridine in plants due to the addition of inactive uridine to the [6-^{14}C]OA solution, a sharp decrease of specific activity of UMP was also observed (Table 2, no. 3). (5) Finally, if uridine were a breakdown product of UMP, then the addition of inactive UMP to the labelled OA should result in a fall of specific activity and in an increase of the amount of uridine in plant material. As can be seen from Table 2, no. 4, despite the addition of inactive UMP to the incubation mixture the specific activity of uridine was even higher than in the control sample and the amount found only about half of the quantity estimated in the tissue fed with [6-^{14}C]OA only.

However, it is not likely that the main synthesis of uridine from OA in plant material follows the path *via* uracil as previously suggested [4] because, as can be seen from Table 1, the specific activity of uracil is always considerably below the activity of uridine. Apparently the plant possesses two independent active enzyme systems responsible for the metabolism of OA to pyrimidine nucleotides: (1) ribosidation and decarboxylation of OA, which supplies uridine for further anabolic processes, and (2) decarboxylation of OA to uracil as suggested previously [4, 5]. A third path may also function, namely ribotidation of OA to OMP, followed by its decarboxylation to UMP as known to occur in animal tissues and microorganisms [9, 6, 3].

The processes of ribosidation and ribotidation of OA in plants still remain to be elucidated. In our experiments we were unable to prove the presence of orotidine or OMP in plant tissues, even when the conditions for their presence were favourable, the medium being supplied with uridine or UMP, respectively. Similarly Hurlbert & Potter [8] could not detect OMP in livers of rats fed with OA despite the fact that the livers very actively synthesized UMP from OA. However, synthesis of OMP was observed by Blair, Stone & Potter [1] *in vitro*.

Uracil in plant material may arise in considerable quantities by way of degradation of UMP. The addition of inactive UMP to the medium caused a fivefold increase of uracil in the tissue together with a marked decrease of its specific activity (Table 2, no. 4). Nevertheless uracil may also arise in plant material through decarboxylation of OA, as it is being synthesized from [6-^{14}C]OA at the same rate as UMP. Furthermore, when the intake of radioactive OA was interrupted and the blades incubated with water, the specific activity of uracil declined considerably, while the activity of UMP remained unchanged (Table 1).

As can be seen from Table 1, CMP in plant material arises from OA *via* UMP. The addition of uracil, uridine and particularly UMP to the medium increased considerably the synthesis of CMP (Table 2).

The addition of uracil to the medium caused a very distinct inhibition of early precursors of pyrimidine compounds prior to the synthesis of OA. As a result of this inhibition a very considerable fall in absolute quantities of uridine and UMP was observed with a corresponding increase of their specific activities (Table 2, no. 2). In fact the specific activity of uridine was very nearly the same as the activity of ingested [6-^{14}C]OA. A very similar effect was caused by the addition of UMP to the medium, which was due to the breakdown of UMP to uracil in the tissue (Table 2, no. 4).

This inhibitory action of uracil was first observed in 1954 by Brooke *et al.* [2] in experiments with *Aerobacter aerogenes* and then explained by Yates & Pardee [11] as "feed-back" mechanism controlling the intensity of pyrimidine biosynthesis.

Finally we would like to draw attention to the fact of extremely rapid metabolism of UMP in plants. 12.8 μmoles of UMP were ingested and completely metabolized within 2 hr. (Table 2, no. 4).

SUMMARY

Excised wheat blades were fed with [6-^{14}C]orotic acid and with a mixture containing [6-^{14}C]orotic acid and inactive uracil, uridine or UMP, respectively. Radioactive acid-soluble pyrimidine derivatives were isolated from plants after various periods of incubation.

It was established that in wheat blades, unlike in microorganisms and animal tissues, uridine is an intermediate product of metabolism of orotic acid to UMP.

Uracil in plants is certainly a product of UMP degradation, but it may also be synthesized by decarboxylation of orotic acid.

The "feed-back" effect of uracil, inhibiting the synthesis of pyrimidine precursors, has been observed in plants.

Uracil, uridine and particularly UMP are precursors of CMP biosynthesis in plants.

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SYNTEZA POCHODNYCH PIRYMIDYNOWYCH W MATERIALE ROŚLINNYM Z ZASTOSOWANIEM KWASU [6-^{14}C]OROTOWEGO

Streszczenie

Odcięte źdźbła pszenicy dokarmiano kwasem [6-^{14}C]orotowym oraz mieszaninami kwasu [6-^{14}C]orotowego z nieznakowanym uracylem, urydyną lub UMP. Radioaktywne kwasorozpuszczalne produkty izolowano z roślin po różnych okresach dokarmiania.

Stwierdzono, że w odróżnieniu od drobnoustrojów i tkanek zwierzęcych, w roślinie produktem pośrednim w przemianie kwasu orotowego do UMP jest urydyna.

Uracyl w roślinach wyższych powstaje niewątpliwie w wyniku metabolizmu UMP oraz prawdopodobnie na drodze dekarboksylacji kwasu orotowego.

Zaobserwowano hamujący wpływ uracylu na wczesne etapy syntezy pirymidyn w roślinie.

Uracyl, urydyna i szczególnie UMP są prekursorami CMP w tkankach roślin.

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