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GRAŻYNA MUSZYŃSKA and I. REIFER

THE ARGINASE INHIBITOR FROM SUNFLOWER SEEDS: PURIFICATION AND INHIBITORY PROPERTIES

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1. The *N*-derivative of chlorogenic acid from sunflower seeds is the natural inhibitor of arginase. 2. The inhibitor is unstable and during the isolation procedure some artifacts arise. 3. Oxidation by chlorogenic acid oxidase results in inactivation of the inhibitor whereas the presence of arginase prevents its oxidation. 4. Organic reducing agents such as L-cysteine, reduced glutathione, 2-mercaptoethanol and ascorbate, reactivated the arginase blocked by the inhibitor and when added together with the inhibitor, prevented the inhibition. These results may indicate that the inhibitor combines with the reduced active centre of the enzyme.

The presence of arginase inhibitor in sunflower seeds has been reported in a previous paper (Reifer & Morawska, 1963). The properties (Morawska-Muszyńska & Reifer, 1965; Muszyńska & Reifer, 1968) and structure (Reifer & Augustyniak, 1968) of the inhibitor were also investigated. In this paper, further results concerning properties of the inhibitor and interaction between arginase and the inhibitor, are reported.

MATERIALS AND METHODS

Preparation of the inhibitor. The first steps of isolation of the inhibitor from kernels of sunflower seeds (100 g.), up to methanol extraction, were carried out as described previously (Morawska-Muszyńska & Reifer, 1965). Following evaporation of methanol, the residue (8 g). was dissolved in 40 ml. of redistilled water and dialysed against 160 ml. of redistilled water. Dialysis was continued for 6 hr., water being changed every hour. The first diffusate was discarded and the subsequent 5 external fluids were pooled and evaporated at 100° to a volume of about 200 ml. On cooling, 100 ml. of a 20% neutral lead acetate solution was added, the precipitate centrifuged and suspended in 5 ml. of water, then 1 M-H_2SO_4 was slowly added up to pH 2.5 - 3.0; $PbSO_4$ was centrifuged off and the supernatant concentrated to a volume of about 1 ml. This solution was placed on the Sephadex G-50 coarse column (2.7×25 cm.) and eluted with water at a rate of 1 drop per 8 - 10 sec. Fractions

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of 10 ml. were collected. The fractions with the highest extinction at 325 mμ were pooled, evaporated to dryness at 100°, placed on a Sephadex G-50 fine column (3.7×120 cm.) and eluted with 1% acetic acid in a cold room. Fractions of 15 - 20 ml. were collected, the rate of flow being 1 ml./80 sec. The inhibitor showing extinction at 325 mμ emerged in three distinct peaks. Each of them was pooled and evaporated under reduced pressure at room temperature. The residues were dissolved in 0.001 N-HCl using 100 μl./1 mg., distributed into several tubes and kept in a desiccator at -5° until required.

Later it was found that the isolation was improved when the following modifications were introduced: (1), evaporations were carried out at room temperature under reduced pressure instead of evaporation at 100°; (2), dialysis was cut down to 4 hr., and, owing to evaporation at reduced pressure, the first diffusate did not have to be discarded; (3), the filtration on Sephadex G-50 fine has been omitted (see Discussion).

Arginase activity assay. The standard incubation mixture contained: 5 μg. of arginase solution in 25 μl. of 0.02 M-K-phosphate buffer of pH 7.2, 0.15 - 1.25 μg. of inhibitor, and 25 μl. of 0.01 M-$MnCl_2$, pH 6.8 - 7.0. The sample was preincubated for 60 min. at 38° at pH 7, then 25 μl. of 0.5 M-L-arginine, pH 10.0, was added. The final volume was 150 μl. and pH 10.0. After 30 min. at 38°, the reaction was stopped by adding 50 μl. of conc. $HClO_4$, the samples were diluted with water to 2 ml., and urea was determined using the Archibald method (1944) as modified by Ratner (1955).

Purification of chlorogenic acid oxidase (*CGA-oxidase*). A simplified procedure of purification based on the method of Filippa & Alberghina (1964b) is described below. Potato tubers, 150 g., were grated on a plastic rasp and ground finely in a porcellain mortar with glass powder. Then 300 ml. of 0.05 M-Na,K-phosphate buffer, pH 6.5, was added, mixed and put through a double layer of cheese-cloth. After centrifugation, the supernatant was treated with ammonium sulphate and the fraction precipitated between 0.25 and 0.55 saturation was collected and dissolved in 5 ml. of 0.05 M-phosphate buffer, pH 6.5. Samples of 5 μl. were used for determination of CGA-oxidase according to the method of Sisler & Evans (1958).

Special reagents. Arginase from plants of blue bitter lupin was prepared as described by Muszyńska & Reifer (1968), L(+)arginine was from Fluka AG (Buchs SG, Switzerland), L(+)cysteine and reduced glutathione from Schuchardt GmbH and Co. (Munich, West Germany), Sephadex G-50 coarse and fine from Pharmacia (Uppsala, Sweden); chlorogenic acid was a kind gift from prof. Nils Nybom (Fruit Breeding Institute, Fijälkestad, Sweden).

RESULTS AND DISCUSSION

As a result of partition of the inhibitor on Sephadex G-50 fine, three fractions were obtained (Fig. 1) which were characterized by different inhibitory activity. The first fraction had the highest activity and the third fraction the lowest (Fig. 2).

The first fraction was found to be the nitrogen derivative of chlorogenic acid

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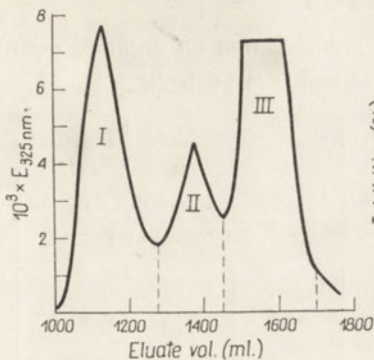

Fig. 1

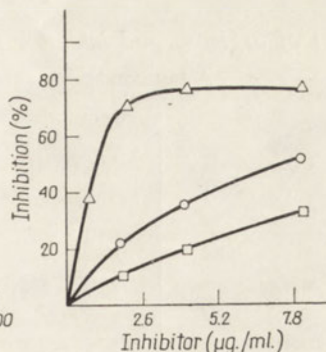

Fig. 2

Fig. 1. Fractionation of arginase inhibitor from sunflower seeds on Sephadex G-50 fine column.

Fig. 2. Inhibition of arginase activity as a function of inhibitor concentration. The arginase was preincubated with Mn^{2+} for 60 min., then the inhibitor and arginine were added. △, Fraction *I*; ○, fraction *II*; □, fraction *III*.

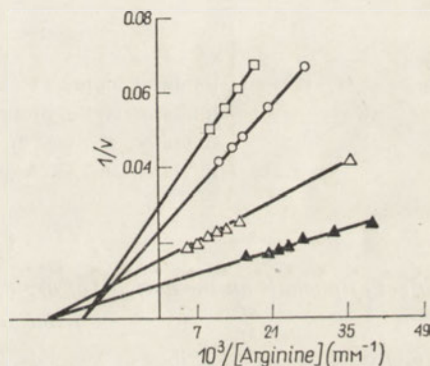

Fig. 3. Graphical determination by the method of Lineweaver-Burk of the type of arginase inhibition. ▲, Arginase alone, and with the addition of: △, fraction *I*, 10 µg./ml.; ○, fraction *II*, 16 µg./ml.; □, fraction *III*, 25 µg./ml. Arginine was applied at concentrations of 30 - 200 mM: *v* is expressed as µmoles of urea formed/mg. protein/30 min.

(*N*-derivative of CGA) as on alkaline hydrolysis it gave NH_3, proving that this product was the *N*-derivative of CGA, which is in agreement with the results of Reifer & Augustyniak (1968). The second fraction showing an intermediate activity has not been identified; however, the UV spectrum seems to suggest that it is an isomer or derivative of chlorogenic acid. The third fraction was found to be pure CGA by comparison of its UV spectrum and inhibitory activity with those of standard CGA.

The effect of arginase preincubation with the three fractions is shown in Table 1. The *N*-derivative of CGA (fraction *I*), in contrast to fraction *II*, did not require preincubation; interaction with enzyme took place immediately. The observed differences of inhibition together with the Lineweaver-Burk plots for the three fractions seem to suggest that different active centres were affected (Fig. 3).

The inhibition with the *N*-derivative of CGA (fraction *I*) was non-competitive,

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Table 1

Effect of arginase and inhibitor preincubation on arginase activity

Assay conditions as described in Methods.

Preincubation (60 min.)	Addition after preincubation	Fraction *I* 1 μg./ml.	Fraction *II* 8.2 μg./ml.	Fraction *III* 8.2 μg./ml.
		Inhibition (%)		
Arginase alone	inhibitor, then substrate	44.5	46.5	40.0
Arginase alone	substrate, then inhibitor	53.2	46.5	20.5
Arginase with inhibitor	substrate	50.0	78.3	33.5

Table 2

Effect of preincubation of inhibitor with CGA-oxidase in presence or absence of arginase

Assay conditions: inhibitor concentration 2 μg./ml., chlorogenic acid oxidase 330 μg./ml., preincubation time 60 min. In control samples arginase was preincubated with chlorogenic acid oxidase.

Preincubated sample	Inhibition (%)
Inhibitor alone	67.3
Inhibitor and arginase	67.5
Inhibitor, arginase and CGA-oxidase	69.0
Inhibitor and CGA-oxidase	22.5

Table 3

Effect of phenols on the activity of arginase alone and arginase inhibited by the inhibitor from sunflower seeds

Assay conditions as described in Methods, inhibitor concentration 4 μg./ml. Arginase activity is expressed in percentages of the activity of arginase without any additions.

Concentration of phenols (μmoles/ml.)	Aminophenol		Dinitrophenol		Phenol	
	arginase alone	with inhibitor	arginase alone	with inhibitor	arginase alone	with inhibitor
None	100	24	100	24	100	24
0.023	95	34	105	24	100	29
0.115	95	34	104	25	94	34
0.460	102	71	98	36	93	35
2.300	103	89	86	63	97	42

with CGA (fraction *III*) and unidentified fraction *II* it was of the mixed type. This may suggest that the centre of inhibition for the *N*-derivative of CGA is somewhat shifted from the centre of the other two compounds. The inhibition centre seems to be closer to the active substrate site on the enzyme surface.

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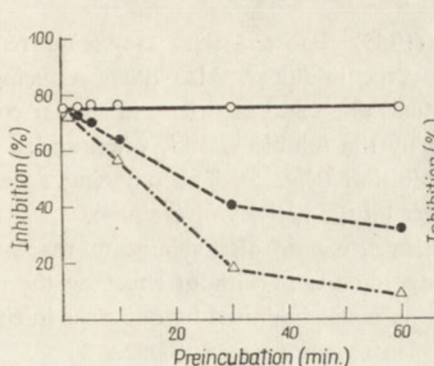

Fig. 4

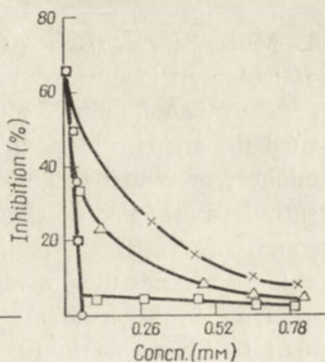

Fig. 5

Fig. 4. Effect of chlorogenic acid oxidase on the activity of arginase inhibitor. The inhibitor (fraction *I*), 4 µg./ml., was preincubated: ○, without oxidase; ●, with 330 µg/ml. oxidase; △, with 825 µg./ml. oxidase; then arginase and arginine were added.

Fig. 5. Effect of reducing compounds on reactivation of the activity of arginase blocked by the inhibitor. Concentration of inhibitor, 2 µg./ml. ×, Cysteine; △, ascorbate; ○, 2-mercaptoethanol; □, reduced glutathione.

The isolated three fractions were extremely unstable; of eight independent preparations, only two showed inhibitor activity. The modification suggested under Materials and Methods (evaporation under reduced pressure, omission of the second filtration on Sephadex) led to a product of high activity, similar to the fraction *I*, with four times higher yield. This compound was the *N*-derivative of CGA, and on a second filtration (on Sephadex G-50 fine) it separated into two fractions, one with the initial activity of *N*-derivative of CGA and the other with much smaller activity, similar to fraction *III*; on electrophoresis and paper chromatography it gave two or three fractions showing little activity. These results seem to suggest that in sunflower seeds the *N*-derivative of CGA is present, and that all other fractions of lower activity are products arising during the purification. For further experiments only the *N*-derivative of CGA was used.

Filippa & Alberghina (1964a) have demonstrated that CGA acts as an inhibitor toward glycerol 3-phosphate and glucose 6-phosphate dehydrogenases. Pierpoint (1966) has shown that CGA may be oxidized by O_2 to quinones and melanines. As our inhibitor preparation was relatively unstable, it was thought that this could be due to the oxidation of the chlorogenic acid moiety of the inhibitor. Therefore the effect of CGA-oxidase on the activity of the inhibitor was studied (Fig. 4). The inhibitor (fraction *I*) was preincubated with the oxidase preparation, then arginase and arginine were added. (It should be noted that CGA-oxidase had no effect on arginase activity). After 30 min. preincubation, the inhibition was diminished very strongly. However, when arginase was present in the preincubation sample, no change in inhibition was observed (Table 2). It is reasonable to assume that following enzyme-inhibitor interaction the group or groups of the inhibitor which may be oxidized, are stabilized. The presence of arginase protected the inhibitor against enzymic oxidation.

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Morawska-Muszyńska & Reifer (1965) reported that L-cysteine reactivated arginase that had been inactivated by the inhibitor. Also other reducing agents such as 2-mercaptoethanol, reduced glutathione and ascorbate in similar concentration reactivated the arginase blocked by the inhibitor, and, when added together with the inhibitor, prevented the inhibition (Fig. 5). The reducing agents alone in the concentrations used had no effect on the activity of arginase.

Phenols, and particularly aminophenol, caused also significant reactivation of arginase (Table 3), whereas these compounds were without effect on the non-inhibited arginase. On the other hand, arginase was inhibited by quinones in concentrations from 0.01 to 1 mM, and by 0.05 mM-*p*-chloromercuribenzoate.

The above presented results may suggest the presence of reduced group or groups in the active centre of arginase which are responsible for the activity of this enzyme.

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REFERENCES

Archibald R. M. (1944). *J. Biol. Chem.* **156**, 121.
Filippa A. & Alberghina M. (1964a). *Life Sci.* **3**, 49.
Filippa A. & Alberghina M. (1964b). *Phytochemistry* **3**, 65.
Morawska-Muszyńska G. & Reifer I. (1965). *Acta Biochim. Polon.* **12**, 187.
Muszyńska G. & Reifer I. (1968). *Acta Biochim. Polon.* **15**, 55.
Pierpoint W. S. (1966). *Biochem. J.* **98**, 567.
Ratner S. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 2, p. 359. Academic Press, New York.
Reifer I. & Augustyniak H. (1968), *Bull. Acad. Polon. Sci. ser. sci. biol.* **16**, 139.
Reifer I. & Morawska G. (1963). *Acta Biochim. Polon.* **10**, 413.
Sisler E. C. & Evans H. J. (1958). *Biochim Biophys. Acta* **28**, 638.

OCZYSZCZANIE I WŁASNOŚCI INHIBITORA ARGINAZY Z NASION SŁONECZNIKA

Streszczenie

1. Azotowa pochodna kwasu chlorogenowego jest naturalnym inhibitorem arginazy obecnym w nasionach słonecznika.

2. Różne frakcje otrzymane podczas izolacji inhibitora wydają się być produktami rozkładu.

3. Utlenienie inhibitora przez oksydazę kwasu chlorogenowego powoduje jego inaktywację, natomiast w kompleksie z arginazą inhibitor nie jest utleniany.

4. Organiczne związki redukujące, takie jak cysteina, 2-merkaptoetanol, zredukowany glutation i askorbinian, reaktywują zahamowaną arginazę, a dodane równocześnie z inhibitorem ochraniają enzym przed inhibicją. Podane wyniki mogą świadczyć, że inhibitor łączy się ze zredukowanymi grupami arginazy.

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ZOFIA KASPRZYK, GRAŻYNA TUROWSKA, ELŻBIETA GRYGIEL
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THE VARIATIONS IN THE CONTENT OF TRITERPENOIDS IN THE DEVELOPING FLOWERS OF *CALENDULA OFFICINALIS* L.

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Changes in the content of individual triterpenoid compounds were investigated in the flowers of *Calendula officinalis* during their development. The free, ester-bound and glycosidic forms were determined separately. It was shown that during the period investigated the content of all compounds is increasing till the stage of full development of flowers. Afterwards the increase ceases due to transport and hydroxylation processes.

It has been shown previously that the following triterpenic compounds occur in the flowers of calendula: monohydroxyalcohols — α- and β-amyrins, ψ-taraxasterol, taraxasterol and lupeol, dihydroxyalcohols — brein, erythrodiol, faradiol with arnidiol and calenduladiol (Kasprzyk & Pyrek, 1968) as well as oleanolic acid (Winterstein & Stein, 1931), together with sterols such as: β-sitosterol, stigmasterol and isofucosterol (Kasprzyk & Turowska, 1969). Mono and dihydroxy alcohols occur in the form of free compounds and as acetates (Kasprzyk, Turowska & Baranowska, 1969), sterols — as free compounds, acetates and glucosides, and finally oleanolic acid in the form of glycosides. The studies by Kasprzyk & Fonberg-Broczek (1967) showed that total amount of triterpenic monols and diols, sterols and oleanolic acid in developing flowers increases rapidly till their full development and then slightly decreases.

The present paper describes the changes in the content of separate forms of individual triterpenes and sterols, occurring in calendula flowers during their development.

MATERIALS AND METHODS

Material. For the present investigation, flowers of field-cultivated calendula were used. The flowers were collected during the first half of Octobeı. They were classified according to age into six stages (the age being indicated in parentheses): buds (5 days), developing flowers (8, 12 and 17 days), fully developed flowers (21 days) and withering ones (27 days). Beginning with the 12-day-old inflorescences, the green parts (involucres) and flowers were investigated separately.

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Extraction and fractionation of the extract. Fresh flowers were exhaustively extracted with methanol. The extract was separated as described by Kasprzyk, Śliwowski & Bolesławska-Kokosza (1970) into the phase containing free triterpenoids and their acetates, and the phase containing compounds in the form of glycosides; the free compounds were separated from their esters by alumina column chromatography.

Hydrolysis of bound terpenoids. The triterpenoid esters isolated by alumina column chromatography were hydrolysed as described previously (Kasprzyk *et al.*, 1970).

Acetylation and oxidation of free triterpenoids. Acetylation was carried out in a tenfold volume of a mixture of acetic acid anhydride-pyridine (1:1, v/v) at room temperature for 24 hr. The solvent was then removed by evaporation at 80°.

The amyrins were oxidized with a tenfold excess of SeO_2 in a solution of acetic acid - benzene (2:1, v/v) at 100° for 1 hr., the reaction products were extracted with hexane and separated by thin-layer chromatography.

Thin-layer chromatography. This was performed as described previously (Kasprzyk *et al.*, 1970), the same standards being applied. The following solvent systems were used: I, light petroleum (b.p. 40 - 60°) - chloroform - methanol (20:10:1, by vol.) for the silica gel and alumina; and II, chloroform - ethyl ether (49:1, v/v) and III, chloroform, for silica gel impregnated with $AgNO_3$. Individual monols as well as acetates of diols were separated in system II, and sterol acetates in system III.

Quantitative determination of triterpenoids. This was carried out using the colorimetric method with $CoCl_2$ described by Fonberg & Kasprzyk (1965). Standard curves were prepared with the compounds tested. Measurements were performed by means of Carl Zeiss VSU-1 spectrophotometer.

RESULTS

The development of inflorescence is characterized by an intensive process of synthesis resulting in an increase of its fresh and dry weight; this was observed in the whole inflorescence till the 8th day, and then in the flowers and involucre analysed separately till full development of the flowers (21st day). At the next stage, however, when the flowers were overblown, both fresh and dry weight of flowers decreased whereas fresh weight of the involucre did not change and its dry weight was still increasing (0.9 g. per 100 involucres). This could be explained by the transport of some compounds from flowers to the seeds which began to develop at this stage, and were analysed together with the involucre. The changes in fresh and dry weight calculated per 100 inflorescences are presented in Table 1.

Thin-layer chromatography of 12-day-old flowers and green parts of inflorescence revealed considerable differences in the content of triterpenoids. The receptaculum and involucre contained only trace amounts of triterpenic monols (β-amyrin) and triterpenic diols (erythrodiol), and greater quantities of sterols and oleanolic acid. On the other hand, flowers contained considerable amounts of triterpenoids of all groups.

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Table 1

Fresh and dry weight of the inflorescences analysed

Age (days)	Number	Fresh weight of 100 inflorescences (g.)		Dry weight of 100 inflorescences (g.)		Dry weight (% of fresh wt.)	
5	329	16		1.8		11.3	
8	222	44		4.4		10.0	
		Flowers	Involucre	Flowers	Involucre	Flowers	Involucre
12	107	41	22	4.5	2.4	10.9	11.0
17	108	48	24	5.0	2.6	10.4	11.9
21	217	51	27	6.3	3.2	12.4	12.0
27	134	40	27	6.1	4.1	15.1	14.9

Variations in the content of individual free sterols (*A*), their acetates (*B*) and glycosides (*C*) during the development of calendula flowers, are presented in Fig. 1. The results for 5 and 8 days are given with respect to whole inflorescence, and those for later stages represent the content of the investigated compounds in flowers. The content of free sterols and their acetates rapidly increased at the stage of bud formation. The increase was then slower till the stage of full development of flowers, and it practically did not change in old flowers. The course of changes of individual sterols was almost parallel in both fractions except for β-sitosterol aeetate. In 5-day-old inflorescences, no quantitative determinations were made of sterol glycosides because of their small concentration. The quantity of each sterol glycoside increased steadily till the stage of overblowing. This indicated the continuous process of glycosylation of all sterols and of esterification of β-sitosterol during flowering, and also in old flowers in which there was no increase of free sterols. The quantitative relations between free sterols, their acetates and glycosides were determined previously in flowers by Kasprzyk *et al.* (1969). The present results indicate similar ratios of individual forms during the whole period of development of flowers, amounting to: 65% of free sterols, 20% of acetates and 15% of glycosides. The fraction of free and ester-bound compounds contained about 55% of stigmasterol, and the fraction of glycosides, about 60% of β-sitosterol. Isofucosterol occurred in all fractions in the lowest concentration (about 14%), this being in agreement with the supposition that this compound is a precursor of β-sitosterol and stigmasterol.

Changes in the content of oleanolic acid and its biosynthetic precursors, β-amyrin and erythrodiol, are presented in Fig. 2. It is noteworthy that both precursors occurred in calendula flowers almost exclusively in the form of free compounds. The content of β-amyrin increased considerably during the first days of development of flowers, followed by a slower increase at further stages, reaching a maximum on the 21st day. The content of erythrodiol was about 8 times lower, and its increase slower, being unchanged in older flowers. The results of determination of oleanolic acid confirmed the previous data (Kasprzyk & Fonberg-Broczek, 1967; Kasprzyk & Wojciechowski, 1967) that great quantities of oleanolic acid accumulate in flowers in the form of glycosides (1.5% of dry weight).

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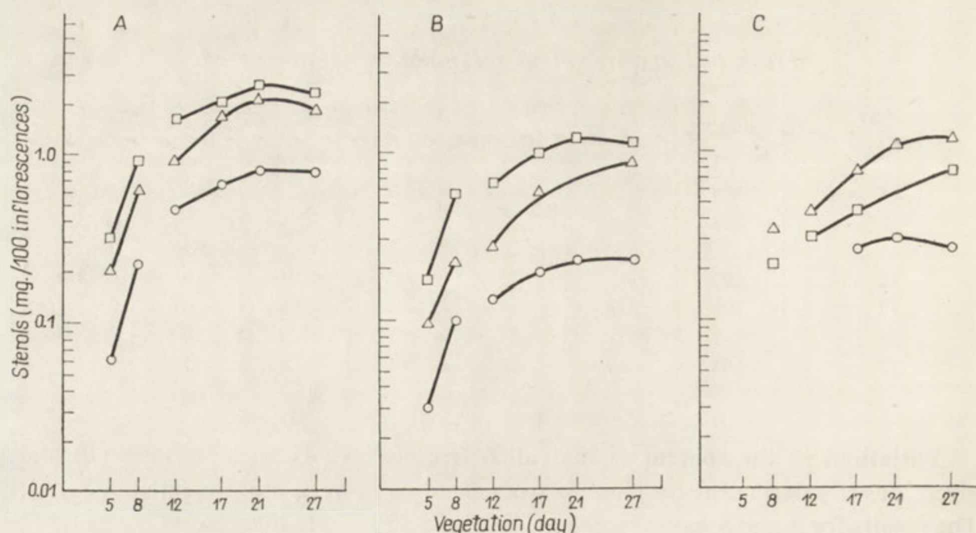

Fig. 1. Changes in the amount of *A*, free sterols; *B*, acetates of sterols; *C*, glycosides of sterols, in the developing flowers of calendula. ○, Isofucosterol; △, β-sitosterol; □, stigmasterol.

Changes in the content of individual monols and diols other than β-amyrin, are shown in Fig. 3. The shape of curves indicates that quantitative relations among individual monols did not vary significantly during the whole period studied. The content of all monols in flowers, both those in free and acetate forms, was considerably increased at the initial stage of flower development, the increase being then much slower, with the maximum at the stage of full development. These results confirmed previous determinations (Kasprzyk *et al.*, 1969) of the quantitative relations between free monols and their acetates in calendula flowers, which showed that free forms amounted to 90% of the sum of monols. Similar quantitative relations were found in the fraction of free monols and acetates, amounting to 40% for ψ-taraxasterol, 30% for lupeol, 17% for taraxasterol and 10% for α-amyrin at all stages of development of the flowers. The only difference was that β-amyrin was found only in the fraction of free compounds (1.7%). The content of diols in developing flowers increased as rapidly as that of oleanolic acid, on average from 0.8 mg. in 100 inflorescence buds to 60 mg. in flowers from 100 fully developed inflorescences, then it slightly decreased. This decrease was the result of lowered content of diols in free form as well as of faradiol with arnidiol and brein acetates, the content of calenduladiol being unchanged till the end of the period studied. In calendula flowers, similarly as in seeds (Kasprzyk *et al.*, 1970), diols occurred mainly in the form of esters (to 98%); the amount of this form being increased about a hundred times during the period of development of the flowers. The quantitative relations among individual free diols and diol esters did not significantly change during the period of investigation, and on average represented in the developed flowers 50% for faradiol and arnidiol acetates (it was impossible to separate these two

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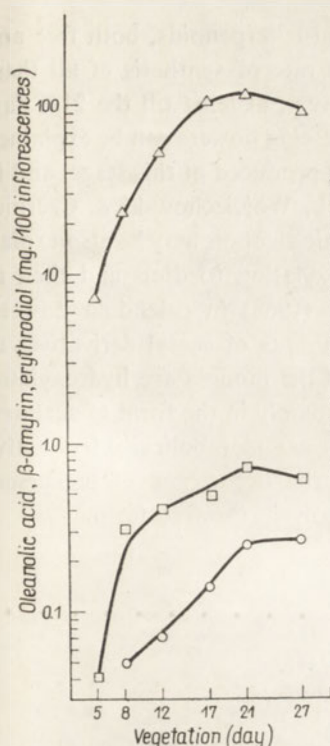

Fig. 2

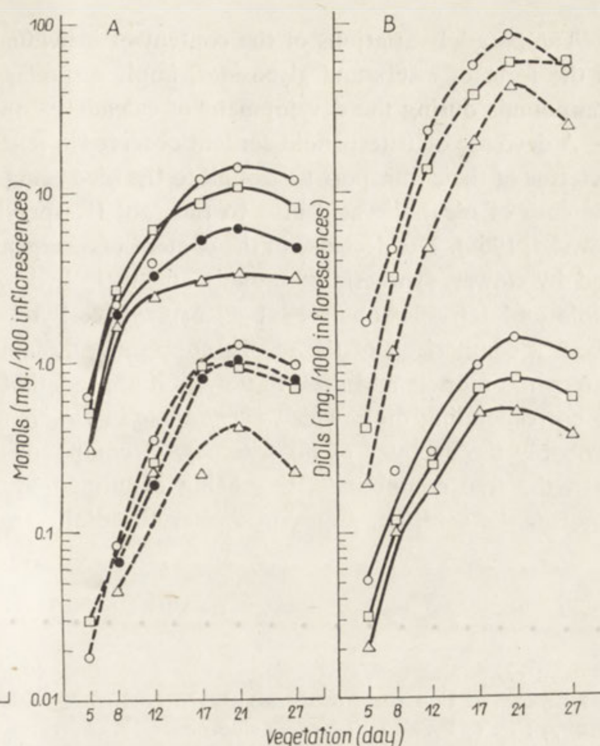

Fig. 3

Fig. 2. Changes in the amount of oleanolic acid and its biosynthetic precursors in the developing flowers of calendula. △, Oleanolic acid; □, β-amyrin; ○, erythrodiol.

Fig. 3. Changes in the amount of *A*, triterpenic monols and their acetates, and *B*, triterpenic diols and their acetates in developing flowers of calendula. (———), Free compounds, (- - -), acetates. *A*, ○, ψ-taraxasterol; □, lupeol; ●, taraxasterol; △, β-amyrin. *B*, ○, faradiol with arnidiol; □, calenduladiol; △, brein.

compounds with the chromatographic method used), 35% for calenduladiol acetate and 15% for brein acetate. Erythrodiol was present only in the fraction of free compounds, its content being on average 5%.

DISCUSSION

The results obtained indicate that the synthesis of free β-amyrin and erythrodiol as well as of oleanolic acid glycoside occurs in flowers till the stage of their full development, similarly as in the green parts of the inflorescence and the shoots of calendula plants (Kasprzyk & Wojciechowski, 1969). The synthesis of sterols occurs also both in the shoots and the green parts of the inflorescence, and in the flowers. The synthesis of triterpenic monols and diols other than β-amyrin and erythrodiol, is limited to the flowers and does not occur in any other part of the calendula plant.

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The parallel variations of the content of individual triterpenoids, both free and in the form of esters and glycosides, imply a similar rate of synthesis of all these compounds during the development of calendula flowers, at least till the 21st day.

A decrease of triterpenoid content observed in senescing flowers can be explained in terms of their transport first of all to the seed being produced at this stage, and in the case of oleanolic acid also to the root (Kasprzyk, Wojciechowski & Czerniakowska, 1968). The decrease in the content of triterpenic alcohols may be also explained by slower synthesis of monols and their hydroxylation to diols and then to triols and tetraols, observed by Kasprzyk & Pyrek (1968) in calendula flowers. From a comparison of the results concerning the amounts of acetyl derivatives of triterpenic monols and diols in flowers it appears that the monols are hydroxylated to corresponding diols which accumulate in flowers mainly in the form of acetates. Probably the acetates of all triterpenoid compounds are metabolically less active than the free compounds, this being confirmed by the occurrence of precursors of oleanolic acid, i.e. β-amyrin and erythrodiol, usually in the free form.

REFERENCES

Fonberg M. & Kasprzyk Z. (1965). *Chemia Analityczna* **10**, 1181.
Kasprzyk Z. & Fonberg-Broczek M. (1967). *Physiol. Plantarum* **20**, 321.
Kasprzyk Z. & Pyrek J. (1968). *Phytochemistry* 7, 1631.
Kasprzyk Z., Śliwowski J. & Bolesławska-Kokosza D. (1970). *Acta Biochim. Polon.* **17**, 11.
Kasprzyk Z. & Turowska G. (1969). *Bull. Acad. Polon. Sci., Ser. sci. chim.* **17**, 397.
Kasprzyk Z., Turowska G. & Baranowska E. (1969). *Bull. Acad. Polon. Sci., Ser. sci. chim.* **17**, 399.
Kasprzyk Z. & Wojciechowski Z. (1967). *Phytochemistry* **6**, 69.
Kasprzyk Z. & Wojciechowski Z. (1969). *Phytochemistry* **8**, 1921.
Kasprzyk Z., Wojciechowski Z. & Czerniakowska K. (1968). *Physiol. Plantarum* **21**, 966.
Winterstein A. & Stein G. (1931). *Z. Physiol. Chem.* **64**, 199.

ZMIANY ZAWARTOŚCI TRÓJTERPENOIDÓW W ROZWIJAJĄCYCH SIĘ KWIATACH *CALENDULA OFFICINALIS* L.

Streszczenie

Badano zmiany zawartości poszczególnych związków trójterpenoidowych w kwiatach *Calendula officinalis* w czasie ich rozwoju. Oddzielnie oznaczano formy wolne, estrowe i glikozydowe. Wykazano, że w badanym okresie ich ilość wzrasta do okresu pełnego rozkwitu; następnie wzrost ustaje, co jest spowodowane transportem i hydroksylacją.

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HYDROLYSIS OF 5-CYANO-, AND DECARBOXYLATION OF 5-CARBOXY-, URACILS: A FACILE ROUTE TO THE SYNTHESIS OF ANALOGUES OF 1-CYCLOHEXYLURACIL AND CYTOSINE

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1. A relatively simple method is described for the preparation of 1-cyclohexyluracil by the simultaneous hydrolysis and decarboxylation of the nitrile group of 1-cyclohexyl-5-cyanouracil in anhydrous phosphoric acid at 160 - 170°. 2. The ease of decarboxylation of 5-carboxyuracil, as compared to the relative resistance under the same conditions of 6-carboxyuracil (orotic acid) has been interpreted as due to the formation in the case of the former of an intramolecular hydrogen bond between the 5-carboxyl group and the C_4 carbonyl. 3. The hydrolysis and decarboxylation reactions have been studied under various conditions. 4. The resulting 1-cyclohexyluracil was used as starting product for the preparation of a variety of 1-cyclohexyl analogues of uracil and cytosine, intended for studies on association of purines and pyrimidines in non-aqueous media, photochemical reactions in non-aqueous media, etc.

Studies on the association by hydrogen bonding of base-pairs of purines and pyrimidines require the use of derivatives which are soluble in non-polar solvents (Kyogoku, Lord & Rich, 1967). For this purpose l-cyclohexyl substituted uracil and cytosine analogues have been found quite satisfactory. Such compounds would also be expected to be useful in studies on the photochemistry of pyrimidine derivatives in non-aqueous media. We describe herein a relatively simple pathway to the preparation of such compounds, applicable both on a small and large scale.

RESULTS AND DISCUSSION

Attempts to obtain l-cyclohexyluracil *via* the Hilbert-Johnson (1930) rearrangement by reaction of cyclohexyl bromide with 2,4-dicyclohexyloxypyrimidine, or directly by treatment of uracil with cyclohexyl bromide, both proved unsatisfactory. Heating at 100° for two days with cyclohexyl bromide of 2,4-dicyclohexyloxypyrimidine (obtained by reaction of 2,4-dichloropyrimidine with sodium cyclohexanolate) resulted in the recovery of starting product. This finding is not unexpected in view of the known steric effects involved in the Hilbert-Johnson rearrangement (Prystaš & Šorm, 1966).

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A variety of l-substituted uracils was prepared by Baker, Kawazu, Santi & Schwan (1967) by heating an excess of the required uracil derivative with an alkyl halide in dimethyl sulphoxide (DMSO) in the presence of K_2CO_3. However, the application of this procedure to reaction of uracil with cyclohexyl bromide yielded only traces of l-cyclohexyluracil, due most likely to the lability of the cyclohexyl bromide under these reaction conditions. In fact, heating of cyclohexyl bromide with K_2CO_3 in DMSO at 80 - 90° in the absence of uracil led to substantial liberation of bromide ion.

Cheng & Lewis (1968) have described the preparation of l-alkyluracils (including l-cyclohexyluracil) *via* the corresponding 5,6-dihydro derivatives, but this method is considerably more arduous and time-consuming than that described below.

The starting point for the present procedure is the method described by Atkinson, Shaw & Warrener (1956) for the synthesis of l-substituted 5-cyanouracils by the condensation of primary amines with α-cyano-β-ethoxy-*N*-ethoxycarbonylacrylamide. With cyclohexylamine, this gives l-cyclohexyl-5-cyanouracil in quantitative yield. We have now found that the nitrile group can be readily removed to give l-cyclohexyluracil in good yield simply by heating the 5-cyano derivative to 160 - 170° in anhydrous phosphoric acid.

Berger & Olivier (1927) and Olivier (1929) had earlier found that *ortho* substituted benzonitriles, which are particularly resistant to hydrolysis, were readily converted to the corresponding benzoic acids on heating in anhydrous orthophosphoric acid. Under analogous conditions the nitriles were unaffected by treatment with 92% phosphoric acid. The foregoing authors concluded that the water present in 92% H_3PO_4 prevented the formation of a presumed adduct of the nitrile and phosphoric acid, which then underwent transformation to carboxylic acid. And, in the case of acetonitrile, they in fact succeeded in isolating the adduct $CH_3CN\cdot$ $\cdot H_3PO_4$, m.p. 51 - 53°, stable under strictly anhydrous conditions. The water essential for hydrolysis is, however, formed in anhydrous orthophosphoric acid by its transformation to pyrophosphate at 160 - 170°. The products of hydrolysis of 2,4-dibromobenzonitrile were, in fact, found to contain, in addition to 2,4-dibromobenzoic acid, traces of the corresponding amide as well as 2,4-dibromobenzene, testifying to partial decarboxylation of the acid formed.

When l-cyclohexyl-5-cyanouracil, prepared by the method of Atkinson *et al.* (1956), was heated in anhydrous H_3PO_4 at 160 - 170°, the nitrile group readily hydrolysed to give the 5-carboxyl analogue which, under these conditions, underwent rapid decarboxylation. This was verified by paper chromatography, which demonstrated the presence in the reaction products of traces of l-cyclohexyl-5-carboxyuracil, identical with an authentic specimen obtained by hydrolysis in boiling alkali of l-cyclohexyl-5-cyanouracil, as well as by the evolvement of CO_2 during the reaction.

In view of the foregoing, an examination was made of conditions for decarboxylation of 5-carboxyuracil in the presence of (*a*) anhydrous H_3PO_4 at 160-170°, (*b*) cyclohexanol, 161°, and (*c*) ethylene glycol, 197°. Decarboxylation proceeded readily in each case, the reaction being followed by paper chromatography (Table 1),

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Table 1

Ascending paper chromatography of 1-cyclohexyl derivatives

Whatman paper no. 3 and the following solvent systems were used: (*A*), water-saturated *n*-butanol with ammonia in gas phase; (*B*), *n*-butanol - glacial acetic acid - water (80:12:30, by vol); (*C*), water-saturated amyl alcohol with ammonia in gas phase.

Derivative	R_F in solvent		
	A	*B*	*C*
1-Cyclohexyl-			
5-cyanouracil	0.52	—	—
5-carboxyuracil	0.19	—	—
uracil	0.88	—	0.75
5-bromouracil	—	—	0.56
5-chlorouracil	0.72	—	—
4-thiouracil	0.87	0.93	—
cytosine	0.96	0.80	—
5-Carboxyuracil	0.05	0.41	—
Orotic acid	0.02	0.21	—
Uracil	0.18	0.50	

but was most efficient in the case of anhydrous H_3PO_4 (2.5 hr. for completion of reaction). The reaction time in ethylene glycol was 4 hr. and in cyclohexanol 15 hr. In contrast to 5-carboxyuracil, orotic acid (6-carboxyuracil) was unaffected after heating for 3 hr. at 160 - 170° in anhydrous H_3PO_4.

The relative ease of decarboxylation of 5-, as compared to 6-, carboxyuracil, may be interpreted on the one hand by the higher electron density at C_5, as compared with C_6, in the uracil ring; and on the other by the possibility of formation of an intramolecular hydrogen bond between the carboxyl hydrogen of the former with $C_4{=}O$. The resulting electron transfer in the hydrogen-bonded six-membered ring leads more readily to carbanion formation on C_5 than in the case of a free carboxyl group. Furthermore, involvement of the carboxyl group in hydrogen bonding renders it more rigid so that it possesses greater symmetry than in the case of a free carboxyl group. In the case of 6-carboxyuracil, the formation of a six-membered ring closed by hydrogen bonding is excluded.

A variety of other observations testify to the relative ease of decarboxylation of 5-carboxyuracil analogues as compared to the corresponding 6-carboxyuracils. For example, 5-carboxyuracil is fully decarboxylated by heating for several minutes in the dry state at 280° (Shaw, 1955); 3-methyl- and 3-phenyl-5-carboxyuracil require 10 - 20 min. at 225° and 243°, respectively (Whitehead, 1952); whereas orotic acid, and its 3-methyl and 3-phenyl-analogues require at least one hour heating in boiling quinoline (238°) in the presence of powdered copper (Atkinson, Maguire, Ralph, Shaw & Warrener, 1957). 4-Amino-5-carboxypyrimidine which, like 5-carboxyuracil, can form an intramolecular hydrogen bond, undergoes complete decarboxylation on heating for several min. at 280° in benzophenone (Brown & Short, 1953).

With the foregoing procedure at our disposal for the preparation of 1-cyclohexyluracil on a large scale (see Experimental), it is a relatively simple matter to

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use this compound as the starting product for the preparation by standard procedures of a variety of other analogues, e.g. 5-halogeno, 5,6-dihydro, 4-thio, 4-chloro, etc. Furthermore, the 4-thio or 4-chloro derivatives may then be aminated to provide cytosine or alkylaminocytosine analogues. The preparation of several typical derivatives is described under Experimental. In particular, the 4-thio derivative is expected to be of considerable value in studies on the photochemistry of 4-thiouracil in non-aqueous media (cf. Pleiss, Ochiai & Cerutti, 1969; Sato & Kanaoka, 1969).

Following completion of the present study, two new publications appeared which are of direct interest in relation to the above. In one of these, Doub, Krolls, Vandeblet & Fisher (1970) describe the preparation of l-alkyluracils essentially by the same method, i.e. simultaneous hydrolysis and decarboxylation of l-alkyl-5-cyanouracils. The hydrolysis medium employed by these authors was a refluxing mixture of water, conc. HCl and glacial acetic acid (1:1:2, by vol.). With this medium the efficiency for the hydrolysis and decarboxylation reactions was considerably lower than in our case and varied from 24 to 168 hr., depending on the nature of the l-alkyl substituent, and was due principally to the lower temperature employed, which markedly influences the rate of decarboxylation.

An entirely different principle was employed by Isono & Suzuki (1970) for the decarboxylation of derivatives of 5-carboxyuracil. These authors profited from the well-known nucleophilic character of the C_6 carbon of uracil (which is even more reactive in the case of 5-carboxyuracil). They demonstrated the nucleophilic addition of $NaHSO_3$ to the 5,6 double bond of l-(5′-amino-5′-deoxy-β-D-allofuranosyl)-uracil-5-carboxylic acid (polyoxin C acid) under very mild conditions (50° and an initial pH of 4.0). Under these conditions the resulting 5,6-dihydro-5-carboxyl-6--sulphonate derivative underwent decarboxylation with the simultaneous partial elimination of the group to regenerate the 5,6 double bond. Elimination of the group was quantitative at pH 11 - 12 at room temperature. Similar reactivity was exhibited by 5-carboxyuracil and 2-thio-5-carboxyuracil. The mild reaction conditions required, and the almost quantitative nature of the reaction, were made use of for the decarboxylation of a number of other polyoxines. The nucleophilic addition of sodium bisulphite to uracil (and cytosine) has been recently studied by others (Hayatsu, Wataya & Kai, 1970).

EXPERIMENTAL

Anhydrous phosphoric acid was obtained by dissolving P_2O_5 in 92% H_3PO_4 (40 g. P_2O_5 to 100 ml. acid). UV absorption spectra were obtained on a Zeiss (Jena) VSU-2P instrument. Melting points were measured with the aid of a Boetius microscope hot stage. Elementary analysis and spectral data are presented in Table 2, R_F values in Table 1.

1-Cyclohexyl-5-cyanouracil: This was prepared as described by Atkinson *et al.* (1956), the crude product being obtained in 100% yield, and used as such for further reactions. A sample for elementary analysis and spectral data was obtained by recrystallization from ethanol, which gave platelets with m.p. 321°, as compared to 324° reported by Atkinson *et al.* (1956).

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Table 2

Elementary analyses and UV spectral data for some 1-cyclohexyluracil derivatives

1-Cyclohexyl derivative of	Elementary analysis (%)						Spectral data					
	Calculated			Observed			0.01 N-HCl			0.01 N-NaOH		
	C	H	N	C	H	N	λ_{max} (mμ)	ε_{max}	λ_{min} (mμ)	λ_{max} (mμ)	ε_{max}	λ_{min} (mμ)
5-Cyanouracil	60.27	5.98	19.17	60.28	6.04	18.71	283	14.5×10^3	241	280	10.5×10^3	248
5-Carboxyuracil	55.45	5.93	11.76	55.91	6.15	11.39	284	13.9×10^3	244	276	8.7×10^3	251
Uracil	61.84	7.27	14.43	62.09	7.15	13.74	268-9	10.9×10^3	233	266	8.1×10^3	241
5-Bromouracil	43.96	4.80	10.26	44.07	5.06	10.50	285	9.9×10^3	245-6	281	7.1×10^3	250
5-Chlorouracil	52.52	5.73	12.25	53.12	5.64	12.11	283-4	10.4×10^3	242-3	279-280	7.1×10^3	249
4-Thiouracil	57.11	6.71	13.32	57.79	6.64	13.24	246 337	4.7×10^3 24.9×10^3	280	317	21.2×10^3	257
Cytosine	62.15	7.82	21.74	62.63	7.52	21.51	286	14.3×10^3	242	275	9.5×10^3	250

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l-Cyclohexyl-5-carboxyuracil: 1.53 g. of l-cyclohexyl-5-cyanouracil was heated under reflux in 20 ml. of 2.5 N-NaOH for 7 hr. The solution was brought to room temperature and, following addition of 6 ml. conc. HCl, left for several hours. The resulting precipitate was filtered off and washed with water to give, following drying over P_2O_5, 347 mg. (21%) of crude product. Crystallization from 50 ml. of acetone-ethanol (4:1, v/v) gave 237 mg. of amorphous product which, during heating, was transformed in the temperature range 280 - 290° to needles which melted at 309 - 310°.

1-Cyclohexyluracil (small scale): 1 g. of 1-cyclohexyl-5-cyanouracil in 10 ml. of anhydrous H_3PO_4 was heated for 3 hr. at 160 - 170° on a Wood's bath, with evolvement of CO_2. The mixture was cooled and 50 ml. of water added; the resulting precipitate was filtered off and washed several times with water to provide, after drying, 586 mg. (66%) of crude product. Crystallization from 5 ml. of ethanol in the presence of active charcoal gave 323 mg. (36%) of white, bipyramid crystals, m.p. 217 - 218°.

The filtrate obtained following removal of the crude product was extracted with 50 ml. of chloroform. Chromatography (Table 1) demonstrated the presence of three products with R_F values of 0.19, 0.52 and 0.88 (solvent *A*) which, chromatographically and spectrally, were identical with 1-cyclohexyl-5-carboxyuracil, 1-cyclohexyl-5-cyanouracil and 1-cyclohexyluracil, respectively.

1-Cyclohexyluracil (large scale): 25 g. of 1-cyclohexyl-5-cyanouracil in 250 ml. anhydrous H_3PO_4 was heated for 6 hr. at 160-170° on a Wood's bath. The mixture was brought to room temperature and added to 1 litre water. After standing overnight, the precipitate was filtered off and washed with several portions of water to give 14.5 g. (66%) of crude product. Crystallization from 50 ml. of ethanol yielded 10.7 g. of white crystals (m.p. 218.5 - 219.5°). The filtrate resulting from removal of the crude product was extracted with 3 portions of 500 ml. chloroform and the extracts dried with anhydrous $MgSO_4$. Chromatography with solvent *A* demonstrated the presence in this extract, as well as in the filtrate following crystallization, of only 1-cyclohexyluracil. The extract and filtrate were combined, dried on a rotary evaporator, and the residue crystallized from ethanol to give 4.3 g. of white crystals, m.p. 218.5 - 219.5°. Cheng & Lewis (1968) report 217 - 218°. Overall yield 15.0 g.(67 %).

Decarboxylation of 5-carboxyuracil: (*a*) 30 mg. of 5-carboxyuracil in 0.5 ml. anhydrous H_3PO_4 was heated for 2.5 hr. at 160 - 170°, then brought to room temperature. Addition of 2 ml. water gave a precipitate, m.p. 329 - 331° (decomp.) as compared to 335° (decomp.) for authentic uracil. Paper chromatography with solvent *B* (Table 1) exhibited one spot corresponding to uracil, confirmed by spectral analysis of the eluate.

(*b*) 30 mg. of 5-carboxyuracil in 3 ml. of cyclohexanol was heated under reflux for 6 hr., following which chromatography showed the presence of 2 spots with solvents *A* (R_F values 0.05 and 0.18) and *B* (R_F values 0.41 and 0.50) corresponding to 5-carboxyuracil and uracil, respectively (Table 1). Complete decarboxylation required heating for 15 hr.

(*c*) 30 mg. of 5-carboxyuracil in 3 ml. of ethylene glycol was heated under reflux

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and progress of decarboxylation followed by chromatography as above. The reaction went to completion in 4 hr.

Attempted decarboxylation of orotic acid: 30 mg. of 6-carboxyuracil in 1.5 ml. of anhydrous H_3PO_4 was heated for 3 hr. at 160 - 170°. No clarification of the suspension occurred, nor was there any evolvement of CO_2. Addition of 10 ml. of water gave a precipitate which was filtered off. Chromatography showed the presence only of orotic acid, with not a trace of uracil.

1-Cyclohexyl-5-bromouracil: To a suspension of 1.94 g. of 1-cyclohexyluracil in 60 ml. of aqueous ethanol (1:1, v/v) was added 0.52 ml. of bromine, followed by vigorous agitation on a shaker. Following clarification of the suspension, the mixture was heated to boiling, and heating continued in an open flask to reduce the volume to one-third. The resulting precipitate, which appeared after 20 min. boiling, was filtered off from the cooled solution. Crystallization from 25 ml. of ethanol with charcoal gave 1.91 g. of white platelets (70%), m.p. 224 - 225°.

1-Cyclohexyl-5-chlorouracil: 1.55 g. of 1-cyclohexyluracil was dissolved in a mixture of 19 ml. of acetic acid and 1 ml. of acetic anhydride containing several crystals of $FeCl_3$. Following addition of 0.71 ml. of SO_2Cl_2, the mixture was heated under reflux for 3 hr. Solvent was then removed almost to dryness under reduced pressure, 20 ml. of 2.5 N-NaOH added, and heating under reflux carried out for 1 hr. Following cooling, addition of 5 ml. of conc. HCl gave a precipitate which was filtered off. Crystallization from ethanol-water (25 ml. plus 50 ml.) yielded 860 mg. (47%) of white rhomboid crystals, m.p. 223 - 224°. (*Note*: in earlier trials the product was contaminated with the 5,6-dihydro derivative, which could not be removed by crystallization. In the procedure described above, the dihydro derivative was destroyed by heating in alkali).

1-Cyclohexyl-4-thiouracil: 5 g. of 1-cyclohexyluracil was dissolved in 300 ml. of hot pyridine previously distilled over KOH. Then 5 g. of P_2S_5 was added portion-wise to the stirred solution, which was then heated under reflux, with constant stirring, for 5 hr. An additional 5 g. of P_2S_5 was added and heating continued for another 5 hr. During the course of the reaction, water was added dropwise (1 - 2 drops/hr.) so that the mixture exhibited an orange turbidity. The reaction mixture was then reduced to about 50 ml. under reduced pressure. To this was added 800 ml. of boiling water and the whole boiled for 30 min. with constant stirring. The solution was then left at room temperature overnight, the resulting precipitate filtered off and the filtrate extracted 3 times with 250 ml. of chloroform. The chloroform extracts were dried with anhydrous $MgSO_4$, reduced to dryness and combined with the foregoing precipitate. Crystallization from about 100 ml. ethanol with charcoal yielded 4 g. of product, m.p. 185 - 195°. Two recrystallizations from 120 ml. portions of benzene gave 2.81 g. (52%) of a yellow product, m.p. 198 - 200°, which is suitable for further syntheses. This product exhibited an R_F of 0.9 with traces of a fluorescing impurity at R_F 0.8 on TLC silica gel GF_{254} (acetone/benzene, 1:1, v/v), a Wood's lamp being used for revealing the spots. Several additional recrystallizations from benzene and from ethanol yielded chromatographically homogeneous 1-cyclohexyl-4-thiouracil in the form of yellow-green square plates, m.p. 202 - 203°.

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1-Cyclohexylcytosine: 1 g. of 1-cyclohexyl-4-thiouracil (m.p. 198 - 200°) in 25 ml. of anhydrous ethanol saturated with ammonia at 0° was heated in a sealed ampoule for 24 hr. at 100°. The reaction mixture was then brought to dryness, the residue boiled in 15 ml. of water and, following addition of 1 ml. of conc. NH_4OH, left overnight at room temperature. The resulting crystals were recrystallized from several millilitres of 75% ethanol with addition of several drops of conc. NH_4OH, and once again from 20 ml. of water with addition of several drops of conc. NH_4OH, to yield finally 480 mg. (47%) of white long needles, m.p. 271 - 274°.

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REFERENCES

Atkinson M.R., Maguire M.H., Ralph R.K., Shaw G. & Warrener R.N. (1957). *J. Chem. Soc.* 2363.
Atkinson M. R., Shaw G. & Warrener R. N. (1956). *J. Chem. Soc.* 4118.
Baker B. R., Kawazu M., Santi D. V. & Schwan T. J. (1967). *J. Med. Chem.* **10**, 304.
Berger G. & Olivier S. C. J. (1927). *Rec. Trav. Chim.* **46**, 600.
Brown D. J. & Short L. N. (1953). *J. Chem. Soc.* 331.
Cheng C. C. & Lewis L. R. (1968). *Synthetic Procedures in Nucleic Acids Chemistry*, vol. 1, p. 109. Interscience Publishers, New York, N.Y.
Doub L., Krolls U., Vandeblet J. M. & Fisher M. W. (1970). *J. Med. Chem.* **13**, 242.
Hayatsu H., Watay a Y. & Kai K. (1970). *J. Am. Chem. Soc.* **92**, 724.
Hilbert G. E. & Johnson T. B. (1930). *J. Am. Chem. Soc.* **52**, 2001.
Isono K. & Suzuki S. (1970). *Tetrahedron Lett.* No. 6, 425.
Kyogoku Y., Lord R. C. & Rich A. (1967). *J. Am. Chem. Soc.* **89**, 496.
Olivier S. C. J. (1929). *Rec. Trav. Chim.* **48**, 568.
Pleiss M., Ochiai H. & Cerutti P. A. (1969). *Biochem. Biophys. Res. Commun.* **34**, 70.
Prystaš M. & Šorm F. (1966). *Coll. Czechoslov. Chem. Commun.* **31**, 1035.
Sato E. & Kanaoka Y. (1969). *Tetrahedron Lett.* No. 40, 334.
Shaw G. (1955). *J. Chem. Soc.* 1834.
Whitehead C. W. (1952). *J. Am. Chem. Soc.* **74**, 4269.

HYDROLIZA 5-CYJANO- I DEKARBOKSYLACJA 5-KARBOKSYURACYLI: PROSTA METODA SYNTEZY 1-CYKLOHEKSYLO POCHODNYCH URACYLU I CYTOZYNY

Streszczenie

1. Opisano stosunkowo prostą metodę otrzymywania 1-cykloheksylouracylu na drodze jednoczesnej hydrolizy i dekarboksylacji grupy cyjanowej 1-cykloheksylo-5-cyjanouracylu w bezwodnym kwasie fosforowym w temperaturze 160 - 170°.

2. Wytłumaczono łatwość — w porównaniu z niereagującym w analogicznych warunkach 6-karboksyuracylem (kwasem orotowym) — dekarboksylacji 5-karboksyuracylu jako wynik tworzenia się wewnątrzcząsteczkowego wiązania wodorowego między grupą 5-karboksylową i grupą karbonylową C_4.

3. Badano w różnych warunkach reakcje hydrolizy i dekarboksylacji.

4. Otrzymany 1-cykloheksylouracyl został użyty jako produkt wyjściowy do przygotowania różnych 1-cykloheksylo pochodnych uracylu i cytozyny, wykorzystywanych w badaniach asocjacji puryn i pirymidyn w rozpuszczalnikach niewodnych, w badaniach reakcji fotochemicznych w rozpuszczalnikach niewodnych, itp.

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RELATIONSHIP BETWEEN SENSITIVITY TO AMYTAL INHIBITION AND THE CONTENT OF NICOTINAMIDE NUCLEOTIDES IN RAT-LIVER MITOCHONDRIA FOLLOWING SOME UNCOUPLING TREATMENTS

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This paper is dedicated to Professor Dr. Irena Chmielewska on the occasion of her 40th year of teaching and research work at the University of Warsaw

1. Sensitivity to amytal as well as the content of nicotinamide nucleotides were studied in the mitochondria treated under uncoupling conditions (by freezing and thawing, incubation with $CaCl_2$, suspension in water) and submitted to ageing in phosphate medium. 2. All the treatments resulted in a decrease of glutamate, β-hydroxybutyrate and proline oxidation. The oxidation was restored on addition of NAD^+. 3. Amytal sensitivity of respiration was markedly decreased in the absence of exogenous NAD^+, whereas in the presence of added NAD^+ low concentrations of amytal inhibited completely the oxidation of glutamate and NADH, but not that of β-hydroxybutyrate and proline. 4. The uncoupling treatments of mitochondria resulted in the release of about 80% of endogenous NAD^+ and decomposition of about 60% of NADPH.

Amytal has long been known to inhibit the NAD-linked oxidation of substrates (Estabrook, 1957; Chance & Hollunger, 1961). It also blocks the electron transport from substrates entering the respiratory chain at the level of ubiquinone, such as: succinate (Chance & Hollunger, 1961), choline (Ernster, Jalling, Löw & Lindberg, 1955; Packer, Estabrook, Singer & Kimura, 1960) and proline (Johnson & Strecker, 1962).

Pumphrey & Redfearn (1963) have shown that some uncoupling treatments of rat-liver mitochondria *i.e.* freezing and thawing the mitochondrial suspension, suspending the mitochondria in water instead of isotonic sucrose, addition of deoxycholate or $CaCl_2$ to the reaction medium, prevented (or reversed) amytal inhibition of succinate oxidation. On the other hand, these treatments were without effect on the susceptibility of NAD-dependent oxidases to amytal. However, Ernster (1956) has shown that oxidation of glutamate was less sensitive to amytal in the presence of $CaCl_2$ than in its absence.

Studies carried out in several laboratories (Ernster, 1956; Hunter, Malison,

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Bridgers, Schutz & Atchison, 1959) have indicated that all the uncoupling treatments mentioned above were associated with a decrease of NAD-linked oxidation. Although these observations have suggested that treatment of the mitochondria with $CaCl_2$ (Ernster, 1956; Greenspan & Purvis, 1965), freezing and thawing (Lusena, 1965) or suspending the mitochondria in water (Caplan & Greenwald, 1966) result in the release of NAD^+ from mitochondria, quantitative data about the content of nicotinamide nucleotides have not been reported.

This paper presents several observations on the effect of various uncoupling treatments on the content of nicotinamide nucleotides in the mitochondria, and on amytal susceptibility of the NAD-dependent oxidation. Some of these results have been reported in preliminary communications (Bryła & Kaniuga, 1967; Bryła, Frąckowiak & Kaniuga, 1967; Frąckowiak, Zajączkowska & Kaniuga, 1968)

MATERIALS AND METHODS

Materials. ADP, NAD^+, NADH and crystalline egg albumin were commercial preparations supplied by Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Phenazine methosulphate was from British Drug Houses (Poole, Dorset, England). Amytal was obtained from Eli Lilly & Co. (Basingstoke, U.S.A.); it was dissolved in 0.1 N-NaOH just before use. Hexokinase (EC 2.7.1.1) from yeast was prepared as described by Darrow & Colowick (1962), omitting the final crystallization step; cytochrome *c* was isolated from beef heart according to Margoliash (1954); alcohol dehydrogenase (EC 1.1.1.1) was prepared from baker yeast by the method of Racker (1955); glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was isolated from beer yeast as described by Kornberg & Horecker (1955).

Mitochondria. Rat-liver mitochondria were isolated in 0.25 M-sucrose as described by Myers & Slater (1957). Freezing of the mitochondrial suspension was carried out overnight at -20°, and thawing at 0°. Water-treated mitochondria were prepared by suspending mitochondria containing 25 - 30 mg. of mitochondrial protein in 20 ml. of water and incubation at 0° for 1.5 hr. NAD-depleted mitochondria were prepared according to Ernster & Navazio (1957).

Measurement of the mitochondrial oxidation. Oxygen uptake was determined polarographically at 25° with Clark electrode according to the procedure of Chappell (1964). The standard reaction medium contained 15 mM-KCl, 2 mM-EDTA, 50 mM-tris-HCl buffer (pH 7.4), 5 mM-$MgCl_2$, 20 mM-sodium phosphate, 0.1 mM-ADP, 60 mM-glucose and 100 - 150 Cori units of yeast hexokinase in a final volume of 1.5 ml. The following concentrations of substrates were used: 1.5 mM-glutamate, 15 mM-L-proline, 15 mM-β-hydroxybutyrate, 9 mM-succinate and 9 mM-NADH.

Respiratory control was assayed in the presence of 2 mM-ADP, both glucose and hexokinase being absent from the reaction medium. When water-treated or frozen and thawed mitochondria were examined, the reaction medium did not contain ADP, glucose and yeast hexokinase. In the experiments in which the effect of $CaCl_2$ (0.2 mM) was assayed, the reaction medium did not contain EDTA and

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$MgCl_2$. Activities of NAD-depleted mitochondria were determined in a mixture containing 10 μM-cytochrome *c* and 0.5 mM-NAD^+.

Extraction and determination of nicotinamide nucleotides. The nicotinamide nucleotides were extracted from the mitochondria according to the method described by Purvis (1960). Reduced forms of the coenzymes were extracted with Na_2CO_3 in a final concentration of 0.1 M at 100°, oxidized forms with trichloroacetic acid in a final concentration of 5%, at room temperature. After being cooled, the extracts were centrifuged for 15 min. at 18 000 *g* at 4°. The supernatants were neutralized in the presence of tris-HCl buffer (pH 7.5), and the amount of nicotinamide nucleotides was determined immediately by the enzymic-polarographic method described by Greenbaum, Clark & McLean (1965).

The oxidized and reduced forms of NAD were estimated in 1.5 ml. of a medium containing: 160 mM-tris-HCl buffer (pH 9.0), 1.2 M-ethanol, 0.06 mM-EDTA, 0.4 mg. of phenazine methosulphate, 0.2 - 0.5 ml. of either acid or alkali extract of mitochondria, and 1500 units of alcohol dehydrogenase.

Oxidized and reduced NADP was estimated in 1.5 ml. of a medium containing: 60 mM-tris-HCl buffer (pH 8.0), 6 M-glucose 6-phosphate, 0.6 mM-EDTA, 0.2 mg. phenazine methosulphate, 0.05 - 0.5 ml. of either acid or alkali extract of mitochondria, and 7.5 units of glucose 6-phosphate dehydrogenase.

In both cases the reaction was started by addition of enzyme solution and the oxygen consumption was recorded. The amount of nicotinamide nucleotides was calculated from the calibration curves obtained for NAD^+ (or NADH) and $NADP^+$ (or NADPH).

Protein content was determined by the biuret method according to Cleland & Slater (1953) using crystalline egg albumin as standard.

RESULTS

Effect of uncoupling treatments on the sensitivity to amytal. In agreement with Pumphrey & Redfearn (1963), the oxidation of succinate by the intact mitochondria was inhibited only by 80% in the presence of 6 mM-amytal, while oxidation of both glutamate and proline was completely blocked by 1 and 1.5 mM-inhibitor, respectively (Fig. 1A). Freezing and thawing the mitochondrial suspension (Fig. 1B), addition of $CaCl_2$ to the reaction medium (Fig. 1C) or suspending the mitochondria in water (Fig. 1D) resulted in a differentiation of sensitivity to amytal of oxidation of the substrates. Similarly as reported by Pumphrey & Redfearn (1963), all these uncoupling treatments abolished completely the response of the succinate oxidation to amytal. On the other hand, proline oxidation in mitochondria treated with $CaCl_2$ or suspended in water was inhibited by only 30% in the presence of 6 mM-amytal, whereas in mitochondria frozen and thawed, by about one-half. Amytal at 6 mM concentration inhibited the glutamate oxidation by about 80% in the mitochondria treated with $CaCl_2$ or water, and only by 30% in the mitochondria frozen and thawed.

Enzymic activity and sensitivity to amytal in the presence of exogenous NAD^+. Ernster (1956) found that preincubation of the mitochondria in the presence of

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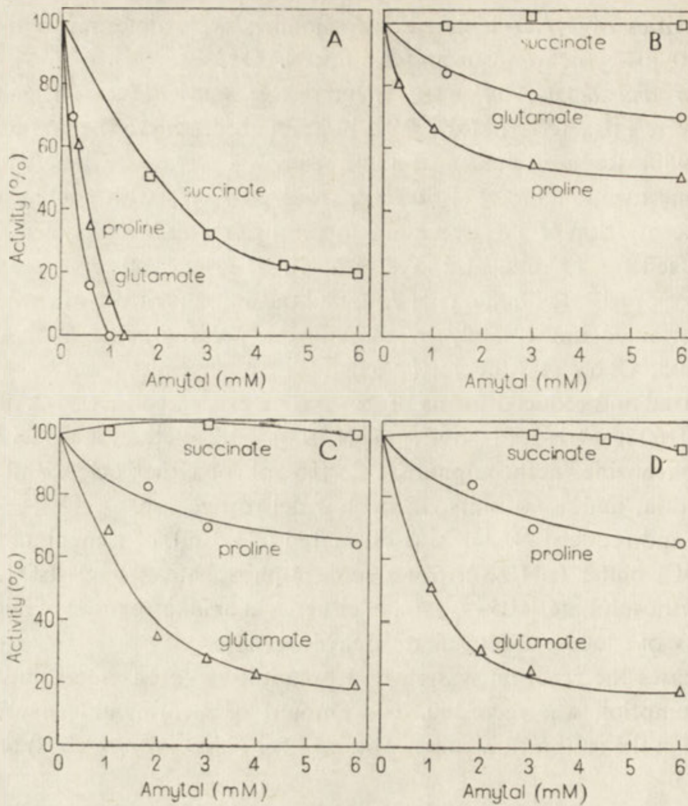

Fig. 1. Effect of uncoupling treatment on the susceptibility to amytal of glutamate, proline and succinate oxidation. *A*, Intact mitochondria (1.6 mg. of protein); *B*, mitochondria frozen and thawed (3.7 mg.); *C*, mitochondria treated with 0.2 mM-$CaCl_2$ (3.1 mg.); *D*, mitochondria suspended in water (4.6 mg.)

Ca^{2+} resulted in a decreased sensitivity of respiration to amytal and antimycin A, tested in the presence of NAD^+ and cytochrome *c* with glutamate as substrate. Since in liver mitochondria the oxidation of exogenous NADH in the presence of added cytochrome *c* is insensitive to amytal and antimycin due to the transfer of electrons by the "external" pathway (Jacobs & Sanadi, 1960), we have studied the susceptibility to amytal in the absence of exogenous cytochrome *c*.

As shown in Table 1, the oxidation of glutamate, β-hydroxybutyrate and proline was decreased in the mitochondria following the uncoupling treatments. The rather high respiratory capacity observed with proline as substrate in the absence of exogenous NAD^+ was probably caused by the fact that the first step of proline oxidation is NAD-independent (Johnson & Strecker, 1962). In agreement with earlier findings (Ernster, 1956; Caplan & Greenwald, 1966), addition of NAD^+ to the medium resulted in a rapid increase of the respiration with glutamate, β-hydroxybutyrate and proline as substrates. Addition of $NADP^+$ instead of NAD^+ was without effect on the glutamate oxidation by mitochondria treated under uncoupling conditions.

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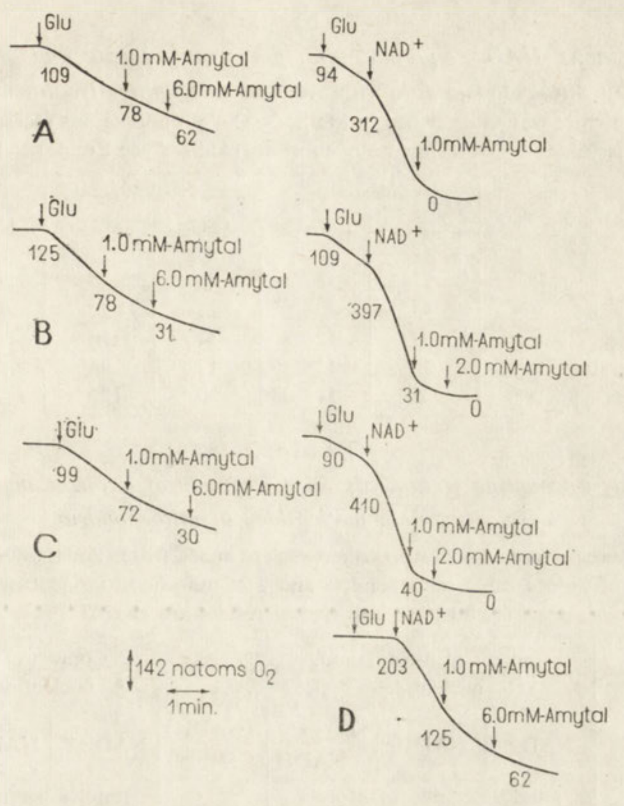

Fig. 2. Effect of exogenous NAD+ on the susceptibility of glutamate oxidation to amytal in the mitochondria following uncoupling treatments. *A*, Mitochondria frozen and thawed; *B*, mitochondria treated with $CaCl_2$; *C*, mitochondria suspended in water; *D*, mitochondria incubated with 50 mM-Na-phosphate for 30 min. In the experiments carried out without added NAD+ (*left*), 3.2, 6.4 and 4.8 mg. of protein were used in *A*, *B* and *C*, respectively. In the experiments carried out in the presence of exogenous NAD+ (*right*), 2.4, 3.2, 3.6 and 1.8 mg. of protein were used in *A*, *B*, *C* and *D*, respectively. In this and subsequent Figures, the numbers on the tracings express the oxygen consumption as natoms per minute.

The sensitivity to amytal of glutamate, β-hydroxybutyrate and proline oxidation by the mitochondria following uncoupling treatments in the absence as well as in the presence of exogenous NAD+ is presented in Figs. 2, 3 and 4. The respiration of the treated mitochondria which, with glutamate as substrate, was not completely inhibited even by 6 mM-amytal, on addition of NAD+ became sensitive again, and 1 or 2 mM-amytal blocked completely the oxidation (Fig. 2A,B,C). However, the respiration of NAD-depleted mitochondria (Fig. 2D) in the presence of exogenous NAD+ was decreased by 6 mM-amytal by only 70%.

The oxidation of β-hydroxybutyrate by mitochondria following uncoupling treatments was blocked by 3 mM-amytal in the absence of exogenous NAD+ but not in its presence (Fig. 3).

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Table 1

Effect of exogenous NAD^+ on glutamate, β-hydroxybutyrate and proline oxidation by mitochondria following various uncoupling treatments

The incubation mixture was as described in Methods. Oxygen uptake was measured with a Clark electrode. The values represent nmoles of substrate oxidized per 1 min. per 1 mg. protein.

Mitochondria	Glutamate		β-Hydroxybutyrate		Proline	
	$-NAD^+$	$+NAD^+$	$-NAD^+$	$+NAD^+$	$-NAD^+$	$+NAD^+$
Intact	57	57	45	45	41	41
Frozen and thawed	35	123	27	72	35	50
$CaCl_2$-treated	20	124	34	110	33	56
Suspended in water	21	126	17	98	21	43
NAD-depleted	6	113	0	129	33	66

Table 2

Effect of various uncoupling treatments on the content of oxidized and reduced forms of nicotinamide nucleotides in mitochondria

Determinations were performed on 5 suspensions each of intact, frozen and thawed or $CaCl_2$-treated mitochondria, 4 of water-treated mitochondria and 2 of mitochondria incubated with phosphate for 30 min. The typical results are given.

Treatment	Content of nicotinamide nucleotides (μmoles per mg. of protein)			$[NAD^+]/[NADH]$ ratio	Content of nicotinamide nucleotide phosphates (μmoles per mg. of protein)		
	NAD^+	NADH	$NAD^+ + NADH$		$NADP^+$	NADPH	$NADP^+ + NADPH$
None	1.60	1.60	3.20	1.0	0.10	2.40	2.50
Freezing and thawing	2.40	0.70	3.10	3.4	0.10	1.50	1.60
None	1.80	1.30	3.10	1.4	0.20	2.70	2.90
0.2 mM-$CaCl_2$	2.90	0.33	3.23	8.8	0.17	0.76	0.93
None	2.57	1.10	3.47	2.3	0.10	3.30	3.40
Water	3.47	0.10	3.57	34.7	0.10	0.60	0.70
None	2.00	1.10	3.10	1.8	0.10	3.20	3.30
50 mM-Phosphate	1.00	0.10	1.10	—	0.00	0.40	0.40

Table 3

Relationship between respiratory control index and $[NAD^+]/[NADH]$ ratio in the intact mitochondria

Expt. no.	Respiratory control with		Content of nucleotides (nmoles per mg. protein)		$[NAD^+]/[NADH]$ ratio
	glutamate	β-hydroxy-butyrate	NAD^+	NADH	
1	7.0	6.1	1.75	1.60	1.1
2	15.0	10.0	2.10	1.80	1.2
3	3.5	2.9	2.73	0.30	9.1
4	4.0	3.0	2.45	0.66	3.7

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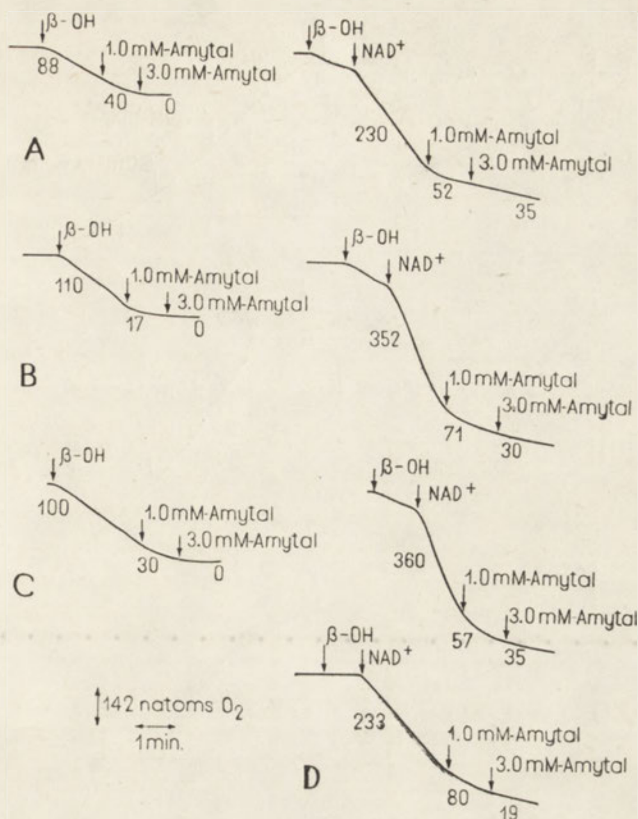

Fig. 3. Effect of exogenous NAD^+ on the sensitivity of β-hydroxybutyrate oxidation to amytal in the mitochondria following uncoupling treatments. *A*, *B*, *C* and *D*, as in Fig. 2. In the experiments carried out without added NAD^+ (*left*), 3.2, 3.2 and 6 mg. of protein were used in *A*, *B* and *C*, respectively; in the experiments carried out in the presence of exogenous NAD^+ (*right*), 3.2, 3.2, 3.6 and 1.9 mg. of protein were used in *A*, *B*, *C* and *D*, respectively.

Quite different results were obtained with proline as substrate (Fig. 4). The oxidation of proline [the second step of which is NAD-dependent (Strecker, 1960)] was completely blocked by 1.5 mM-amytal in intact mitochondria, whereas in the treated ones it was hardly affected, either in the absence or presence of exogenous NAD^+. Similarly, added NAD^+ had no effect on the degree of inhibition by 6 mM-amytal.

Since following uncoupling treatments of the mitochondria, susceptibility to amytal of the glutamate oxidation differed from that of β-hydroxybutyrate oxidation, we have studied the amytal sensitivity of NADH oxidation. It was found that 2 mM-amytal inhibited completely the oxidation of exogenous NADH by the treated mitochondria (Fig. 5A,B,C). On the other hand, in NAD-depleted mitochondria 6 mM-amytal did not cause complete inhibition of NADH oxidation.

Effect of uncoupling treatments on the content of nicotinamide nucleotides. The phenomenon of release of NAD from the mitochondria due to the incubation in a phosphate-containing medium has been studied rather extensively by Hunter

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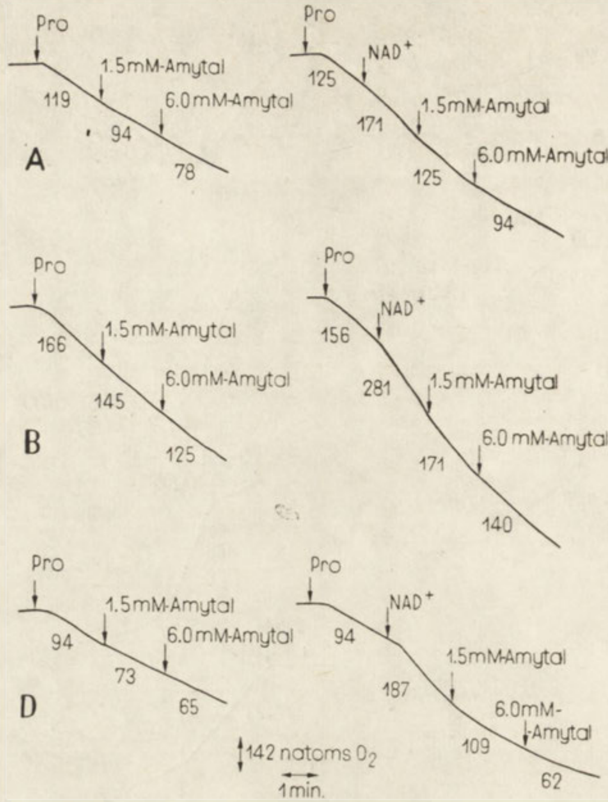

Fig. 4. Effect of exogenous NAD+ on the sensitivity to amytal of proline oxidation. *A*, *B* and *D*, as in Fig. 2; the amount of protein used being, respectively, 3.4, 5.0 and 2.8 mg.

et al. (1959), Ernster (1956, 1967) and McLennan & Tzagoloff (1966). In Table 2 the content of nicotinamide nucleotides in the mitochondria treated under uncoupling conditions and in mitochondria depleted of NAD by incubation with phosphate is presented. The treated mitochondria lost less of their nicotinamide nucleotides than those aged in the presence of phosphate, in which most of the reduced as well as the oxidized forms were decomposed. It was found that in mitochondrial suspensions which were frozen and thawed, treated with $CaCl_2$ or treated with water, the NAD^++NADH content was the same as in the suspensions of intact mitochondria. However, more NAD^+ than NADH was present. On the other hand, the $NADP^+$+NADPH content was much lower in comparison with that of the suspension of intact mitochondria, about 40 - 80% being decomposed.

The [NAD^+]/[NADH] ratio, which was 1.0 - 2.3 for the intact mitochondria, was much increased (usually 5 - 10 times) for the mitochondrial suspension treated under uncoupling conditions. The [NAD^+]/[NADH] ratio seemed to be dependent on the integrity of the fresh mitochondria. As shown in Table 3, this ratio was equal to 1.1 - 1.2 only for the tightly coupled mitochondria which have rather high respiratory control index, as tested with both glutamate and β-hydroxybutyrate. On the

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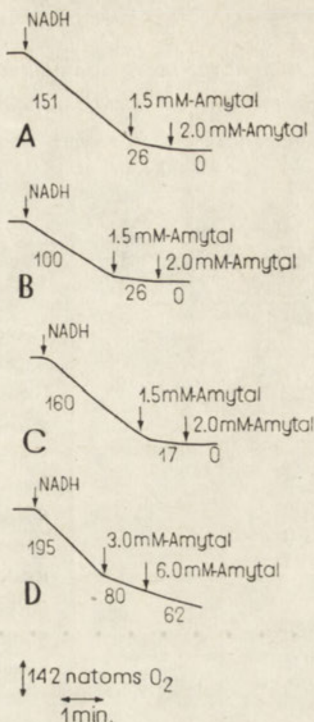

Fig. 5. Amytal sensitivity of NADH oxidation. *A*, *B*, *C* and *D*, as in Fig. 2, the amount of protein used being, respectively, 1.4, 0.8, 1.6 and 2.9 mg.

contrary, the [NAD^+]/[NADH] ratio was much higher (3.7; 9.1) for the loosely coupled mitochondria with low respiratory control index.

The effect of various uncoupling treatments on the distribution of the nicotinamide nucleotides in the fractions obtained following centrifugation of the mitochondrial suspension, is presented in Table 4. It can be seen that after centrifugation of the suspension of intact mitochondria, the oxidized as well as the reduced forms of nicotinamide nucleotides remained in the residue. On the other hand, following uncoupling treatment the main part of NAD^+ (about 80%) was released to the supernatant, while NADH and NADPH were retained in the residue, and $NADP^+$ was decomposed.

DISCUSSION

The content of NAD^++NADH determined in the mitochondrial suspension after freezing and thawing, treatment with $CaCl_2$ or water was found to be the same as in intact mitochondria, but the amount of NAD^+ was markedly higher than that of NADH. All these treatments did not result in decomposition of NAD while, in agreement with the results of Hunter *et al.* (1959) and McLennan & Tzagoloff (1966), the incubation of mitochondria in the phosphate-containing medium did. In tightly coupled mitochondria the [NAD^+]/[NADH] ratio was low, but it became much higher in loosely coupled mitochondria as well as in mitochondria treated under uncoupling conditions (Tables 3 and 2). This higher ratio was of the same order

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Table 4

Distribution of the nicotinamide nucleotides in the fractions obtained after centrifugation of the mitochondrial suspension following various uncoupling treatments

Supernatant and residue fractions were obtained after centrifugation of the mitochondrial suspension for 30 min. at 18 000 *g* in the presence of 50 mM-nicotinamide.

Mitochondria (mg. of protein/3 ml.)	Fraction	Content of nicotinamide nucleotides (nmoles/sample)			
		NAD^+	NADH	$NADP^+$	NADPH
Intact (29.1)	Suspension	65	50	14	70
	Supernatant	4	0	0	1
	Residue	60	49	12	68
Frozen and thawed (31.2)	Suspension	98	17	0	12
	Supernatant	80	8	0	0
	Residue	20	10	0	10
$CaCl_2$-treated (41.1)	Suspension	160	8	0	30
	Supernatant	140	0	0	0
	Residue	19	8	0	30
Suspended in water (24.0)	Suspension	76	0	0	13
	Supernatant	61	0	0	0
	Residue	16	0	0	12

of magnitude as that calculated by Krebs & Veech (1969) from the content of free NAD^+ and NADH.

The $NADP^+$+NADPH content in mitochondria following treatment under uncoupling conditions or incubation in the phosphate-containing medium was markedly decreased due to decomposition of NADPH. The breakdown of NADPH seems not to be mediated by NAD(P) glycohydrolase since the mitochondria do not show any activity of that enzyme (Waravdekar & Griffin, 1964). Our observations are different from those of Slater (1967) who found that freezing of intact rat liver has no effect on the amount of $NADP^+$+NADPH, only about 30% of NADPH is converted to $NADP^+$.

Krebs & Veech (1969) postulate that β-hydroxybutyrate dehydrogenase (located in the cristae) and glutamate dehydrogenase (located in the matrix) share a common pool of the nicotinamide nucleotides. However, in our experiments on the mitochondria treated under uncoupling conditions, the amytal sensitivity of β-hydroxybutyrate oxidation was quite different from that of glutamate oxidation, both in the presence and absence of exogenous NAD^+ (see Figs. 2 and 3). β-Hydroxybutyrate dehydrogenase was inhibited by amytal in the absence of exogenous NAD^+, while the glutamate system was less sensitive in the absence of exogenous NAD^+ and fully sensitive after its addition. The different behaviour of the two systems toward amytal may have been due to differences in the release of β-hydroxybutyrate and glutamate dehydrogenases as postulated by Lusena (1965) for frozen and thawed mitochondria. However, the differences in the accessibility of the two dehydrogenases to exogenous NAD^+ cannot be excluded. Amytal sensitivity of

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oxidation of the exogenous NADH was similar to that of glutamate oxidation in the presence of added NAD^+.

All the studied uncoupling treatments resulted in the loss of structural integrity of mitochondria, as indicated by the release of nicotinamide nucleotides, by the decreased sensitivity to amytal, as well as by oxidation of glutamate only in the presence of NAD^+ but not of $NADP^+$. The latter observation is in agreement with Klingenberg & Schollmeyer (1963) and Tager & Papa (1965) who demonstrated that glutamate dehydrogenase is specific for $NADP^+$ only within the intact mitochondria.

The results concerning the proline oxidation seem to be of interest. The markedly decreased sensitivity to amytal in the mitochondria following uncoupling treatments, in the presence of exogenous NAD^+, seems to indicate that the two steps of proline oxidation are not so tightly coupled in the treated mitochondria as in the intact ones.

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REFERENCES

Bryła J. & Kaniuga Z. (1967). *4th FEBS Meet., Oslo. Abstr. of Commun.* p. 86, no. 344.

Bryła J., Frąckowiak B. & Kaniuga Z. (1967). *5th Meet. Polish Biochem. Soc., Kraków. Abstr. of Commun.* p. 62, no. C 43.

Caplan A. & Greenwald J. (1966). *J. Cell Biol.* **31**, 455.

Chance B. & Hollunger G. (1961). *Fed. Proc.* **20**, 50.

Chappell J. B. (1964). *Biochem. J.* **90**, 225.

Cleland K. W. & Slater E. C. (1953). *Biochem. J.* **53**, 547.

Darrow R. A. & Colowick S. P. (1962). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 5, p. 226. Academic Press, New York.

Ernster L. (1956). *Exp. Cell Res.* **10**, 704, 721.

Ernster L. (1967). In *Mitochondrial Structure and Compartmentation* (E. Quagliariello, S. Papa, E. C. Slater & J. M. Tager, eds.) p. 341. Adriatica Editrice, Bari.

Ernster L., Jalling L., Löw H. & Lindberg O. (1955). *Exp. Cell. Res. Suppl.* **3**, 124.

Ernster L. & Navazio F. (1957). *Biochim. Biophys. Acta* **26**, 408.

Estabrook R. W. (1957). *J. Biol. Chem.* **227**, 1093.

Frąckowiak B., Zajączkowska M. & Kaniuga Z. (1968). *6th Meet. Polish Biochem. Soc. Olsztyn, Abstr. of Commun.* p. 113.

Greenbaum A. L., Clark J. B. & McLean P. (1965). *Biochem. J.* **95**, 161.

Greenspan M. D. & Purvis J. L. (1965). *Biochim. Biophys. Acta* **99**, 167, 191.

Hunter F. E., Malison R., Bridgers W. F., Schutz B. & Atchison A. (1959). *J. Biol. Chem.* **234**, 693.

Jacobs E. E. & Sanadi D. R. (1960). *Biochim. Biophys. Acta* **38**, 12.

Johnson A. B. & Strecker H. J. (1962). *J. Biol. Chem.* **237**, 1876.

Klingenberg M. & Schollmeyer P. (1963). In *Proc. 5th Intr. Congr. Biochem. Moscow*, 1961. *Intracellular Respiration: Phosphorylating and Non-Phosphorylating Oxidation Reactions* (E. C. Slater, ed.) vol. 5, p. 46. Pergamon Press, Oxford.

http://rcin.org.pl

Kornberg A. & Horecker B. L. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 323. Academic Press, New York.
Krebs H. A. & Veech R. L. (1969). In *Energy Level and Metabolic Control in Mitochondria* (S. Papa, J. M. Tager, E. Quagliariello & E. C. Slater, eds.) p. 329. Adriatica Editrice, Bari.
Lusena C. V. (1965). *Can. J. Biochem.* **43**, 1787.
McLennan D. H. & Tzagoloff A. (1966). *J. Biol. Chem.* **241**, 1933.
Margoliash E. (1954). *Biochem. J.* **56**, 529, 535.
Myers D. K. & Slater E. C. (1957). *Biochem. J.* **67**, 558.
Packer L., Estabrook R. W., Singer T. P. & Kimura T. (1960). *J. Biol. Chem.* **235**, 535.
Pumphrey A. M. & Redfearn E. R. (1963). *Biochim. Biophys. Acta* **74**, 317.
Purvis J. L. (1960). *Biochim. Biophys. Acta* **38**, 435.
Racker E. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 500. Academic Press, New York.
Slater T. F. (1967). *Biochem. J.* **104**, 833.
Strecker H. J. (1960). *J. Biol. Chem.* **235**, 3218.
Tager J. M. & Papa S. (1965). *Biochim. Biophys. Acta* **99**, 570.
Waravdekar V. S. & Griffin C. C. (1964). *Exp. Cell Res.* **103**, 514.

ZWIĄZEK MIĘDZY WRAŻLIWOŚCIĄ NA INHIBICJĘ AMYTALEM A ZAWARTOŚCIĄ NUKLEOTYDÓW NIKOTYNAMIDO-ADENINOWYCH W MITOCHONDRIACH WĄTROBY SZCZURA PODDANYCH DZIAŁANIU CZYNNIKÓW ROZPRZĘGAJĄCYCH

Streszczenie

1. Badano wrażliwość na Amytal oraz zawartość nukleotydów nikotynamido-adeninowych w mitochondriach wątroby szczura po zamrożeniu i odtajaniu, inkubowanych z $CaCl_2$, zawieszanych w wodzie oraz inkubowanych w środowisku zawierającym fosforan.

2. Wymienione warunki powodowały spadek szybkości utleniania glutaminianu, β-hydroksymaślanu i proliny. Przywracanie utleniania obserwowano po dodaniu egzogennego NAD^+.

3. Wrażliwość oddychania na Amytal znacznie malała, jeśli mieszanina reakcyjna nie zawierała NAD^+.

4. W obecności dodanego NAD^+, niskie stężenia Amytalu hamowały całkowicie utlenianie glutaminianu i NADH, natomiast nie hamowały utleniania β-hydroksymaślanu i proliny.

5. Poddawanie mitochondriów działaniu czynników rozprzęgających powodowało uwalnianie około 80% endogennego NAD^+ oraz rozpad około 60% NADPH.

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SOME PROPERTIES OF PURIFIED PORCINE PANCREATOPEPTIDASE E

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1. Porcine pancreatopeptidase E was purified 700-fold from a commercial pancreas powder. 2. Molecular weight of the purified preparation (21 000) as well as K_m (0.95 mg./ml.) and the dependence of the velocity of elastolysis on the kind of buffer, pH, and ionic strength, were studied. 3. Activity of the enzyme towards four different substrates was assayed. Inhibition of these reactions by soya bean trypsin inhibitor and normal human serum was investigated. The results do not support the hypothesis on the multi-center character of the enzyme.

Pancreatopeptidase E, called also "elastoproteinase" (EC 3.4.4.7), is the main component of the "elastase complex" of pancreas. Even the purest preparations of this enzyme exhibit a general proteolytic activity in addition to the activity towards elastin. It has been postulated that these two functions of the enzyme are related to different catalytic sites. This hypothesis was based on the observations of a selective inhibition by soya bean trypsin inhibitor (SBTI)[1] (Walford & Kickhöfen, 1962) and Trasylol (Solyom & Tolnay, 1965) of casein and haemoglobin degradation catalysed by elastoproteinase. These inhibitors were practically without effect on the elastolytic activity of the enzyme. On the other hand, the latter function could be selectively inhibited by high ionic strength (Walford & Kickhöfen, 1962). However, the possibility that the elastase preparations used may have been contaminated with other pancreatic proteases, was not excluded. Therefore, the aim of the present work was to purify pancreatopeptidase E from crude elastase and to study the homogeneity and some properties of the resulting preparation, mainly to test the hypothesis of the double-center character of this enzyme.

MATERIALS AND METHODS

Elastin. This was obtained according to the method of Partridge, Davis & Adair (1955) from ox nuchal ligament. A final preparation was ground mechanically and the fraction below 0.1 mm. in diameter was collected.

[1] Abbreviations used: SBTI, soya bean trypsin inhibitor; DIP-, diisopropoxy-; EU, elastolytic unit; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; DFP, diisopropylfluorophosphate.

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Pancreatopeptidase E. Crude enzyme preparation was obtained from a commercial porcine pancreatin according to the method of Hall & Czerkawski (1959) as modified by Loeven (1960). The obtained preparation represented a material that has precipitated in the course of exhaustive dialysis against water of acid pancreatin extract. It is also called "euglobulins" of pancreas or AEI, following the terminology of Hall & Czerkawski (1959).

Crude elastase was further purified by means of DEAE-Sephadex chromatography, according to the method of Loeven (1963), except that carbonate buffer of ionic strength (I) of 0.2 instead of 0.13 was used. Fractions with elastolytic activity were combined and acidified to pH 5 - 6 with 2 M-hydrochloric acid. The enzyme solution was then exhaustively dialysed against water and freeze-dried.

Another elastase preparation was obtained according to the method of Bagdy & Banga (1957, 1958).

Paper electrophoresis. This was performed in 0.05 M-veronal buffer of pH 8.6 on Whatman no. 1 paper (4×15 cm.); 0.04 ml. of 1% enzyme solution being applied on a strip. Electrophoresis was performed for 5 hr. using 16 V/cm. The electrophoretograms were stained with 0.2% Amido Black 10B.

Starch gel electrophoresis. Vertical electrophoresis, essentially according to Smithies (1959) was employed. 20% gels were made in 40% urea acidified to pH 3.8 with concentrated formic acid. Gels were poured into apparatus of dimensions: $25 \times 13 \times 0.6$ cm. and 0.1 ml. samples of 1 - 3% enzyme solutions were applied. Electrophoresis was carried out for 20 hr. with 0.3 M-ammonium formate buffer, pH 3.8, as an electrode solution, using 4 V/cm. Gels were stained with 0.5% Amido Black 10B.

Determination of molecular weight. Approximate molecular weight of diisopropoxy--derivative of purified pancreatopeptidase E (DIP-enzyme) was established with the use of molecular sieving in Sephadex G-100 gel. Cytochrome *c*, DIP-trypsin, egg albumin, and ox serum albumin were used as standards, assuming the molecular weights of 12 500, 24 000, 45 000 and 69 000, respectively. Fractions of 1 ml. were collected at a flow rate of 0.4 ml./min. Molecular weight of DIP-pancreatopeptidase E was calculated from the plot of elution volumes of the standard proteins against logarithms of their molecular weights. DIP-enzymes were prepared in the following manner: 10 mg. sample of the enzyme was dissolved in 1.0 ml. of the solution containing 0.9 ml. of the carbonate buffer, pH 8.7, and 0.1 ml. of 10 mM-diisopropylfluorophosphate in isopropanol. This mixture was incubated for 10 min. and then introduced into the column.

Determination of elastolytic activity. This was carried out by the two methods described previously. The first (Ardelt, Księżny & Niedźwiecka-Namysłowska, 1970) is based on the spectrophotometric determination at 276 nm of desmosine, isodesmosine and tyrosine among the soluble elastolysis products. In the second method (Banga & Ardelt, 1967), an elastin preparation stained with resorcin fuchsin is used as a substrate, and the dye released into solution together with elastin degradation products is determined colorimetrically.

The activity is expressed in elastolytic units (EU), one unit being defined as

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the amount of enzyme that releases 1 mg. of soluble elastolysis products during 30 min. under the conditions described. Specific activity is expressed as EU/mg. of enzyme preparation.

Determination of the activity towards casein. The substrate solution was made in the following manner: 3 g. sample of casein was suspended in 80 ml. of borate buffer of pH 8.7, *I* 0.12, and mixed mechanically for 10 min. The pH was then adjusted to 8.7 with 2 M-sodium hydroxide. The mixture was heated in a boiling water bath for 10 min., pH was corrected again and the buffer was added to make the final volume 100 ml.

For the assay, to 1 ml. of casein solution 1 ml. of the appropriate buffer containing 2 - 7.5 μg. of pancreatopeptidase E (or trypsin) was added, and the mixture was incubated at 37° for 30 min., then 3 ml. of 15% trichloroacetic acid was added. After 30 min. the samples were centrifuged at 10 000 *g*, and the extinction at 280 nm was read in the supernatants. The "zero-time" sample was used as a blank. The results are expressed in terms of caseinolytic units (Cas. U) per 1 mg. of the enzyme preparation. One Cas. U. is defined as the amount of the enzyme that releases trichloroacetic acid-soluble products giving the increase of extinction at 280 nm of 1.0 per minute.

Determination of esterase activity. Determination of the activity towards *N*-*α*-benzoyl-L-arginine ethyl ester (BAEE) was carried out according to the method of Schwert & Takenaka (1955) as modified by Rick (1963), except that the substrate concentration of 0.83 mM, instead of 1.0 mM was used.

The activity towards *N*-acetyl-L-tyrosine ethyl ester (ATEE) was measured according to slightly modified method of Schwert & Takenaka (1955). Instead of phosphate buffer the 0.05 M-tris-HCl buffer of pH 7.0, containing 0.02 M-calcium chloride was employed, and 1.5 M-substrate solution was used. The activity is expressed arbitrarily as the relationship between the change of extinction at 237 nm and the enzyme concentration in mg. per sample.

Extinction coefficient. The purified preparation of pancreatopeptidase E was dissolved in 0.12 mM-hydrochloric acid; concentration of the enzyme solution was determined spectrophotometrically at 282 nm using the extinction coefficient of 16.0 for 1% solution at 1 cm. light path. For trypsin the extinction coefficient at 280 nm was taken as 17.1 (Kunitz, 1947), and for *α*-chymotrypsin 20.0 (Laskowski, 1955).

Inhibition. The inhibition by SBTI was investigated at a weight ratio enzyme: inhibitor of 1:2. The enzymes were preincubated with SBTI in appropriate buffer for 10 min. at room temperature, then the activity was determined.

Special reagents. Porcine pancreatin was a commercial preparation manufactured by Polfa (Warszawa, Poland). Potato starch "Superior" was a Polish commercial product. DEAE-Sephadex A-50 and Sephadex G-100 (Pharmacia, Uppsala, Sweden); elastase (Mann Research Lab., New York, U.S.A., and Sigma, St. Louis, Mo., U.S.A.); Amido Black 10B (G. T. Gurr, London, England); *α*-chymotrypsin, 4 times crystallized (British Drug Houses, Poole, England); *N*-*α*-benzoyl-L-arginine ethyl ester hydrochloride, cytochrome *c* and diisopropylfluorophosphate (Koch-Light,

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Colnbrook, Bucks., England); casein, Hammersten (E. Merck, Darmstadt, West Germany); tris, purum, and crystallized serum albumin (Fluka A. G., Buchs, Switzerland); trypsin, crystalline (Spofa, Prague, Czechoslovakia); soya bean trypsin inhibitor, crystalline (Worthington Biochem. Corp., Freehold, N. J., U.S.A.).

RESULTS AND DISCUSSION

Figure 1 shows the elution diagram of DEAE-Sephadex chromatography of the crude elastase preparation. Elastolytic activity appeared in the first peak, well separated from the others. The active protein separated from the chromatogram, represented the final pancreatopeptidase E preparation. This migrated as a single

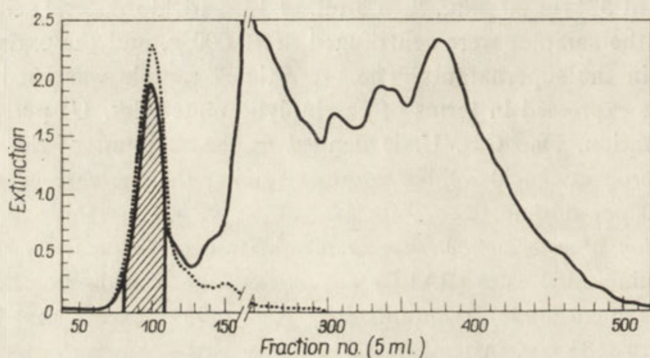

Fig. 1. DEAE-Sephadex chromatography of crude elastase. To the column (4×40 cm.) equilibrated with carbonate buffer of pH 8.7 and *I* 0.2, 3.0 g. of protein (20 ml.) was applied and eluted with the buffer. The flow rate was 0.5 ml./min. (——), Protein, E at 282 nm; (·····), elastolytic activity, E at 560 nm. Active samples were separated as marked on the Figure.

band in paper electrophoresis at pH 8.6, but was inhomogeneous in urea-formate starch gel electrophoresis at pH 3.8. Figure 2 shows the electrophoretic patterns in starch gel of our preparation as well as of several other elastase preparations, crystalline trypsin and four times crystallized *a*-chymotrypsin. All the preparations tested were inhomogeneous under the conditions employed. Elastase preparations and trypsin were contaminated, among others, by a protein migrating to the cathode much faster than the main band. Our preparation E_{20} was contaminated with this protein only, whereas commercial elastases E_M and E_S as well as the preparation E_3 obtained according to the method of Bagdy & Banga (1957, 1958), represented a much lower degree of purity. Our preparation seems to be not contaminated by other enzymes of the "elastase complex", which were found to exhibit distinctly different electrophoretic mobility (Loeven, 1964).

The purification of pancreatopeptidase E from commercial pancreatin is presented in Table 1; a 700-fold purification was achieved. The activity of the final product determined with the use of resorcin fuchsin-elastin as substrate, was 71.7 EU/mg.; the activity determined by the spectrophotometric method was higher and amounted to 97.5 EU/mg. It should be noted that the change of ionic strength

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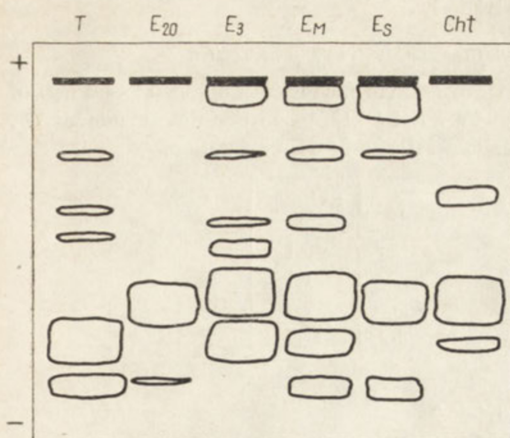

Fig. 2

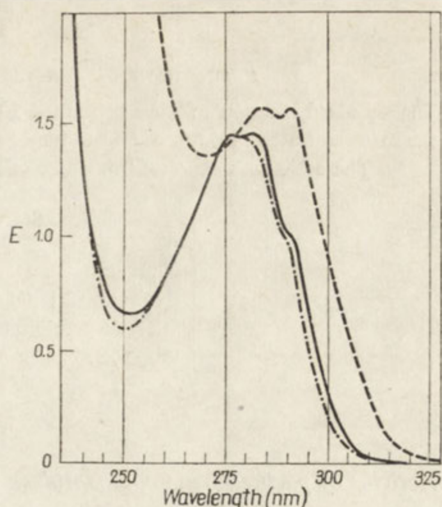

Fig. 3

Fig. 2. Urea-formate starch gel electrophoresis of T, crystalline trypsin; E_{20}, pancreatopeptidase E purified as described under Materials and Methods; E_3, elastase obtained according to the method of Bagdy & Banga (1957, 1958); E_M, elastase preparation purchased from Mann; E_S, elastase purchased from Sigma; Cht, four times crystallized α-chymotrypsin.

Fig. 3. Ultraviolet absorption spectra of purified pancreatopeptidase E (967 μg./ml.) in (——), borate buffer, pH 8.7, *I* 0.12; (- - -), in 0.2 M-sodium hydroxide; (-·-·), in 0.05 M-hydrochloric acid.

of the buffer used for DEAE-Sephadex chromatography permitted to obtain a preparation of higher homogeneity and higher activity.

Ultraviolet spectra of the purified pancreatopeptidase E preparation are presented in Fig. 3. In the spectrum recorded in borate buffer of pH 8.7 and *I* 0.12, the maxima at 277 and 282 nm as well as the shoulder at 291 - 292 nm are visible. The extinction coefficient at 282 nm ($E^{1\%}_{1\,cm.}$) of the purified enzyme, amounted to 16.0. In 0.05 M-hydrochloric acid, the single peak at 278 nm could only be observed. The spectrum in 0.2 M-sodium hydroxide shows two peaks at 284 and 291 nm. Distinct red shift in this spectrum can be due to a considerable contribution of tyrosyl residues which ionize in strong alkaline medium.

By gel filtration on Sephadex G-100, molecular weight of 21 000 was estimated for the purified pancreatopeptidase E treated previously with DFP to prevent autolysis (Fig. 4.). DIP-pancreatopeptidase E was active neither towards elastin nor towards casein, BAEE or ATEE.

The purpose of the next three experiments was to establish the optimum conditions for elastolytic activity of the purified enzyme preparation. The highest activity was found in carbonate buffer (Table 2). The activity with other buffers decreased in the following order: borate, tris-HCl, glycine-NaOH, veronal-acetate. In carbonate buffer, however, the pH of the reaction mixture could not be maintained during 30 min. of incubation. Therefore borate buffer was used.

The optimum enzyme activity was found to be at pH 8.7 (Fig. 5A). A well known inhibiting effect of the increasing ionic strength on the elastolytic activity of the

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Table 1

Purification of pancreatopeptidase E from pancreatin

The standard reaction mixture contained 30 mg. of resorcin fuchsin-elastin and 0.1 - 4.0 mg. of the material tested in 6 ml. of borate buffer of pH 8.7 and *I* 0.12. Incubation time 30 min. at 37°. The activity is expressed in elastolytic units per 1 mg. of the preparation.

Step	EU/mg.
Pancreatin	~0.1
Extraction at pH 4.7	1.2
Dialysis precipitation	9.7
DEAE-Sephadex chromatography	71.7

Table 2

Activity of purified pancreatopeptidase E determined in various buffers of pH *8.7 and* I *0.12*

The standard incubation mixture contained 30 mg. of the substrate and 50 µg. of the enzyme in 6 ml. of the appropriate buffer. Incubation time 30 min. at 37°.

Buffer	EU/mg.	
	with unstained substrate	with resorcin fuchsin-elastin
Carbonate (Na_2CO_3-HCl)	109.7	84.4
Borate ($Na_2B_4O_7$-H_3BO_3)	97.5	71.7
Tris-HCl	90.9	65.2
Glycine-NaOH	79.7	55.5
Veronal-acetate-HCl	—	12.4

Table 3

Activities of two different elastase preparations and trypsin, determined with elastin and casein as substrates

E_{20}, pancreatopeptidase E purified as described under Materials and Methods; E_3, elastase obtained according to the method of Bagdy & Banga (1957, 1958); T, crystalline trypsin. The reaction mixture for the determination of elastolytic activity contained 50 µg. of the enzyme preparation and 30 mg. of unstained elastin in 6 ml. of borate buffer, pH 8.7 and *I* 0.12. The incubation mixture for the determination of activity towards casein contained 2 - 7.5 µg. of the enzyme preparation and 30 mg. of casein in 2 ml. of borate buffer of pH 8.7. Determination of inhibition by SBTI was performed as described under Materials and Methods. Incubation time 30 min. at 37°.

Enzyme	Activity towards elastin		Activity towards casein	
	EU/mg.	Inhibition by SBTI (%)	Cas. U/mg.	Inhibition by SBTI (%)
E_{20}	97.5	9.2	1.3	8.2
E_3	34.2	28.0	0.9	44.0
T	0.8	—	0.6	96.2

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enzyme was confirmed (Fig. 5B). The maximum activity was observed at the ionic strength 0.12. In the buffer solutions of ionic strength below this value (broken line on the Figure), the pH values of the incubation mixtures were unstable in the course of the reaction. An inhibition in this region was therefore due to acidification of the media during the enzyme action.

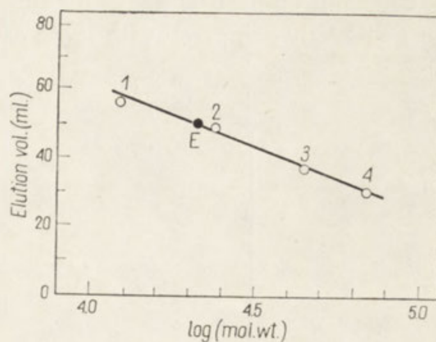

Fig. 4. Determination of molecular weight by Sephadex G-100 gel filtration. The column (1.7 × 30 cm.) was equilibrated with 0.05 M-sodium carbonate buffer, pH 8.7. Standard proteins, 10 mg. each (1 ml.): *1*, cytochrome *c*; *2*, diisopropoxy- derivative of trypsin; *3*, egg albumin; *4*, ox serum albumin. *E*, diisopropoxy-derivative of purified pancreatopeptidase E. Elution volumes were assayed by protein determination at 282 nm.

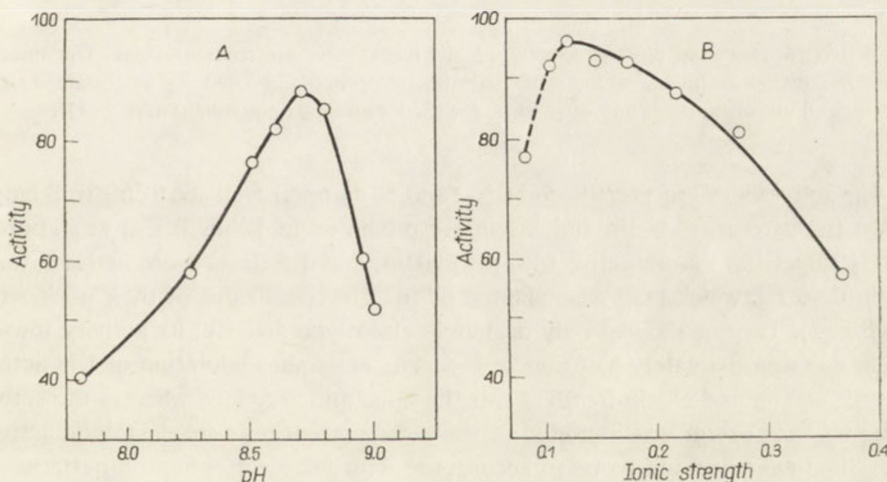

Fig. 5. The effect of: *A*, pH and *B*, ionic strength on the elastolytic activity of purified pancreatopeptidase E. The incubation mixture contained 50 µg. of the enzyme preparation and 30 mg. of unstained elastin in 6 ml. of borate buffer, in *A* of *I* 0.2, and in *B*, of pH 8.7. Incubation time 30 min. at 37°.

On the basis of these results, borate buffer of pH 8.7 and ionic strength 0.12 was chosen for further experiments. Under these conditions, the relationship between velocity of elastolysis and enzyme concentration was found to be linear up to 20 µg./ml. Time-course was linear until 80% of the substrate was taken into solution.

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Lineweaver-Burk plot of velocity against the initial concentration of elastin is presented in Fig. 6. From this plot the apparent Michaelis-Menten constant of 0.95 mg./ml. was calculated. It is expressed in mg./ml. because of insolubility of the substrate and therefore its unknown molecular weight. Another consequence of insolubility of elastin is the dependence of the results of specific activity determinations and values of the kinetic constants on particle size of the substrate preparation used. Therefore, these can not be directly compared with those of other authors.

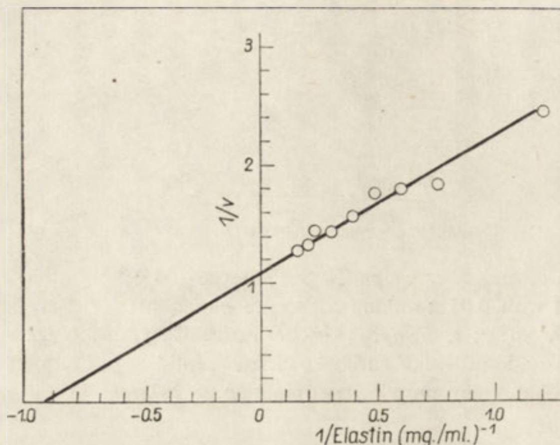

Fig. 6. Double-reciprocal plot of velocity against initial substrate concentrations. The standard incubation mixture contained 50 µg. of the enzyme preparation and 5 - 40 mg. of unstained elastin in 6 ml. of borate buffer of pH 8.7, *I* 0.12. *V* represents mg./ml./30 min. at 37°.

The activities of our preparation (E_{20}) and of that prepared according to Bagdy & Banga (E_3), towards elastin and casein are presented in Table 3. E_{20} was approximately three times more active towards elastin, and 1.5 times more active towards casein than E_3, which may be explained by the different degree of their purification (cf. Fig. 2). Trypsin showed only negligible elastolytic activity; its activity towards casein was approximately half that of E_{20}. The maximum inhibition of E_{20} activity towards elastin and casein by SBTI was the same and very low, whereas the activity of E_3 towards casein was inhibited to the higher extent than its elastolytic activity. These findings appeared to be in accordance with the electrophoretic patterns and supported the supposition that E_3 was highly contaminated with trypsin. To test this supposition, the activity of both elastase preparations towards the specific substrate for trypsin (BAEE) has been determined and compared with that of crystalline trypsin (Table 4). E_{20} had only negligible activity, whereas the activity of E_3 was relatively high and corresponded to 40% contamination with trypsin. Moreover, the esterase activity of both elastase preparations, like that of trypsin, was nearly completely inhibited by SBTI. Therefore it seems reasonable to ascribe this activity of the elastases to contaminating trypsin. The amount of this enzyme in E_{20} was very small and could not be demonstrated by electrophoresis.

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Table 4

Activity of two different elastase preparations and of crystalline trypsin towards N-α-benzoyl-L-arginine ethyl ester

Enzyme preparations as in Table 3. The standard reaction mixture contained 2.49 μmoles of the substrate and 150 μg. of E_{20} (or 50 μg. of E_3, or 15 μg. of T) in 3.2 ml. of 0.05 M-tris-HCl buffer containing 0.02 M-calcium chloride. Readings were made every 15 sec. at 25°.

Enzyme	Activity (μmoles/ /min./mg.)	% of trypsin activity	Inhibition by SBTI (%)
T	31.3	100.0	98.5
E_{20}	0.2	0.6	97.0
E_3	12.4	39.6	98.0

Table 5

Activity of two different elastase preparations and of α-chymotrypsin towards N-acetyl-L-tyrosine ethyl ester

Elastase preparations as in Table 3; Cht, α-chymotrypsin. The reaction mixture contained 4.5 μmoles of the substrate and 100 μg. of E_{20} (or 50 μg. of E_3, or 7.0 μg. of Cht) in 3.2 ml. of 0.05 M tris-HCl, pH 7.0. Readings were made every 15 sec. at 25°.

Enzyme	Activity (E_{237}/min./ /mg.)	% of chymo-trypsin activity	Inhibition by SBTI (%)
Cht	18.2	100.0	43.0
E_{20}	0.4	2.1	4.2
E_3	2.7	14.7	42.0

The activity towards ATEE, the specific substrate of α-chymotrypsin, is presented in Table 5. E_{20} showed very small activity in comparison with that of E_3. Moreover, the latter enzyme was inhibited by SBTI to the same extent as α-chymotrypsin, while the inhibition of E_{20} was ten times lower. Thus it appears that E_{20} was probably not contaminated with α-chymotrypsin, and its activity towards ATEE seems to be an inherent property of the enzyme.

As it is known that serum proteolytic inhibitors exhibit a higher affinity to trypsin than to elastase (Graham, 1960), inhibition of E_{20} and E_3 by normal human serum was tested (Fig. 7). E_{20} was inhibited to a higher degree when relatively small amounts of serum were used, whereas both elastase preparations were completely inhibited when an excess of serum was applied. This result can also be explained by the presence of trypsin in the E_3 preparation. Small amounts of serum had negligible effect on elastolytic activity of E_3 because the inhibitors were nearly fully bound by trypsin. A considerable inhibition of elastase could take place only after nearly all contaminating trypsin had been saturated with the inhibitors.

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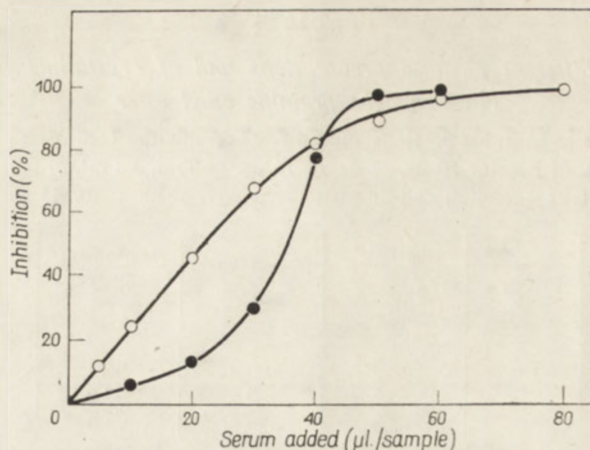

Fig. 7. The effect of the amount of human serum added to the reaction mixture on the elastolytic activity of (●), E_3 and (○), E_{20}. The standard incubation mixture contained 50 μg. of the enzyme preparation, 30 mg. of resorcin fuchsin-elastin, and normal human serum in 6 ml. of borate buffer of pH 8.7 and *I* 0.12. Incubation time 30 min. at 37°. The enzymes were preincubated with serum for 10 min. at room temperature.

On the basis of the presented results, the higher inhibition by SBTI of caseinolysis catalysed by E_3 in comparison with that of elastolytic activity of this enzyme, can be explained as follows. E_3 was heavily contaminated by trypsin and perhaps by *α*-chymotrypsin. Its activity towards casein was therefore the result of simultaneous action of elastase and of both contaminants. Elastolytic activity of this preparation was due practically only to elastase action, as trypsin was found to exhibit very small activity. In the presence of SBTI, the activity of the purified elastase towards casein was only negligibly reduced, whereas trypsin and *α*-chymotrypsin were highly inhibited. Thus their presence in the preparation E_3 was probably the cause of the observed higher inhibition of casein degradation by E_3.

The data presented do not support the concept of the existence of two active centres in the pancreatopeptidase E molecule.

The finding that elastolytic, general proteolytic and esterolytic activity of the enzyme are fully inhibited by DFP, a known inhibitor of serine proteases, seems to indicate that all these activities are related to the same active centre in which the serine residue is involved.

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REFERENCES

Ardelt W., Księżny S. & Niedźwiecka-Namysłowska I. (1970). *Analyt. Biochem.* **34**, **180**.
Bagdy D. & Banga I. (1957). *Acta Physiol. Acad. Sci. Hung.* **11**, 371.
Bagdy D. & Banga I. (1958). *Experientia* **14**, 64.
Banga I. & Ardelt W. (1967). *Biochim. Biophys. Acta* **146**, 284.
Graham G. N. (1960). *Biochem. J.* **75**, 14P.
Hall D. A. & Czerkawski J. W. (1959). *Biochem. J.* **73**, 356.
Kunitz M. (1947). *J. Gen. Physiol.* **30**, 291.
Laskowski M. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 2, p. 8. Academic Press, New York.
Loeven W. A. (1960). *Acta Physiol. Pharmacol. Neerl.* **9**, 44.
Loeven W. A. (1963). *Acta Physiol. Pharmacol. Neerl.* **12**, 57.
Loeven W. A. (1964). *Proc. of NATO Conference on the Structure and Function of Connective and Skeletal Tissues, St. Andrews, Scotland*, p. 109.
Partridge S. M., Davis H. F., & Adair G. S. (1955). *Biochem. J.* **61**, 11.
Rick W. (1963). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) p. 815. Academic Press, New York & London.
Schwert G. W. & Takenaka Y. (1955). *Biochim. Biophys. Acta* **16**, 570.
Smithies O. (1959). *Biochem. J.* **71**, 585.
Solyom A. & Tolnay P. (1965). *Enzymologia* **28**, 152.
Walford R. L. & Kickhöfen B. (1962). *Arch. Biochem. Biophys.* **98**, 191.

NIEKTÓRE WŁASNOŚCI OCZYSZCZONEJ WIEPRZOWEJ PANKREATOPEPTYDAZY E

Streszczenie

1. Pankreatopeptydazę E izolowano z handlowego proszku trzustki i oczyszczono 700-krotnie.

2. Oznaczono masę cząsteczkową enzymu (21 000), stałą Michaelisa (0,95 mg./ml.) oraz zbadano zależność szybkości elastolizy od rodzaju użytego buforu, pH i siły jonowej.

3. Oznaczono aktywność enzymu wobec czterech różnych substratów oraz jej hamowanie przez sojowy inhibitor trypsyny i normalną ludzką surowicę krwi. Wyniki nie potwierdzają hipotezy o występowaniu w cząsteczce pankreatopeptydazy E więcej niż jednego centrum katalitycznego

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A C T A B I O C H I M I C A P O L O N I C A

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EFFECT OF MALEATE ON GLUTAMATE OXIDATION BY RAT LIVER MITOCHONDRIA

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The effect of maleate and 2,4-dinitrophenol (DNP) on the oxidation of glutamate by rat liver mitochondria was studied in the presence of arsenite. It was found that maleate, similarly as malonate, stimulated the oxidation of glutamate measured by the formation of total α-oxoglutarate, and it decreased the amount of α-oxoglutarate within the mitochondria. On the other hand, maleate had no effect on glutamate oxidation by sonicated mitochondria. Stimulation of glutamate oxidation by DNP was observed also in the presence of maleate. The mechanism of action of maleate and DNP on glutamate oxidation by rat liver mitochondria, is discussed.

Among the tissues studied, liver is the only one in which the oxidation of α-oxoglutarate, pyruvate and oxoisocaproate is not inhibited by maleate. It has been even reported that maleate, at concentrations of about 1 mM, stimulated the oxidation of these metabolites by liver mitochondria (Angielski & Rogulski, 1962; Angielski, Łysiak & Rogulski, 1966; Angielski, Rogulski, Pacanis, Szutowicz & Wójcikowski, 1968).

De Haan & Tager (1966, 1968) and Papa *et al.* (1967a,b) have demonstrated that malonate and L-malate stimulated the reductive amination of α-oxoglutarate in the presence of ammonia, and the oxidation of glutamate in the presence of arsenite. These authors postulated that malonate and malate exerted their stimulatory effect by increasing the permeability of mitochondrial membranes for α-oxoglutarate. Recently, Meyer *et al.* (1970) suggested that also the stimulating effect of the compounds which uncouple oxidative phosphorylation, on glutamate oxidation in the presence of arsenite, may be explained by changes in the permeability of the mitochondrial membrane for α-oxoglutarate.

The aim of this and accompanying work (Wójcikowski & Angielski, 1970) was to study the effect of maleate on the outflow of α-oxoglutarate from mitochondria. It was found that the mechanism of action of maleate on the oxidation of glutamate by rat liver mitochondria is different from that of 2,4-dinitrophenol (DNP) but is similar to that of malonate. A preliminary communication from these experiments has been presented (Wójcikowski, Angielski & Rogulski, 1967).

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MATERIALS AND METHODS

Preparation of mitochondria. Rat liver mitochondria were prepared according to Hogeboom (1955) using 250 mM-sucrose - 1 mM-EDTA solution. The mitochondria were washed twice, then suspended in 250 mM-sucrose at a concentration of 20 - 25 mg. of protein per 1 ml.

Disintegration of mitochondria. This was done by ultrasonic treatment at 20 kHz for 1 min. in MSE disintegrator. During this time the vessel containing the mitochondria was kept in ice. The suspension of disintegrated mitochondria was used without subsequent centrifugation.

Measurement of glutamate oxidation and accumulation of α-oxoglutarate in mitochondria. The mitochondria were incubated at 23° in a medium consisting of: 190 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 10 mM-KCl, 5 mM-$MgCl_2$, 2 mM-L-glutamate and 1 mM-sodium arsenite; where indicated, sodium maleate, sodium malonate or DNP were added. The final volume was 2 ml. For determinations of glutamate and total α-oxoglutarate, the reaction was stopped with perchloric acid. For determination of α-oxoglutarate present within the mitochondria, the reaction was stopped by adding 20 ml. of ice-cold 250 mM-sucrose - 1 mM-EDTA solution. The mitochondria were sedimented by centrifuging at 9000 *g* for 5 min. at 0 - 4° and suspended in 1.0 ml. of cold water, then perchloric acid was added.

The solutions deproteinized with perchloric acid were neutralized with 30% KOH in the presence of 30 mM-K-phosphate buffer, pH 7.4. The precipitated potassium perchlorate was centrifuged off and the supernatant was used for determinations. α-Oxoglutarate accumulated within the mitochondria was determined fluorimetrically according to Goldberg, Passonneau & Lowry (1966) in a Farand type fluorimeter. In other experiments, in which the total amount of α-oxoglutarate formed was determined, the spectrophotometric method according to Bergmeyer & Bernt (1965) was used. L-Glutamate was determined enzymically according to Bernt & Bergmeyer (1965), and protein by the biuret method as described by Gornall, Bardawill & David (1949).

Enzymes and reagents. L-Glutamate dehydrogenase (EC 1.4.1.2) from ox liver was obtained from Sigma Chem. Co. (St. Louis, Mo., U.S.A.) as a suspension in 50% glycerol. In some experiments, the enzyme from ox liver obtained according to Warren, Carr & Grisolia (1964), including the crystallization step, was used.

NADH, NAD^+ and ADP were from C. F. Boehringer & Soehne G.m.b.H. (Mannheim, West Germany), maleic acid from v/o Sojuzchimexport (Moscow, U.S.S.R.). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

Effect of maleate on glutamate oxidation and α-oxoglutarate accumulation in liver mitochondria. The oxidative deamination of glutamate to α-oxoglutarate in the presence of arsenite was enhanced by 1 mM-maleate, as measured by the amount

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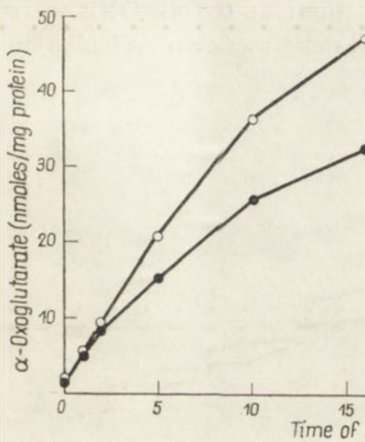
of total *a*-oxoglutarate formed (Fig. 1). The effect of maleate increased with time of incubation and after 15 min. the oxidation exceeded the control value by about 40%. This effect could be explained by formation of oxaloacetate from maleate, and subsequent formation of *a*-oxoglutarate v*ia* transamination of glutamate. However, as it has been demonstrated previously (Taggart, Angielski & Morell, 1962) liver mitochondria, unlike kidney mitochondria, exhibit but very slight activity of maleate hydratase (Angielski *et al.*, 1962; Britten, Morell & Taggart, 1969) and D-2-hydroxy acid dehydrogenase (EC 1.1.99.6) (Tubbs & Greville, 1961), the enzymes which catalyse the conversion of maleate to oxaloacetate *via* D-malate.

From the results presented in Fig. 2 it appears that *a*-oxoglutarate formed from glutamate, accumulated within the mitochondria reached after 5 min. of incubation a concentration of 10 nmoles per 1 mg. of mitochondrial protein. Longer incubation led to a decrease of the intramitochondrial *a*-oxoglutarate. In the presence of 1 mM-maleate, notwithstanding the stimulation of glutamate oxidation, the amount of *a*-oxoglutarate in mitochondria did not exceed 4 nmoles per 1 mg. of mitochondrial protein.



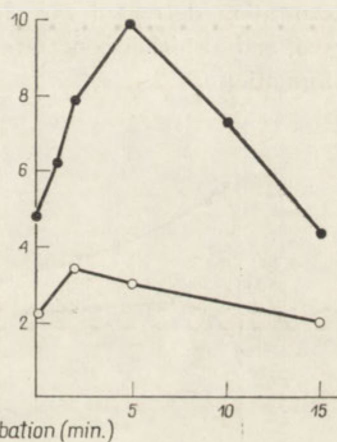


Fig. 1

Fig. 2

Fig. 1. The effect of maleate on glutamate oxidation by rat liver mitochondria. The medium was as described under Methods and contained 4 mg. of mitochondrial protein/ml. (●), Control; (○), with 1 mM-maleate. The results are expressed as nmoles of total *a*-oxoglutarate/mg. of mitochondrial protein.

Fig. 2. The effect of maleate on accumulation of *a*-oxoglutarate in rat liver mitochondria. Conditions of the experiment were as described under Methods; the medium contained 15 mg. of mitochondrial protein per 1 ml. (●), Control; (○) with 1 mM-maleate. The results are expressed as nmoles of *a*-oxoglutarate in mitochondria/mg. of mitochondrial protein.

Effect of malonate and DNP on glutamate oxidation in liver mitochondria. The uncouplers of oxidative phosphorylation, including 2,4-dinitrophenol (DNP), are known to stimulate glutamate oxidation *via* oxidative deamination (De Haan, Tager & Slater, 1967; Papa *et al.*, 1967a; Meyer *et al.*, 1970). In our experiments, DNP stimulated glutamate oxidation but, in contrast to maleate, it lowered only

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slightly the concentration of α-oxoglutarate within the mitochondria (Table 1). After 5 min. of incubation in the presence of 0.05 mM-DNP, there was 7.4 nmoles of α-oxoglutarate per 1 mg. of mitochondrial protein, whereas only 3.6 nmoles were found after incubation in the presence of 1 mM-maleate. Addition of DNP together with maleate caused increased stimulation of glutamate oxidation, and under these conditions the concentration of α-oxoglutarate in mitochondria was found to be very low. The effect of malonate was similar to that of maleate; glutamate oxidation was stimulated, and the concentration of α-oxoglutarate in mitochondria decreased. Addition of DNP together with malonate caused a still greater increase in glutamate oxidation, whereas the effects of malonate and maleate added together were not additive.

As it may be seen from the results presented in Table 1, both in the presence of maleate and DNP the amount of oxidized glutamate corresponded to the amount of total α-oxoglutarate formed.

The effect of DNP on glutamate oxidation was dependent on its concentration (Fig. 3), the highest stimulation being observed at 0.02 mM-DNP. The increase of DNP concentration decreased the stimulation; at 0.5 mM-DNP no stimulation was observed, and at higher concentrations there was even an inhibition of α-oxoglutarate formation.

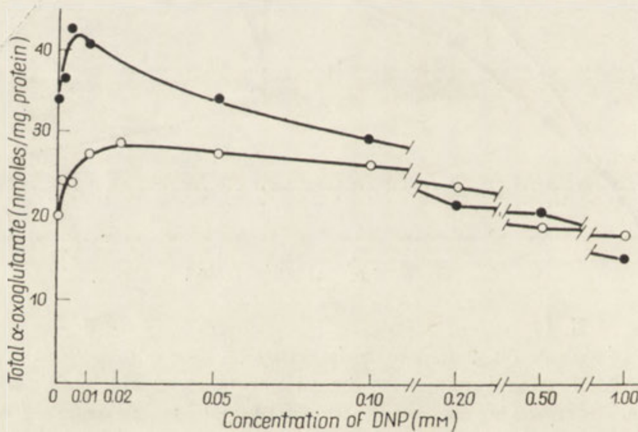

Fig. 3. The effect of DNP concentration on glutamate oxidation by rat liver mitochondria, (○) in the absence, and (●), in the presence of 1 mM-maleate. Conditions of the experiment were as described under Methods; the medium contained 7.0 mg. of mitochondrial protein per 1 ml. The results are expressed as nmoles of total α-oxoglutarate/mg. of mitochondrial protein/15 min.

Effect of maleate and DNP on sonicated mitochondria. Glutamate oxidation by disintegrated mitochondria was practically unaffected by 1 mM-maleate (Table 2). However, DNP added together with maleate, raised the amount of α-oxoglutarate formed by about 50%, similarly as it has been found with intact mitochondria,

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Table 1

Effect of maleate, malonate and DNP on glutamate oxidation and α-oxoglutarate concentration in rat liver mitochondria

Conditions of the experiment were as described under Methods. The amount of mitochondria corresponded to 6.0 mg. of mitochondrial protein per 1 ml. of the medium. Maleate and malonate were used at concentrations of 1 mM, and DNP at 0.05 mM.

Addition	Decrease in glutamate content after 15 min.	Increase in total α-oxoglutarate content after 15 min.	Amount of intra-mitochondrial α-oxoglutarate after 5 min.
	nmoles/mg. of mitochondrial protein		
None	28	26	9.2
Maleate	47	41	3.6
DNP	41	39	7.4
Maleate+DNP	65	63	2.6
Malonate	—	35	2.8
Malonate+DNP	—	49	—
Malonate+maleate	—	38	—
Malonate+maleate+DNP	—	57	—

Table 2

Effect of maleate and DNP on glutamate oxidation by disintegrated rat liver mitochondria

Conditions of the experiment were as described under Methods. The amount of mitochondria disintegrated by sonication corresponded to 6 mg. of mitochondrial protein per 1 ml. of the medium. Final concentration of maleate was 1 mM, and that of DNP 0.05 mM.

Addition	α-Oxoglutarate formed (nmoles/mg. of mitochondrial protein/15 min.)
None	27
Maleate	32
Maleate+DNP	47

DISCUSSION

Maleate, similarly as DNP, stimulates, in the presence of arsenite, glutamate oxidation by intact rat liver mitochondria, the effect of these two compounds being additive. However, their mechanism of action seems to be different. Maleate has no effect on glutamate oxidation by sonicated mitochondria, whereas the stimulatory effect of DNP is maintained. The amount of α-oxoglutarate within the mitochondria is lowered by maleate whereas DNP, when applied at the most effective concentrations, has but little influence.

Both the action of uncouplers of oxidative phosphorylation and the effect of

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malonate on oxidative deamination of glutamate, were extensively studied by De Haan & Tager (1966, 1968), Papa *et al.* (1967a,b) and De Haan *et al.* (1967); they concluded that stimulation of glutamate oxidation by uncouplers would be to some extent dependent on stimulation of NADPH oxidation due to inhibition of energy-linked transhydrogenase. A similar effect was obtained by stimulating NADPH oxidation in the presence of 2-methyl-1,4-naphthoquinone (Papa *et al.*, 1967b). However, Meyer *et al.* (1970) suggested another mechanism of action of these compounds: dicoumarol, which stimulates the oxidative deamination of glutamate, partially overcomes the inhibition of α-oxoglutarate oxidation by arsenite. This results in formation of malate, which in an exchange diffusion with the extra-mitochondrial orthophosphate moves out of the mitochondria. Then the extra-mitochondrial malate is exchanged for intramitochondrial α-oxoglutarate by an exchange-diffusion reaction mediated by a specific carrier (De Haan & Tager, 1968). The lowering of α-oxoglutarate concentration within the mitochondria leads to stimulation of glutamate oxidation. The interpretation offered by Meyer *et al.* (1970) for stimulation by dicoumarol of glutamate oxidation, does not explain the observations reported in the present work concerning the effect of DNP on sonicated mitochondria or the synergistic effect of DNP and maleate or malonate. The inhibition of glutamate oxidation by high concentrations of DNP seems to be due to the inhibition of entry of glutamate into mitochondria. Van Dam & Tsou (1968), Kraayenhof & Van Dam (1969) and Lofrumento, Meyer, Tager, Papa & Quagliariello (1970) demonstrated that the transport of the anionic form of DNP can be mediated by anion carriers and that DNP acts competitively on specific carrier systems. This behaviour of DNP could explain the observed lack of effect of maleate on glutamate oxidation in the presence of DNP concentrations exceeding 0.1 mM (Fig. 3); under those conditions the rate of glutamate oxidation would be limited by the entry of glutamate into mitochondria, and not by the exit of α-oxoglutarate.

Thus it could be supposed that the factors which inhibit oxidative deamination of glutamate are the reaction products accumulating within the mitochondria, mainly α-oxoglutarate and NADPH. Addition of maleate or malonate would decrease the concentration of intramitochondrial α-oxoglutarate by exchange diffusion, whereas DNP would cause as well stimulation of NADPH oxidation.

REFERENCES

Angielski S., Łysiak W. & Rogulski J. (1966). *III FEBS Meeting, Warszawa, Abstr. of Commun.* M 71.

Angielski S. & Rogulski J. (1962). *Acta Biochim. Polon.* 9, 357.

Angielski S., Rogulski J., Pacanis A., Szutowicz A. & Wójcikowski Cz. (1968). *V FEBS Meeting Praha. Abstr. of Commun.* FC 391.

Bergmeyer H. U. & Bernt E. (1965). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) p. 324. Academic Press, New York.

Bernt E. & Bergmeyer H. U. (1965). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) p. 384. Academic Press, New York.

http://rcin.org.pl

Britten J. S., Morell H. & Taggart J. V. (1969). *Biochim. Biophys. Acta* **185**, 220.
De Haan E. J. & Tager J. M. (1966). *III FEBS Meeting, Warszawa. Abstr. of Commun.* M 77.
De Haan E. J. & Tager J. M. (1968). *Biochim. Biophys. Acta* **153**, 98.
De Haan E. J., Tager J. M. & Slater E. C. (1967). *Biochim. Biophys. Acta* **131**, 1.
Goldberg N. D., Passonneau J. V. & Lowry O. H. (1966). *J. Biol. Chem.* **241**, 3997.
Gornall A. G., Bardawill C. J. & David M. M. (1949). *J. Biol. Chem.* **177**, 751.
Hogeboom G. H. (1955), In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 16. Academic Press, New York.
Kraayenhof R. & Van Dam K. (1969). *Biochim. Biophys. Acta* **172**, 189.
Lofrumento N. E., Meyer A. J., Tager J. M., Papa S. & Quagliariello E. (1970). *Biochim. Biophys. Acta* **197**, 104.
Meyer A. J., Papa S., Paradies G., Zanghi M. A., Tager J. M. & Quagliariello E. (1970). *Biochim. Biophys. Acta* **197**, 97.
Papa S., De Haan E. J., Francavilla A., Tager J. M. & Quagliariello E. (1967a). *Biochim. Biophys. Acta* **143**, 438.
Papa S., Tager J. M., Francavilla A., De Haan E. J. & Quagliariello E. (1967b). *Biochim. Biophys. Acta* **131**, 14.
Taggart J. V., Angielski S. & Morell H. (1962). *Biochim. Biophys. Acta* **58**, 141.
Tubbs P. K. & Greville G. D. (1961). *Biochem. J.* **81**, 104.
Van Dam F. & Tsou C. S. (1968). *Biochim. Biophys. Acta* **162**, 301.
Warren J. C., Carr D. O. & Grisolia S. (1964). *Biochem. J.* **93**, 409.
Wójcikowski Cz. & Angielski S. (1970). *Acta Biochim. Polon.* **17**, 299.
Wójcikowski Cz., Angielski S. & Rogulski J. (1967). *V Symp. Pol. Tow. Biochem., Kraków* C 44.

WPŁYW MALEINIANU NA UTLENIANIE GLUTAMINIANU PRZEZ MITOCHONDRIA WĄTROBY SZCZURA

Streszczenie

Badano, w obecności arseninu, wpływ maleinianu i 2,4-dwunitrofenolu (DNP) na utlenianie glutaminianu przez mitochondria wątroby szczura. Stwierdzono, że maleinian, podobnie jak i malonian, stymuluje utlenianie glutaminianu, mierzone przyrostem α-ketoglutaranu i obniża poziom α-ketoglutaranu wewnątrz mitochondrii. Maleinian nie ma jednak wpływu na utlenianie glutaminianu przez mitochondria rozbite działaniem ultradźwięków. Stymulacja utleniania glutaminianu przez DNP występuje również w obecności maleinianu. Przedyskutowano mechanizm działania maleinianu i DNP na utlenianie glutaminianu w mitochondriach wątroby szczura.

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THE STIMULATION BY MALEATE OF α-OXOGLUTARATE EXIT FROM RAT LIVER MITOCHONDRIA

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1. The exit from mitochondria of previously accumulated α-oxoglutarate was studied in the presence of a "trapping system" consisting of NH_4Cl, NADH and L-glutamate dehydrogenase. It was found that L-malate, succinate, maleate, malonate and oxaloacetate stimulated markedly the exit of α-oxoglutarate from mitochondria. In the presence of maleate, V_{max} was 7.1 nmoles/min./mg. of mitochondrial protein, and the energy of activation +17.4 Kcal/mole. 2. The exit of α-oxoglutarate was accompanied by the entrance of an equimolar amount of [2,3-^{14}C]maleate, L-malate or oxaloacetate but no changes were observed in the content of K^+ in mitochondria. 3. The mechanism of the activating effect of some organic anions on the exit of α-oxoglutarate from mitochondria is discussed.

Chappell & Haarhoff (1967) reported that the membrane of liver mitochondria is impermeable to maleate. This is distinctly at variance with the data obtained by Ferguson & Williams (1966) on the activating effect of maleate on the metabolism of isocitrate, as well as with the results reported in the accompanying paper (Wójcikowski & Angielski, 1970) on the stimulating effect of maleate on glutamate oxidation.

The aim of the present work was to study the mechanism of action of maleate and other organic anions on the exit of the accumulated α-oxoglutarate from rat liver mitochondria. The outflow of α-oxoglutarate was studied using glutamate dehydrogenase, NH_4Cl and NADH as a trapping system. The obtained results indicate that maleate penetrates the mitochondrial membrane and is exchanged for intramitochondrial α-oxoglutarate at an equimolar ratio.

MATERIALS AND METHODS

Preparation of rat liver mitochondria. This was done as described in the accompanying paper (Wójcikowski & Angielski, 1970), and the washed mitochondria were suspended in 250 mM-sucrose- 1 mM-EDTA solution at a ratio of about 40 mg. of mitochondrial protein per 1 ml.

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Loading of mitochondria with α-oxoglutarate. Freshly prepared mitochondria, about 100 mg. of mitochondrial protein, were incubated in 10 ml. of a medium consisting of: 190 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 10 mM-KCl, 5 mM-$MgCl_2$, 10 mM-sodium α-oxoglutarate, 1 mM-sodium arsenite and 0.25 mM-EDTA (the last one originating from the mitochondrial suspension). The incubation was carried out at 23° on a thermostated water bath with a shaker, at about 60 oscillations/min., and was stopped after 5 min. by adding 3 volumes of ice-cold 250 mM-sucrose - 1 mM-EDTA solution. The mitochondria were immediately centrifuged at 0 - 4° at 5000 *g* for 5 min., washed twice and suspended in the above sucrose-EDTA solution.

Determination of the rate of exit of α-oxoglutarate from mitochondria. The mitochondria loaded with α-oxoglutarate were incubated at 10° in the medium composed of 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.2 mM-EDTA (originating from mitochondrial suspension), and 2.5 mM-NH_4Cl, 0.3 mM-NADH and glutamate dehydrogenase (100 - 150 μg. protein/ml. of the medium) (the "trapping system"). The reaction was stopped by adding 45% perchloric acid to a final concentration of 6%.

To study the possible exchange of intra- and extra-mitochondrial anions, the loaded mitochondria (12 - 16 mg. of protein) were incubated at 10° in a medium composed of 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.2 mM-EDTA and 0.2 mM-sodium salt of the tested anion. The final volume was 3 ml. After 2 min., the reaction was stopped by filtration under reduced pressure through Millipore filter (HA 0.45 μ) which was covered with one layer of cellite and one layer of cellulose, each 2 - 3 mm. thick, to prevent occlusion of the filter by the large amount of mitochondria. The mitochondria on the filter were washed with 1 ml. of ice-cold sucrose. The filtrate was collected into a tube containing perchloric acid. The time of filtration, together with washing, did not exceed 20 seconds.

Analytical methods. The deproteinized solutions were neutralized with 30% KOH or 2 M-K_2CO_3, in the presence of 30 mM-K-phosphate buffer of pH 7.4. The precipitated potassium perchlorate was centrifuged off and the supernatant was used for the assays. α-Oxoglutarate was determined enzymically according to Bergmeyer & Bernt (1965), L-malate according to Hohorst (1965) and oxaloacetate according to Hohorst & Reim (1965). Protein was determined by the biuret method as described by Gornall, Bardawill & David (1949). Potassium was determined with a flame photometer. For radioactivity measurements an end-window G-M counter was used.

Reagents. Two preparations of L-glutamate dehydrogenase were the same as in the accompanying paper (Wójcikowski & Angielski, 1970). Malate dehydrogenase (EC 1.1.1.37) was prepared according to Ochoa (1965). [2,3-^{14}C]Maleic anhydride (spec. act. 5.3 mc/m-mole) was obtained from the Radiochemical Centre (Amersham, Bucks., England); cellite for chromatography no. 545, tris, propionic acid and isobutyric acid were from British Drug Houses Ltd. (Poole, Dorset, England); NADH from Boehringer & Soehne, GmbH (Mannheim, West Germany); L(—)malic acid, D(+)malic acid, oxaloacetic acid, sodium pyruvate and gramicidin, from Sigma

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Chem. Co. (St. Louis, Mo., U.S.A.); maleic, isovaleric and caproic acids from v/o Sojuzchimexport (Moscow, U.S.S.R.); succinic acid from Reanal (Budapest, Hungary); succinimide from A. G. Fluka (Buchs S. G., Switzerland); thiomalic acid from Koch-Light Ltd. (Colnbrook, Bucks, England). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

Incubation of mitochondria in a medium containing NH_4Cl, NADH and glutamate dehydrogenase permits to determine changes in the intramitochondrial content of α-oxoglutarate, without the necessity of separating the mitochondria from the incubation medium. The α-oxoglutarate accumulated within the mitochondria is inaccessible to the action of exogenous NADH and glutamate dehydrogenase. The α-oxoglutarate released from mitochondria, immediately undergoes reductive amination to glutamate, and the amount of α-oxoglutarate determined in the deproteinized incubation mixture corresponds to that present within the mitochondria.

The effect of time and temperature on reductive amination of α-oxoglutarate. For the assay of the rate of exit of α-oxoglutarate from mitochondria, in the system studied, it was necessary that the rate of the extramitochondrial reductive amination of α-oxoglutarate should be greater than the rate of its exit from mitochondria. Therefore in preliminary experiments the rate of reductive amination of α-oxoglutarate added to the medium, which was to be used in further experiments, was determined.

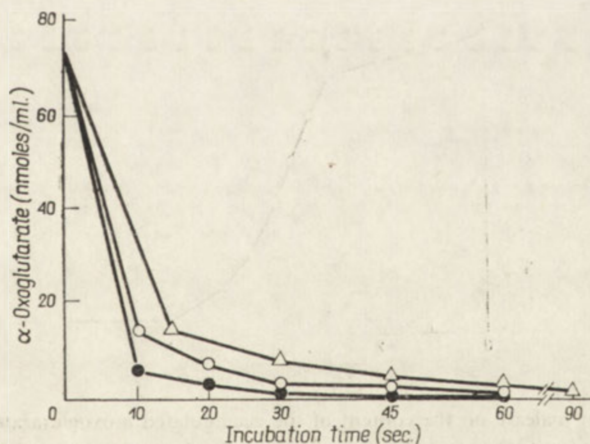

Fig. 1. Effect of time and temperature on the rate of reductive amination of α-oxoglutarate. To the medium consisting of: 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.2 mM-EDTA, 2.5 mM-NH_4Cl and glutamate dehydrogenase (150 μg. of protein/1 ml.), was added α-oxoglutarate (75 nmoles/1 ml.). The temperature of incubation was: △, 0°; ○, 10°; and ●, 23°. The reaction was stopped with perchloric acid. The results represent the amount of unreacted α-oxoglutarate.

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α-Oxoglutarate added in the amount of 75 nmoles per 1 ml. of the medium, at 0° was completely converted to glutamate within less than 60 sec., and at 10° and 23°, in less than 30 sec. (Fig. 1). As it will be described below, the rate of α-oxoglutarate exit from mitochondria was much smaller than its reductive amination.

Effect of maleate on the exit of α-oxoglutarate from mitochondria. The average amount of α-oxoglutarate present in mitochondria which had been incubated with α-oxoglutarate and arsenite, was 11.8 ± 1.6 nmoles/mg. of mitochondrial protein (average from 26 determinations). When the mitochondria were suspended in 250 mM-sucrose - 1 mM-EDTA at 0°, the accumulated α-oxoglutarate flowed out at a rate of about 0.05 nmole/min./mg. of protein (Table 1); when the mitochondria were suspended in the "trapping system" at 10° the rate was greater, 0.4 nmole/min./mg. Addition to the medium of 1 mM-maleate stimulated greatly the outflow of α-oxoglutarate, the rate being 5.0 ± 0.8 nmoles/min./mg. protein.

The effect of maleate on the amount of α-oxoglutarate in mitochondria in relation to the time of incubation, is presented in Fig. 2. During 3 min. of incubation without maleate, the exit of α-oxoglutarate was very small; the addition of 1 mM-maleate caused a rapid decrease of intramitochondrial α-oxoglutarate, which was linear during 1 min., and after 3 min. the amount of α-oxoglutarate in the mitochondria was only 0.5 nmole/mg. of protein.

To exclude the possible stimulating effect of maleate on intramitochondrial metabolism of α-oxoglutarate, the mitochondria were incubated without glutamate dehydrogenase. Under these conditions, α-oxoglutarate could be converted only within the mitochondria, and the α-oxoglutarate determined would correspond to the sum of the extra- and intra-mitochondrial α-oxoglutarate. The results presented

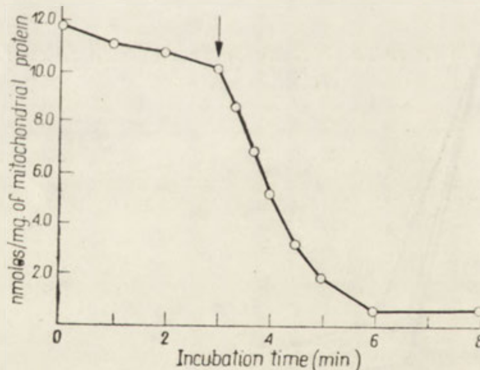

Fig. 2. The effect of maleate on the content of the accumulated α-oxoglutarate in rat liver mitochondria. The loaded mitochondria (4.0 mg. of protein/1 ml.) were incubated at 10° in a medium consisting of: 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.2 mM-EDTA, 2.5 mM-NH_4Cl, 0.3 mM-NADH, and glutamate dehydrogenase (150 µg./ml.). The incubation mixture was stirred electromagnetically in a thermostated vessel. After 3 min. of incubation, tris-maleate was added to a final concentration of 1 mM. At indicated time, 1.0 ml. samples of the incubation mixture were removed and added to perchloric acid. The results represent the amount of α-oxoglutarate in mitochondria.

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Table 1

Effect of the medium on the exit of a-oxoglutarate from rat liver mitochondria

The mitochondria loaded with α-oxoglutarate were incubated at 10° as described in Methods, except-that: in Expt. I, the "trapping system" composed of L-glutamate dehydrogenase, NADH and NH_4Cl, was added after 30 min. of incubation, and after 30 sec. the incubation mixture was deproteinized with perchloric acid; in Expt. IV, instead of 25 mM-tris-HCl buffer of pH 7.4, 20 mM-K-phosphate buffer of the same pH was used. The decrease of α-oxoglutarate is calculated in Expts. I and II in relation to the values found at zero time incubation, and in Expts. III and IV in relation to the values determined in samples incubated without maleate. Mean values ±S.D. are given, the number of determinations being indicated in parentheses.

Expt. no.	Incubation			Decrease of α-oxoglutarate content in mitochondria (nmoles/min./mg. of mitochondrial protein)
	medium	temperature (°C)	time (min.)	
I	250 mM-Sucrose - 1 mM-EDTA	0	30	0.05±0.03 (6)
II	With "trapping system"	10	3	0.4 ±0.2 (10)
III	With "trapping system" and 1 mM-maleate	10	1	5.0 ±0.8 (20)
IV	With "trapping system", maleate and P_i	10	1	5.5 ±0.9 (5)

Table 2

The effect of organic anions on the rate of outflow from rat liver mitochondria of the accumulated a-oxoglutarate

The reaction was started by adding the mitochondria (3.6 mg. of protein/1 ml.) to the medium described for Fig. 2. Time of incubation 1 min., temperature 10°. All the compounds tested were added as tris salts to a final concentration of 1 mM, except 1 mM-potassium pyruvate and 5 mM-potassium phosphate. The results are expressed as decrease (—) or increase (+) of α-oxoglutarate in mitochondria.

Addition	nmoles/min./mg. of mitochondrial protein
Maleate	—5.0
L-Malate	greater than — 11
Succinate	greater than — 11
Malonate	—3.7
Oxaloacetate	—3.4
Glutarate	—0.8
Fumarate	—1.2
D-Malate	+1.3
Itaconate	—1.6
n-Butyrate	—0.3
Isobutyrate	—0.6
n-Caproate	—0.4
Thiomalate	—1.2
Formate	+0.3
Oxalate, dihydroxymaleate, acetate, propionate, isovalerate, citrate, pyruvate, succinimide, phosphate	0

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in Fig. 3 show that 1 mM-maleate did not increase the rate of intramitochondrial metabolism of α-oxoglutarate. Without maleate, in the presence of 2.5 mM-NH_4Cl, the disappearance of α-oxoglutarate was about 0.7 nmole/min./mg. of protein, and after the addition of maleate, 0.8 nmole. However, the rate of metabolism of α-oxoglutarate was dependent on NH_4Cl concentration. In the absence of NH_4Cl, the disappearance of α-oxoglutarate amounted to 0.1 nmole/min., in the presence of 2.5 mM-NH_4Cl it was 0.7 nmole, and at 10 mM-NH_4Cl, 1.5 nmoles.

The effect of concentration of glutamate dehydrogenase, NH_4Cl *and NADH in the presence of maleate.* The effect of glutamate dehydrogenase concentration was

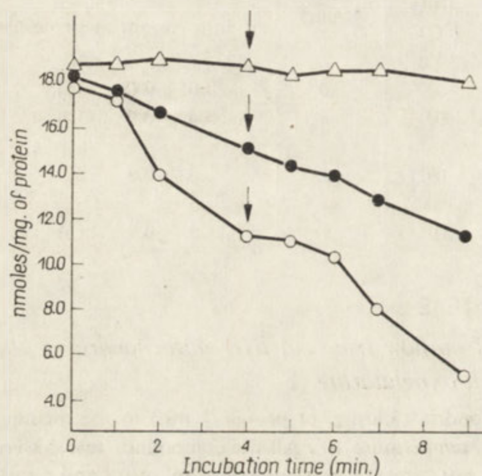

Fig. 3. The effect of maleate on the metabolism of α-oxoglutarate in rat liver mitochondria. The mitochondria (3.5 mg. of protein/1 ml.) loaded with α-oxoglutarate were incubated in a medium consisting of: 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.3 mM-NADH and 0.2 mM-EDTA, △, in the absence of NH_4Cl; and in the presence of ●, 2.5 mM-NH_4Cl and ○, 10 mM-NH_4Cl. Tris-maleate was added at the 4th minute of incubation to a final concentration of 1 mM. The results represent the amount of total α-oxoglutarate.

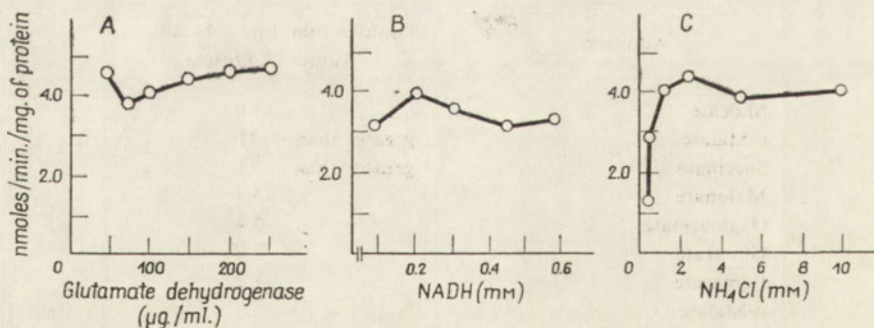

Fig. 4. The effect of concentration of glutamate dehydrogenase, NH_4Cl and NADH on the rate of exit of α-oxoglutarate from the loaded mitochondria, in the presence of maleate. The incubation medium consisted of: 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.2 mM-EDTA, 1 mM-tris-maleate and in: *A*, 0.3 mM-NADH, 2.5 mM-NH_4Cl and varying amounts of glutamate dehydrogenase; *B*, 2.5 mM-NH_4Cl, 150 μg./ml. of glutamate dehydrogenase, and varying amounts of NADH; *C*, 0.3 mM-NADH, 150 μg./ml. of glutamate dehydrogenase, and varying amounts of NH_4Cl. The reaction was started by adding the mitochondria (3.5 mg. of protein/ml.). The time of incubation was 40 sec. at 0°. The results represent the amount of α-oxoglutarate which moved out of mitochondria.

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studied in the presence of 0.3 mM-NADH and 2.5 mM-NH_4Cl (Fig. 4A). The rate of exit of α-oxoglutarate from mitochondria was practically constant, 3.8 - 4.5 nmoles/min., with 50 to 250 mg. of glutamate dehydrogenase. Similarly, the increase of NADH from 0.1 to 0.6 mM did not change the rate of exit (Fig. 4B).

The effect of NH_4Cl concentration was studied in the presence of 0.3 mM-NADH and 150 μg. of glutamate dehydrogenase per 1 ml. (Fig. 4C). The disappearance of α-oxoglutarate from mitochondria increased with increasing NH_4Cl concentration over the range 0.25 - 1.25 mM. Further increase in NH_4Cl concentration was without effect. The low decrease of α-oxoglutarate at NH_4Cl concentrations lower than 1.25 mM seems to be due to the fact that the rate of exit of α-oxoglutarate from mitochondria was then higher than the rate of its reductive amination.

Effect of organic anions. Among the 21 organic anions tested, listed in Table 2, only five showed a distinct ability to stimulate the translocation of α-oxoglutarate from mitochondria. In addition to maleate, these are: L-malate, succinate, malonate and oxaloacetate. The two first compounds stimulated this process very markedly. The rate of α-oxoglutarate exit from mitochondria in their presence exceeded 11 nmoles/min. The other two compounds, malonate and oxaloacetate, similarly to maleate, had a weaker but still considerable effect. Itaconate, fumarate, thiomalate and glutarate gave some stimulation, whereas the other organic anions studied had no effect.

Kinetics of exit of α-oxoglutarate from mitochondria in the presence of maleate. The effect of maleate concentration on the rate of exit of α-oxoglutarate was studied under conditions when the rate of α-oxoglutarate disappearance was linear with the time of incubation. Half of the maximum velocity was obtained at a maleate concentration of 0.5 mM. The maximum velocity of the exit amounted to 7.1 nmoles of α-oxoglutarate/min./mg. of protein (Fig. 5).

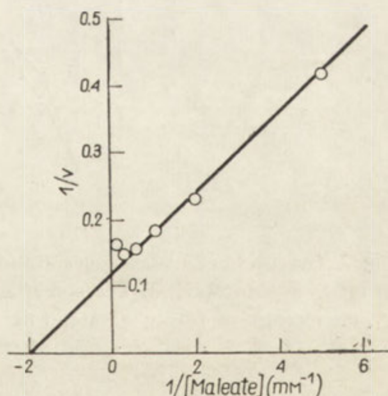

Fig. 5. Double-reciprocal plot of the rate of exit of α-oxoglutarate from mitochondria *versus* maleate concentration. The medium was as described for Fig. 2. The reaction was started by the addition of the mitochondria (4.0 mg. of protein/ml.) loaded with α-oxoglutarate. Incubation time 40 sec.; *v* represents nmoles/min./mg. of mitochondrial protein.

The rate of exit of α-oxoglutarate from mitochondria was temperature-dependent (Fig. 6A). In the presence of 1 mM-maleate, the rate at 0° was 1.8 nmoles/min./mg. of protein, and at 5°, 10° and 15°, respectively, 3, 6, and 10 nmoles. The temperature coefficient, Q_{10}, calculated from these values, was 3.3. The Arrhenius plot (Fig. 6B)

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was a straight line over the range of temperatures studied. The energy of activation was +17.2 Kcal/mole.

Translocation of α-oxoglutarate, [2,3-^{14}C]*maleate,* L-*malate and oxaloacetate.* To determine simultaneously with the exit of α-oxoglutarate from mitochondria,

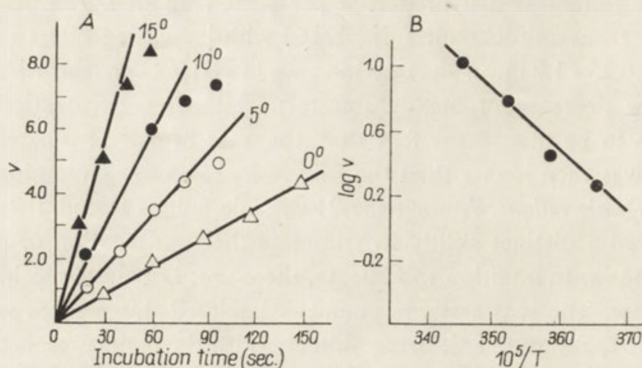

Fig. 6. *A*, The effect of temperature on the rate of exit of α-oxoglutarate from the loaded mitochondria in the presence of maleate. The conditions were as described for Fig. 2. Tris-maleate (final concentration 1 mM) was added following 2 min. preincubation of mitochondria in the medium. The results, *v*, represent the amount of α-oxoglutarate which moved out of mitochondria in nmoles/mg. of protein. *B*, The Arrhenius plot calculated from the same data.

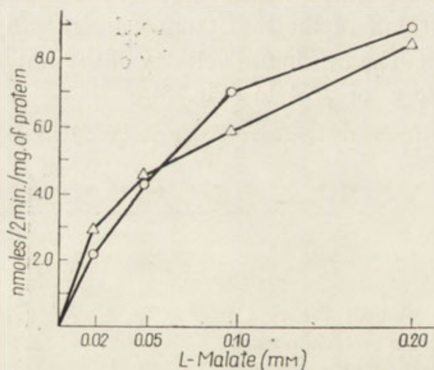

Fig. 7. The effect of L-malate concentration on the exchange diffusion of α-oxoglutarate and L-malate in rat liver mitochondria. The conditions were as described for Table 3. The results represent: △, the increase of α-oxoglutarate in the medium; and ○, the decrease of L-malate in the medium after 2 min. of incubation.

the possible entrance into mitochondria of the anions studied, it was necessary to separate rapidly the mitochondria from the incubation medium. For this purpose, the technique of filtration on Millipore filters was used. In the medium, following removal of mitochondria, determinations were made of the increase of α-oxoglutarate and decrease of the anions entering the mitochondria.

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The data presented in Table 3 show that the amount of α-oxoglutarate which appeared in the medium was almost equal to the amount of the respective disappearing anion, their molar ratios being: maleate:α-oxoglutarate 0.83; L-malate:α-oxoglutarate 0.94; oxaloacetate:α-oxoglutarate 1.00. At different concentrations of L-malate in the medium, a close relationship was observed between the exit of α-oxoglutarate from, and entry of L-malate into, the mitochondria (Fig. 7). The molar ratio L-malate:α-oxoglutarate ranged from 0.76 to 1.06.

Table 3

Translocation of α-oxoglutarate, [2,3-^{14}C]*maleate,* L-*malate and oxaloacetate in rat liver mitochondria*

Liver mitochondria (12 - 16 mg. of protein) loaded with α-oxoglutarate, were incubated for 2 min. in a medium consisting of 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.2 mM-EDTA and 0.2 mM sodium salt of the indicated anion. The mitochondria were separated by filtration on Millipore filter, and in the medium the respective compounds were determined.

Addition (0.2 mM)	Increase of α-oxoglutarate content	Decrease of the anion added
	nmoles/2 min./mg. of mitochondrial protein	
[2,3-^{14}C]Maleate (0.03 μc)	4.8	4.0
L-Malate	8.1	7.5
Oxaloacetate	4.1	4.1

Table 4

The effect of maleate, acetate, and gramicidin on the content of α-oxoglutarate and potassium in rat liver mitochondria

The mitochondria (6.5 mg. of protein) loaded with α-oxoglutarate were incubated at 10° in a medium consisting of 225 mM-sucrose, 25 mM-tris-HCl buffer of pH 7.4, 0.2 mM-EDTA, 2.5 mM-NH_4Cl, and the indicated compounds; final volume 1.0 ml. Tris-maleate and tris-acetate were used. Gramicidin (0.08 μg./mg. of mitochondrial protein) was added as solution in 70% ethanol. After 3-min. incubation, the reaction was stopped with 5 ml. of ice-cold sucrose, the mitochondria were immediately centrifuged for 3 min. at 10 000 *g*, suspended in 1.0 ml. of cold water, thoroughly mixed, then 0.5 ml. was transferred to perchloric acid, and α-oxoglutarate determined. The remaining part of the mitochondrial suspension was boiled, the denatured protein centrifuged off, and in the supernatant potassium was determined.

Addition	nmoles/mg. of protein			
	α-oxoglutarate in mitochondria	Δ	potassium in mitochondria	Δ
None	13.1		73	
1 mM-Maleate	4.2	—8.9	73	0
1 mM-Acetate	13.0	—0.1	70	—3
Gramicidin	13.0	—0.1	59	—14
1 mM-Maleate and gramicidin	4.8	—8.3	63	—10
1 mM-Acetate and gramicidin	13.8	+0.7	57	—16

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Effect of gramicidin and maleate on the translocation of potassium and α-oxoglutarate. To maintain electroneutrality, the exit of α-oxoglutarate from mitochondria should be accompanied by a corresponding movement from mitochondria of an equivalent amount of cation, or by the movement into mitochondria of an equivalent amount of anion.

From the data presented in Table 4 it follows that the decrease of the amount of α-oxoglutarate in mitochondria in the presence of 1 mM-maleate, was not accompanied by a change in the content of K^+ ion in mitochondria. Addition of gramicidin (0.08 μg./mg. of mitochondrial protein) caused a decrease of K^+ by 14 nmoles/mg. of protein without affecting the content of α-oxoglutarate. Simultaneous addition of gramicidin and maleate decreased the content in mitochondria of both K^+ and α-oxoglutarate. Acetate had no effect on the content of K^+ or α-oxoglutarate, either in the presence or absence of gramicidin.

DISCUSSION

The mitochondria which had been loaded with α-oxoglutarate, then washed and suspended in 250 mM-sucrose - 1 mM-EDTA solution, maintained the accumulated α-oxoglutarate against its high concentration gradient across the mitochondrial membrane. The decrease in the amount of α-oxoglutarate in mitochondria was slight and amounted to about 0.05 nmole/min./mg. of mitochondrial protein. This indicates that the mitochondrial membrane was impermeable to α-oxoglutarate and that, under the conditions applied, i.e. without arsenite in the medium, there was practically no metabolism of α-oxoglutarate. However, it should be noted that some arsenite could be present in mitochondria as this compound had been added during loading of mitochondria with α-oxoglutarate.

Although the mitochondria, after being loaded with α-oxoglutarate, had been washed twice, the mitochondrial suspension contained some extramitochondrial α-oxoglutarate. This is evidenced by the fact that in the system containing no glutamate dehydrogenase, the amount of total α-oxoglutarate was higher than 18 nmoles/mg. protein (Fig. 3), as compared to 11.8 nmoles in samples containing the enzyme.

The reductive amination of extramitochondrial α-oxoglutarate by the applied system, exceeded severalfold the highest velocity of its outflow, even at much lowered concentrations of glutamate dehydrogenase, NADH and the amount of NH_4Cl present in the medium. Glutamate formation was only limited by the amount of α-oxoglutarate moving out of mitochondria.

Simultaneously with the exit of α-oxoglutarate from mitochondria, an entrance of a dicarboxylic anion occurred. The molar ratio of the exchange of α-oxoglutarate for maleate, L-malate or oxaloacetate was close to unity. Therefore it may be supposed that the rate of entry of maleate was the factor limiting the rate of α-oxoglutarate exit. This exit, in the presence of maleate, was not accompanied by a translocation of potassium ion. Similarly, the exit of potassium from mitochondria in the presence

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of gramicidin, was not accompanied by exit of α-oxoglutarate, even in the presence of acetate, an anion which under these conditions penetrates the mitochondrial membrane.

The compounds which stimulate the exit of α-oxoglutarate, are not structurally similar to α-oxoglutarate; glutarate, the structure of which bears the greatest similarity, had practically no effect on the transport of α-oxoglutarate from mitochondria.

According to Chappell & Haarhoff (1967), transport of anions through the mitochondrial membrane occurs with participation of carriers. The carrier responsible for transport of α-oxoglutarate into mitochondria is activated, according to Robinson & Chappell (1967), in the presence of orthophosphate, by L-malate, succinate, maleate, malonate, itaconate or mesotartrate. In our experiments, the same compounds stimulated also the exit of α-oxoglutarate. Succinate, however, which is a stronger activator of the exit of α-oxoglutarate than either maleate or malonate, is a weaker activator of entry of α-oxoglutarate into mitochondria. Since the same compounds activate the translocation of α-oxoglutarate both into and out of mitochondria, it could be supposed that the same carrier is involved in the transport of this compound in either direction. It should be noted that phosphate had no effect on the exit of α-oxoglutarate from mitochondria, even in the presence of maleate (Table 1).

The activating effect of maleate on the exit of α-oxoglutarate from mitochondria may be explained on the basis of the mechanism postulated by De Haan & Tager (1968) concerning the translocation of α-oxoglutarate by an exchange-diffusion reaction with some dicarboxylates, mediated by specific carriers. It may also be supposed that the mechanism of action of the other organic anions studied which activate the transport of α-oxoglutarate, is similar.

The carrier α-oxoglutarate-dicarboxylate would mediate, without participation of orthophosphate, the transport into mitochondria of the anion activating the exit of α-oxoglutarate. According to Meijer *et al.* (1970) this carrier is not susceptible to the inhibitory effect of such inhibitors of transport as mersalyl or *n*-butylmalonate. On the other hand, it is inhibited, but only in its function in the transport of α-oxoglutarate into mitochondria, by L-aspartate (Mc Givan, Bradford & Chappell, 1969).

REFERENCES

Bergmeyer H. U. & Bernt E. (1965). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) p. 324. Academic Press, New York.

Chappell J. B. & Haarhoff K. (1967). In *Biochemistry of Mitochondria* (E. C. Slater, Z. Kaniuga & L. Wojtczak, eds.) p. 75. Academic Press, London; Polish Scientific Publishers, Warszawa.

De Haan E. J. & Tager J. M. (1968). *Biochim. Biophys. Acta* **153**, 98.

Ferguson S. M. F. & Williams G. R. (1966). *J. Biol. Chem.* **241**, 3696.

Gornall A. G., Bardawill C. J. & David M. M. (1949). *J. Biol. Chem.* **177**, 751.

Hohorst H. J. (1965). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) p. 328. Academic Press, New York.

Hohorst H. J. & Reim M. (1965). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) p. 335. Academic Press, New York.

http://rcin.org.pl

Mc Givan J. B., Bradford N. M. & Chappell J. B. (1969). *FEBS Letters* **4**, 247.
Meijer A. J., Lofrumento N. E., Papa S., Tager J. M. & Quagliariello E. (1970). *Biochem. J.* **116**, 35p.
Ochoa S. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 735. Academic Press, New York.
Robinson B. H. & Chappell J. B. (1967). *Biochem. Biophys. Res. Commun.* **28**, 249.
Wójcikowski Cz. & Angielski S. (1970). *Acta Biochim. Polon.* **17**, 291.

AKTYWUJĄCE DZIAŁANIE MALEINIANU NA WYJŚCIE KETOGLUTARANU Z MITOCHONDRII WĄTROBY SZCZURA

Streszczenie

1. Badano wyjście nagromadzonego α-ketoglutaranu z mitochondrii w obecności układu składającego się z NH_4Cl, NADH i dehydrogenazy kwasu L-glutaminowego ("trapping system"). Stwierdzono, że L-jabłczan, bursztynian, maleinian, malonian i szczawiooctan wybitnie aktywują wyjście α-ketoglutaranu z mitochondrii. W obecności maleinianu V_{max} wynosiło 7.1 nmola/min./mg. białka mitochondrialnego, a energia aktywacji wynosiła (+) 17.4 Kcal/mol.

2. Wykazano, że wyjściu α-ketoglutaranu towarzyszy wejście do mitochondrii ekwimolarnej ilości [2,3-^{14}C]L-jabłczanu lub szczawiooctanu, natomiast nie stwierdzono zmian w zawartości jonów K^+ w mitochondriach.

3. Przedyskutowano mechanizm aktywującego działania niektórych kwasów organicznych na wyjście α-ketoglutaranu z mitochondrii.

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EVIDENCE FOR TIGHTLY-BOUND ZINC IN LEUCINE AMINOPEPTIDASE FROM PIG KIDNEY

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1. Leucine aminopeptidase (EC 3.4.1.1.) from pig kidney has been isolated in a homogeneous form and shown to contain as much as five gram atoms of tightly bound zinc per molecular weight of 300 000. 2. Zinc can be removed by dialysis against chelating agents to yield inactive, metal-free enzyme. Reactivation of the latter could not be obtained by restoration of zinc ions; however, it could be achieved with manganese ions in the presence of 2-mercaptoethanol. 3. The native enzyme contains 12 titratable sulphydryl groups, and the metal-free protein, 24 groups. A disappearance of sulphydryl groups is observed when the metal-free protein is incubated with zinc ions, implicating these groups in the role of zinc-protein ligands. 4. Gel filtration on Sephadex G-200 indicates that upon removal of zinc by dialysis against chelating agents the protein tends to aggregate into high molecular weight components which can be disaggregated by means of sulphydryl reagents. 5. The data suggest that both metal ions and sulphydryl groups may act in concert in the aggregation-disaggregation phenomenon of metal-free enzyme and thereby dictate the activity of leucine aminopeptidase.

Several bacterial and mammalian metal-containing peptidases have been described in which zinc plays a direct role in the catalytic activity of the enzyme (Vallee & Neurath, 1954; Prescott & Wilkes, 1966; Hopsu, Makinen & Glenner, 1966; Campbell, Lin, Davis & Ballew, 1966). Although the presence of zinc in kidney leucine aminopeptidase has not been observed, a number of other metals, notably magnesium and manganese, have been implicated in its enzymic activity (Spackmann, Smith & Brown, 1955; Smith & Spackmann, 1955; Davis & Smith, 1957). Investigations conducted in this laboratory on a particle-bound zinc-aminopeptidase from pig kidney (Lisowski & Stein, 1967) have prompted a reexamination of the role of metal

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ions in the activity of leucine aminopeptidase from this tissue. Previous reports (Smith & Spackmann, 1955; Bryce & Rabin, 1964) on leucine aminopeptidase have indicated that preincubation of the enzyme with manganese or magnesium ions enhanced enzymic activity, whereas metal-binding agents were found to be inhibitory. The above authors proposed that addition of manganese ions mediates the binding of the substrate to the enzyme with the possible involvement of secondary interactions between substrate and protein.

A preliminary report from this laboratory (Lisowski & Stein, 1967) on the metal content of leucine aminopeptidase has been confirmed recently by Himmelhoch & Peterson (1968) who reported significant but unspecified amounts of zinc in this enzyme. In the present study, the existence of tightly bound zinc in leucine aminopeptidase has been documented. Although a functional role for this metal could not be demonstrated conclusively, the possibility that zinc is involved both in catalysis and in maintaining the oligomeric structure of the enzyme has been investigated.

MATERIAL AND METHODS

Chemicals. All chemicals were of the highest available analytical grade. Reagent solutions were made in metal-free water obtained from sodium phosphate-treated tap water by double distillation in a Jena still made of Duran glass. Wherever necessary, buffers were extracted with dithizone in carbon tetrachloride and glassware was cleaned as described by Thiers (1956). Visking seamless dialysis tubings were boiled for 15 min. in 0.001 M-ethylenediaminetetraacetic acid, pH 8.0, and rinsed several times with metal-free water before use. Spectrographically pure salts of zinc and manganese purchased from Johnson Matthey Company Ltd. (London, England) were used throughout. *p*-Mercuribenzoate and 5,5′-dithiobis-(2-nitrobenzoic acid)[1] were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and L-leucylamide was a product of Calbiochem (Los Angeles, Calif., U.S.A.). DEAE-Sephadex A-50, coarse grade, and Sephadex G-200 were obtained from Pharmacia (Uppsala, Sweden).

Purification of the enzyme. Purification of leucine aminopeptidase from pig kidney was carried out by the procedure of Hill, Spackmann, Brown & Smith (1958) or Moseley & Melius (1967) up to the acetone fractionation step. Further purification was achieved by column chromatography on DEAE-Sephadex and sucrose density gradient centrifugation according to the general procedure of Martin & Ames (1961). To this effect the 0 - 30% acetone fraction of Hill *et al.* (1958) or the acetone precipitate -2 of Moseley & Melius (1967) was dialysed against 0.01 M-tris - 0.005 M-$MgCl_2$, pH 8.3; up to 1500 mg. were applied to a DEAE-Sephadex column (40×2.5 cm.) previously equilibrated with the same buffer. The column was eluted with a linear gradient of 0 - 0.4 M-NaCl (800 ml. in each reservoir) containing the initial buffer. The flow rate was 60 ml. per hour and fractions of

[1] Abbreviations used: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); SDS, sodium dodecyl sulphate.

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approximately 8 ml. were collected and assayed for enzymic activity and extinction at 280 nm. Fractions with a specific activity above 300 units were combined into one pool and the less active fractions into another. Both pools were concentrated by ultrafiltration in 8/32 in. Visking tubings under reduced pressure and dialysed against the tris-$MgCl_2$ buffer. The concentrated fractions of low specific activity from several DEAE-Sephadex columns were combined and stored for rechromatography.

The concentrated fractions of high specific activity were submitted to sucrose density gradient centrifugation at 4°, using 5% to 20% sucrose (w/v) in 0.01 M-tris-5 mM-$MgCl_2$, pH 8.3. The protein solution was layered on top of the gradient and centrifuged at 2° in a Spinco Model L-2 ultracentrifuge using a SW 25.2 head at 25 000 rev./min. for 36 hr. At the end of the run, 40-drop fractions were collected and assayed for protein and activity. The active fractions were combined, concentrated by ultrafiltration, and dialysed as before.

Enzyme assay. Enzyme activity was assayed routinely at 25° against L-leucylamide by the method of Bryce & Rabin (1964), with or without preincubation with 2 mM-manganese chloride. Titrations were performed at pH 8.4 with 0.01 N-HCl (Titrisol) using a Metrohm Combi-Titrator 3 D. One unit of activity is defined as the amount of protein catalysing the hydrolysis of 1 micromole of substrate per minute at 25°.

Dialysis against metal-binding agents. In order to prepare metal-free leucine aminopeptidase, stock enzyme solutions were diluted to 4 - 5 mg./ml. with 0.1 M-EDTA in 0.05 M-sodium phosphate buffer, pH 7.0, or 0.05 M-tris-acetate, pH 8.0, and dialysed at 25° against 200 volumes of the corresponding buffer solutions. Samples withdrawn at various time intervals were further dialysed for 24 - 48 hr. against four changes of 200 volumes of metal-free 0.05 M-tris-acetate, pH 8.3 (4°), to remove the excess of chelator. Enzymic activity, zinc content and protein concentrations were determined on samples obtained in this manner.

Determination of sulphydryl groups. Sulphydryl groups were assayed by the *p*-mercuribenzoate method of Boyer (1954) utilizing cysteine as a standard. To 400 µl. of 6.6×10^{-6} M solution of protein (2.0 mg./ml.) were added 25 µl. portions of a 1.75×10^{-6} M-*p*-mercuribenzoate solution in 0.05 M-sodium phosphate, pH 7.0, with or without 8 M-urea; 3 min. after each addition, extinction at 250 nm was measured. As an alternate method, the reagent developed by Ellman (1959) was used. Ten µl. of 0.01 M-DTNB solution was added to 400 µl. of 6.6×10^{-6} M-protein solution (or 3.3×10^{-6} M in the case of metal-free enzyme) in 0.1 M-sodium phosphate buffer, pH 7.0. The reaction was followed to completion at 412 nm (25°) both in the presence and in the absence of 0.48% sodium dodecyl sulphate (SDS).

Protein concentration was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Metal analyses were performed with a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Zinc was also determined by the dithizone titration method of Malmstrom (1956), except that instead of using trichloroacetic acid, the protein was co-precipitated with zinc-free bovine serum albumin by heating in a boiling water bath for 20 min.

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Gel filtration was carried out according to Andrews (1965). The enzyme (1.5 mg. in 0.04 ml.) was layered onto a column of Sephadex G-200 (2×38 cm.) equilibrated at 25° with a solution containing 0.1 M-tris - 0.065 M-glycine - 0.1 M-EDTA - 0.1 M-KCl, pH 8.3. The column was eluted with the same buffer, and fractions of 20 drops each were collected. The void volume was measured with the Dextran blue 2000. The column was standardized with apo-ferritin, γ-globulin, ovalbumin and myoglobin, purchased from Mann Research Laboratories (New York, U.S.A.).

Polyacrylamide gel electrophoresis was performed according to Davis (1964) in 7% gel at pH 8.8; EDTA (1 mM) was added to protein samples and buffers. Amido Black was used as a stain.

Ultracentrifugation was carried out in a Spinco Model E ultracentrifuge at a protein concentration of approximately 5 mg/ml. in 0.05 M-tris-acetate - 0.7 M-NaCl, pH 8.3. Sample was centrifuged at 186 000 *g* at 20°.

RESULTS

Enzyme purification. The highly active concentrated preparation of leucine aminopeptidase was submitted to sucrose density gradient centrifugation and two fractions were obtained (Fig. 1). Of the total protein applied to the sucrose gradient, more than 60% of the protein with enzymic activity was associated with the heavier fraction. The remainder of the protein (35 - 40%) was recovered in the lighter inactive fraction. Both fractions were checked for the zinc content. The heavier fraction contained almost all (about 88%) protein-bound zinc. The enzyme was obtained in approximately 20% yield with respect to the acetone powder extract and had a specific activity between 500 - 550 when prepared by the modified method of Hill *et al.* (1958) and above 800 when the initial steps of isolation were carried out according to Moseley & Melius (1967). The product exhibited one sharp protein band upon polyacrylamide gel electrophoresis (Fig. 2) and one major peak in the analytical ultracentrifuge (Fig. 3).

Metal analyses. The specific activity and metal content at the various steps of the purification are presented in Table 1. The zinc content of the preparation did not change appreciably until chromatography on DEAE-Sephadex and sucrose density gradient centrifugation; at these stages both the specific activity and zinc content showed concomitant increases. In contrast, the other metals, including manganese, were either practically absent or decreased in concentration during purification.

Table 2 presents spectrographic and chemical analyses of different preparations of leucine aminopeptidase. Analyses were performed on samples with the highest specific activity obtained after sucrose density gradient centrifugation and following 48 - 72 hr. dialysis against 0.05 M-tris-acetate buffer, pH 8.3. As can be seen, while the specific activity of the preparations varied nearly twofold, all preparations had comparable zinc contents. The molar ratios of zinc to protein range from 4.1 to 5.2 gram atoms per mole, based on a molecular weight of 300 000 (Spackmann *et al.*, 1955). Magnesium was the only other metal consistently present; according

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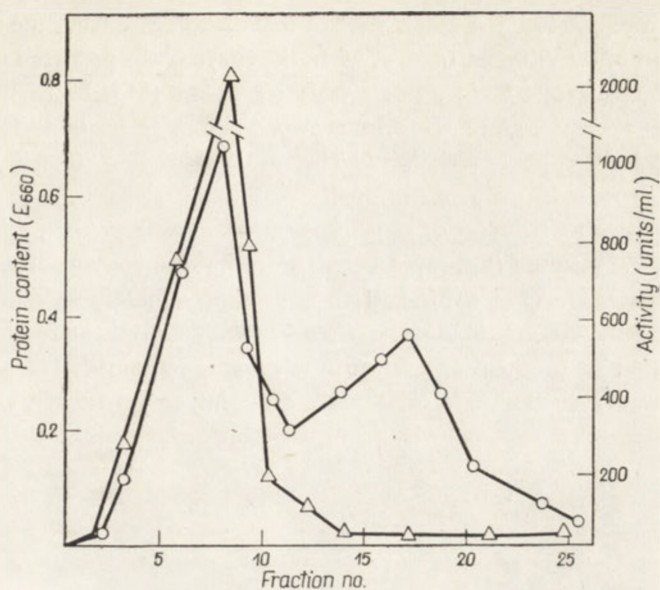

Fig. 1. Sucrose density gradient centrifugation of high activity fractions obtained by DEAE-Sephadex chromatography. Approximately 95 mg. of protein was applied to each gradient and centrifuged in a Spinco Model L-2 ultracentrifuge as described in the text. ○, Extinction at 660 nm (protein); △, enzymic activity.

Fig. 2. Polyacrylamide gel electrophoresis of native leucine aminopeptidase. Pattern displayed by active fractions obtained after sucrose gradient centrifugation. Protein, 100 μg., was applied on the gel and run for 2 hr. at 2 mA per tube in 7% gel at pH 8.8.

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to Spackmann *et al.* (1955), a major part of this metal arose from the use of $MgCl_2$ in the isolation of the enzyme. Indeed, when the enzyme was prepared in the absence of $MgCl_2$, the extract obtained after heat treatment (Moseley & Melius, 1967) contained less than 0.3 gram atom magnesium per mole protein. The content of manganese, cobalt, copper, nickel, and chromium were less than 0.3 gram atom per mole of protein in all preparations.

Preparation and reactivation of metal-free leucine aminopeptidase. Dialysis against EDTA at pH 7.0 resulted in a rapid decrease in the zinc content of leucine aminopeptidase preparations (Fig. 4); in half an hour approximately 80% of the zinc was removed under these conditions while after 12 hr. of dialysis at room temperature only 5% of the zinc originally present in the enzyme remained. The activity of the enzyme followed a similar pattern, rapidly dropping to zero when assayed in the

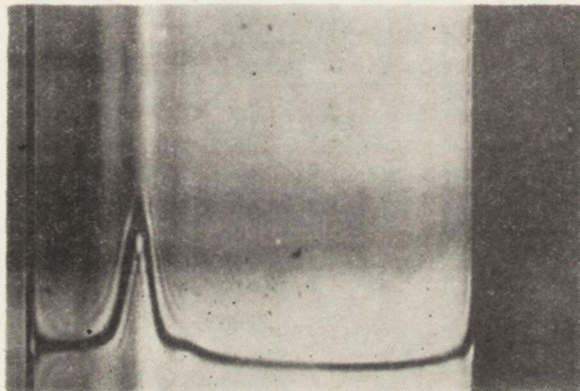

Fig. 3. Ultracentrifuge pattern of native leucine aminopeptidase. Centrifugation was carried out at 186 000 *g* at 20°, bar angle 65°. Enzyme concentration was 5 mg./ml. in 0.05 M-tris-acetate - 0.1 M-NaCl, pH 8.3. Sedimentation is from left to right.

absence of added metal ions. Reactivations of the metal-free enzyme with 0 to 500-fold molar excess of zinc could not be achieved between pH 7.0 and 9.0, the limits of stability of the enzyme. Incubation with zinc for 0 to 3 hr., both in the presence and in the absence of 2-mercaptoethanol (50 mM), at concentrations of substrate ranging from 0.5 mM to 25 mM, also failed to reactivate the metal-free enzyme.

A correlation in the loss of activity and zinc in leucine aminopeptidase became apparent when the dialysis data were corrected for the losses observed in control samples dialysed against 50 mM-tris-acetate, pH 8.0. Thus, dialysis of the enzyme for 12 hr. against this buffer resulted in the loss of 12% of the activity and about 90% of magnesium but no zinc. This large loss in magnesium probably reflects a low binding affinity of the enzyme for this metal. However, when the dialysis was performed against the same buffer containing 0.1 M-EDTA (Fig. 5) the enzyme lost all of its activity and approximately 50% of the zinc. Reactivation with zinc of the zinc-depleted enzyme prepared at pH 8.0 also was unsuccessful under the variety of conditions mentioned before. In this respect, it should be pointed out

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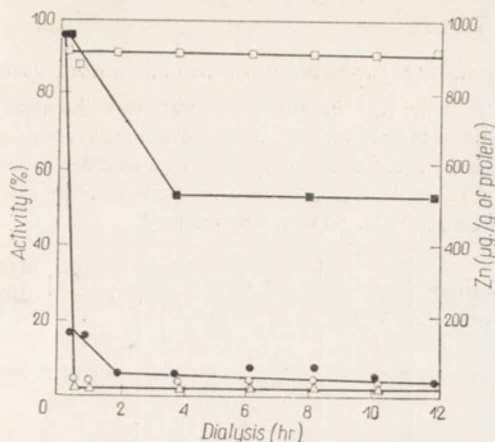

Fig. 4

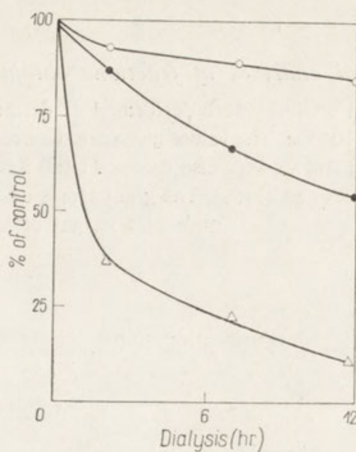

Fig. 5

Fig. 4. Dialysis of the leucine aminopeptidase preparation against 0.1 M-EDTA - 0.05 M-sodium phosphate, pH 7.0, at 25°. Further details in Methods. ●, Zinc content. Activity of the metal-free enzyme: △, in the absence of metal ions, and ○, in the presence of 0 to 500-fold molar excess of added zinc ions; ■, in the presence of 2 mM-manganese; □, in the presence of 2 mM-manganese and 5 mM-mercaptoethanol.

Fig. 5. Dialysis of the leucine aminopeptidase preparation against 0.1 M-EDTA - 0.05 M-tris-acetate pH 8.0. ●, Zinc content; ○, magnesium content; △, activity in the absence of added metal ions. The control represents enzyme dialysed against 0.05 M-tris-acetate, pH 8.0.

that the activity of native, zinc-containing enzyme was inhibited approximately 40% by a 300-fold molar excess of zinc ions.

Incubation of metal-free enzyme with 2 mM-magnesium ions reactivated the enzyme to about 25% of the activity of a control sample. Addition of magnesium in conjunction with 2 mM-zinc or 5 mM-2-mercaptoethanol did not affect the extent of reactivation of metal-free leucine aminopeptidase.

The most intriguing observation concerned the results obtained with manganese, a metal which activates the native enzyme two- to threefold (Smith & Spackmann, 1955) but which was not found in significant quantities (less than 0.3 gram atom per mole) in the purified enzyme. Incubation for one hour with 2 mM-manganese ions restored 50 - 60% of the activity of the metal-free enzyme. This reactivation by manganese ions was essentially complete when 5 mM-2-mercaptoethanol was included in the incubation mixture and during the enzymic assay (Fig. 4). In the presence of 2 mM-zinc, reactivation of metal-free enzyme by manganese was inhibited; the recovery of activity was decreased to 30% in the absence of 5 mM-2-mercaptoethanol and approximately 70% in the presence of this reagent.

Sulphydryl groups. The native enzyme displayed a total of 12 titratable sulphydryl groups (Table 3) three of which were less readily available for reaction with sulphydryl reagents since they were titrated only upon exposure of the enzyme to 8 M-urea or SDS. The number of titratable sulphydryl groups of the native enzyme was unchanged when the protein was incubated with zinc or manganese in the absence

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Table 1

Metal analyses of fractions obtained during purification of leucine aminopeptidase

Metal analyses were performed as described in the text. Protein concentration in the assay was 6.6×10^{-6} M. The values given are the averages of at least ten different preparations. Trace amounts of Cu and Cr were also detected in all fractions. The enzyme was prepared by a modified procedure of Hill *et al.* (1958). The gram atoms of zinc per mole were calculated on the basis of an assumed molecular weight of protein, 300 000. ND, not determined.

Purification step	Activity (units/ /mg. protein)	Yield (%)	Zinc		Manganese	Magnesium
			µg./g. protein	gram atoms/ /mole	µg./g. protein	
Acetone powder extract	2.9	100	285	1.3	305	580
Ammonium sulphate 40 - 80%	5.8	83	300	1.4	ND	ND
Ammonium sulphate 50 - 70%	13.0	67	280	1.3	250	420
Mg^{2+} + heat	47.0	52	230	1.1	120	470
Acetone fraction 0 - 30%	74.0	45	305	1.4	ND	ND
DEAE-Sephadex	344.0	34	1000	4.6	0	385
Sucrose density gradient	515.0	21	1100	5.1	0	100 - - 200

Table 2

Metal content and activity of leucine aminopeptidase

For determinations, the preparations obtained by sucrose density gradient centrifugation were used. The first steps of enzyme isolation were carried out: for preparations 1 - 4 after Hill *et al.* (1958), and for preparations 5 and 6, after Moseley & Melius (1967). ND, not determined.

Preparation no.	Activity (units/mg. protein)	Dithizone method	Spectrographic data			
			Zinc		Manganese	Magnesium
		Zinc (µg./g. protein)	µg./g. of protein	gram atoms/ mole	µg./g. of protein	
1	540	ND	1120	5.1	30	90
2	550	ND	1130	5.2	0	150
3	460	ND	1090	5.0	10	200
4	470	1040	1060	4.9	18	100
5	820	1100	970	4.5	0	130
6	850	800	897	4.1	11	120

of SDS. The removal of metals from the native enzyme resulted in the appearance of additional sulphydryl groups. Furthermore, the 24 sulphydryl groups in the metal-free protein were available for titration both in the presence and absence of denaturing agents. Incubation of metal-free enzyme with a 35-fold molar excess of zinc resulted in a decrease in the number of titratable sulphydryl groups from

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Table 3

Number of titratable sulphydryl groups in leucine aminopeptidase

Sulphydryl groups were determined in Expt. I by the procedure of Boyer (1954), and in Expt. II, by the procedure of Ellman (1959). The zinc-free enzyme employed contained 0.2 - 0.4 gram atom zinc per mole of protein. ND, not determined.

Determination in the presence of	Expt. I		Expt. II	
	Enzyme		Enzyme	
	Native	Zn-free	Native	Zn-free
	SH groups/mole protein			
No additions	8	23	10	24
0.48% SDS	12	23	12	25
8 M-urea	13	21	11	23
2.3×10^{-4} M-$ZnSO_4$	8	11	ND	ND
2.3×10^{-4} M-$MnCl_2$	8	14	ND	ND

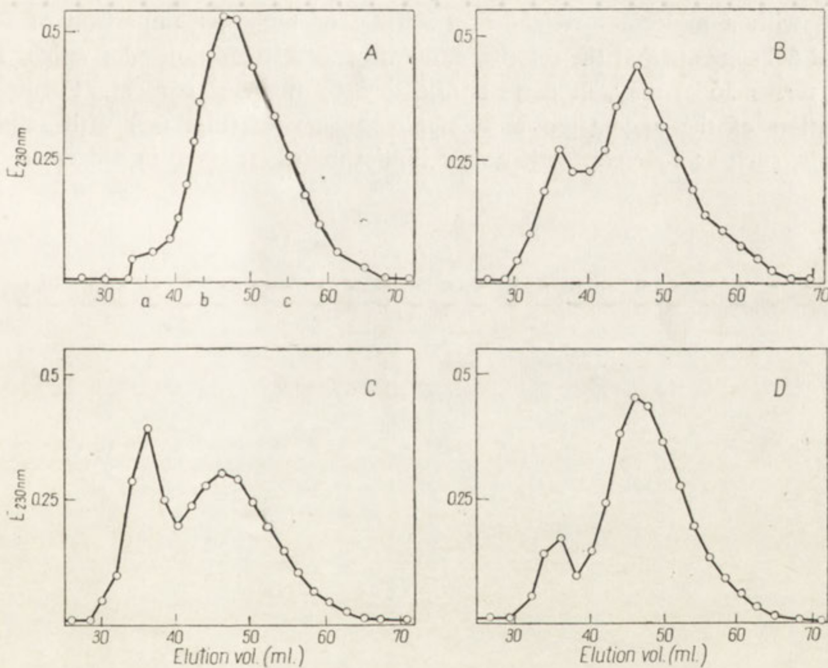

Fig. 6. Elution diagram from Sephadex G-200 columns. The gel filtration was performed as described in the text and the fractions detected by measurement of extinction at 230 nm. *A*, Native leucine aminopeptidase containing 5 gram atoms of zinc per mole protein; *a*, position of blue dextran (mol. wt. $> 10^6$); *b*, apo-ferritin (mol. wt. 4.8×10^5); *c*, γ-globulin (mol. wt. 1.6×10^5). *B*, Enzyme after 2 hr. dialysis against 0.1 M-EDTA - 0.05 M-tris-acetate, pH 8.0, and containing 4 gram atoms of zinc. *C*, Enzyme after dialysis for 24 hr. against the same buffer and containing 1 gram atom of zinc. *D*, preparation *C* incubated for 6 hr. with 2-mercaptoethanol (0.05 M).

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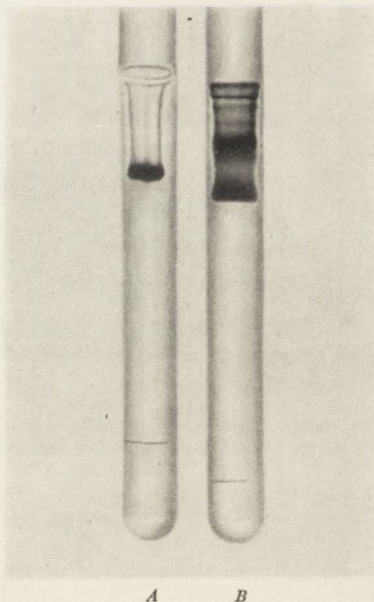
24 to 11, whereas, when manganese ions were added at the same concentration, 14 sulphydryl groups could be titrated. Preincubation of the native enzyme with a 20-fold molar excess of *p*-mercuribenzoate for 2 hr. or with DTNB for 5 hr. (9 moles of DTNB were incorporated) resulted in approximately 50% inhibition of enzymic activity. In addition, titration of metal-free enzyme with sulphydryl reagents prevented the reconstitution of the inactive protein with manganese ions. Similar results have been also reported with magnesium ions (Bryce & Rabin, 1964).

Gel filtration and polyacrylamide gel electrophoresis. The apparent inability of zinc to restore enzymic activity to preparations of metal-free leucine aminopeptidase under the conditions employed, prompted an examination of its importance as a structural component. To investigate whether tightly bound zinc in leucine aminopeptidase plays a structural role, gel filtration on Sephadex G-200 was performed as described by Andrews (1965). The native enzyme afforded a single major peak with an elution volume to void volume ratio (V_e/V_0) of 1.26 - 1.30. This value supports the molecular weight of 300 000 reported by Spackmann *et al.* (1955) (Fig. 6A). In contrast, the metal-depleted enzyme revealed two peaks, one with V_e/V_0 of 1.0, and the other with a value comparable to that of the native enzyme: 1.2 - 1.3 (Fig. 6B and C). The peak that emerged with the void volume corresponded to species with a molecular weight of 1 000 000 or higher. Comparison of Figures 6B and 6C suggests that the relative amount of these high molecular weight species (25% *versus* 36%) may, in part, be dictated by the zinc content. Furthermore, incubation of the aggregated metal-depleted enzyme (Fig. 6C) with sulphydryl reagents, such as 2-mercaptoethanol or dithiothreitol, resulted in a decrease in the



A *B*

Fig. 7. Polyacrylamide gel electrophoresis of *A*, native and *B*, metal-free leucine aminopeptidase. Experimental details were similar to those in Fig. 2, except that 1 mM-EDTA was added to protein samples and buffers. 100 μg. of each protein was applied on the gel.

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proportion of the high molecular weight component (Fig. 6D). Evidence for dissociation of native or metal-free enzyme was not obtained in these studies.

Aggregation of the metal-free enzyme was observed upon polyacrylamide gel electrophoresis in EDTA (Fig. 7). Thus, in addition to a component corresponding to the native enzyme, several slower migrating bands, presumably consisting of high molecular weight components, were also visible. A similar picture was obtained when the native enzyme was incubated for 1 hr. in the presence of 8 M-urea at pH 8.3 and subjected to electrophoresis in urea-containing acrylamide gels.

DISCUSSION

The inclusion of a sucrose density gradient centrifugation step in the purification of leucine aminopeptidase resulted in a significant increase in the purity of the enzyme. A maximum enrichment of activity of 290-fold obtained here compares with a value of 200-fold and 140-fold for the enzyme described by Moseley & Melius (1967) and Hill *et al.* (1958), respectively. Highly purified preparations of leucine aminopeptidase displayed one protein-staining band when subjected to polyacrylamide gel electrophoresis (Fig. 2) and one major peak upon sedimentation in the ultracentrifuge (Fig. 3). Although this enzyme originated from pooled pig kidneys, no evidence was obtained for the existence of multiple forms of the enzyme as reported by Himmelhoch & Peterson (1968).

Purified preparations of leucine aminopeptidase contain 4 - 5 gram atoms of zinc per mole of protein, assuming a molecular weight of 300 000 (Spackmann *et al.*, 1955). This represents a 4 to 5-fold enrichment of zinc during purification of the enzyme, as compared to a 160 to 290-fold increase in activity. This discrepancy probably reflects the relatively high concentration of zinc found in kidney extracts. The lack of direct correlation between zinc content and enzymic activity (Table 2) observed in leucine aminopeptidase prepared by two different procedures indicates that a proportion of the zinc found in the purified enzyme is not essential for function. Similar observations have been reported for alkaline phosphatase from *E. coli* (Simpson, Vallee & Tait, 1968) and for horse liver alcohol dehydrogenase (Drum, Li & Vallee, 1969).

Other possibilities that might account for the lack of relationship between zinc and activity of the enzyme may include differences inherent in the isolation procedures as a result of which certain preparations contain inactive zinc-leucine aminopeptidase. Ammonium sulphate fractionation as well as the high concentrations of magnesium ions added to stabilize the enzyme during purification may displace the intrinsically bound zinc leading to alterations in the zinc content (Simpson *et al.*, 1968). Uncertainties in the molecular weight of the enzyme, an improper selection of representative samples for analysis and considerations of isozymic forms may all bear upon the varied stoichiometry of zinc (Drum *et al.*, 1969).

Besides zinc, the only other metal present in purified preparations of leucine aminopeptidase was magnesium, the content of which varied from 1.2 to 2.4 gram

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atoms per mole of protein. However, magnesium might be of extrinsic origin, since, as discussed above, substantial amounts of magnesium chloride are used during purification of the enzyme. Although the catalytic significance of the magnesium associated with the protein is at present uncertain, it is well known that this metal confers stability to the enzyme when added in high concentrations (Spackmann *et al.*, 1955; Smith & Spackmann, 1955; Hill *et al.*, 1958). The concentration of metals other than zinc (Mn, Cu, Co, Cr) decreased during the course of isolation of leucine aminopeptidase, less than 0.3 gram atoms per mole of purified protein being detected.

Dialysis of leucine aminopeptidase against EDTA resulted in the production of inactive metal-free enzyme. A correlation in the loss of zinc and activity was seen during the initial 12 - 15 hr. of dialysis at pH 8.0. However, the loss of only 2 - 3 gram atoms of zinc per mole protein was sufficient to cause a complete loss of enzymic activity (Fig. 5), suggesting that, at most, only a portion of the zinc in leucine aminopeptidase is directly associated with activity. The activity of the metal-free enzyme could not be restored upon addition of zinc ions under a variety of conditions. Interpretation of these results was complicated by the observation that zinc is capable of inhibiting the activity of the native enzyme.

Manganese was capable of restoring partial activity to metal-free leucine aminopeptidase. Complete reactivation was obtained only in the presence of manganese and 2-mercaptoethanol (Fig. 4). This stimulation by 2-mercaptoethanol implicates sulphydryl groups, in addition to metal ions, in the catalytic activity of the enzyme although it is conceivable that the sulphydryl reagent only helps to maintain manganese ions in an active manganous (II-valent) form.

The postulate that sulphydryl groups participate directly or indirectly in metal binding and activity of leucine aminopeptidase is supported by: (1), the exposure of 12 additional sulphydryl groups on removal of metal from the native enzyme; (2), their resistance to titration after incubation with zinc or manganese, and (3), the inability to reconstitute the metal-free enzyme after its treatment with sulphydryl blocking reagents. Chemical evidence for zinc binding at sulphydryl groups has been obtained for yeast alcohol dehydrogenase (Vallee & Hoch, 1955) and horse liver alcohol dehydrogenase (Oppenheimer, Green & McKay, 1967).

In several instances, metals have been shown to stabilize the quarternary structure of a molecule by establishing cross-links between subunits. Thus, removal of metal from bacterial α-amylase (Stein & Fischer, 1960), yeast alcohol dehydrogenase (Kagi & Vallee, 1960), and beef liver glutamic dehydrogenase (Frieden, 1958) results in dissociation of the molecule. In contrast, the metal-free preparations of leucine aminopeptidase observed in this study exhibited a tendency to form high molecular weight species. Presumably, this phenomenon is an outcome of the aggregation either of metal-free leucine aminopeptidase or of dissociated metal--free subunits. Aggregation of metal-free enzyme was substantiated by the results obtained from gel filtration experiments on Sephadex G-200 (Fig. 6) and acrylamide gel electrophoresis (Fig. 7). As expected, the process of aggregation was concentration-dependent; higher concentrations of proteins favouring aggregation.

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Aggregates of molecular weight 1 000 000 have been observed with leucine aminopeptidase from both bovine dental pulp (Schwabe, 1969) and bovine lens (Kretschmer & Hanson, 1968).

The influence of 2-mercaptoethanol on the aggregation-disaggregation phenomenon (Fig. 6) suggests that aggregation of metal-free protein is a consequence of sulphydryl interaction and formation of disulphide bonds. A similar situation of disulphide bond formation is observed in horse liver alcohol dehydrogenase (Oppenheimer *et al.*, 1967). The tendency of sulphydryl reagents to favour disaggregation of the metal-free enzyme may be related to the 2-mercaptoethanol-dependent stimulation of enzymic activity discussed above, but, in this situation, one would have to assume that the aggregated enzyme is inactive.

In the instance of the native enzyme, aggregation by random interaction of free sulphydryl groups is precluded probably by steric restrictions (Cecil, 1963). Nevertheless, such interactions have been visualized for maintenance of the subunit structure of native leucine aminopeptidase from bovine dental pulp (Schwabe, 1969).

Although a definitive role for the zinc in leucine aminopeptidase could not be demonstrated in this study, the available evidence suggests that this metal is involved in both the structural and functional integrity of the enzyme. Further definition of the role of metals in this enzyme must await an exhaustive examination of its subunit structure, conditions of reconstitution of metal-free enzyme, the effect of zinc on the native enzyme, and the participation of sulphydryl groups both in metal-binding and in the aggregation-disaggregation phenomenon.

When the presented work was finished and prepared for press, a paper by Himmelhoch (1969) was published. The author has also proved that leucine aminopeptidase from pig kidney is a zinc-containing enzyme with 4 to 6 gram atoms of Zn per mole of protein. These results are in fairly good agreement with those presented in our work. However, in contrast to the results of Himmelhoch (1969), our attempts to reactivate with Zn the metal-free enzyme were unsuccessful.

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REFERENCES

Andrews P. (1965). *Biochem. J.* **96**, 595.

Boyer P. D. (1954). *J. Am. chem. Soc.* **76**, 4331.

Bryce G. K. & Rabin E. R. (1964). *Biochem. J.* **90**, 513.

Campbell B. J., Lin A., Davis R. V. & Ballew E. (1966). *Biochim. biophys. Acta* **118**, 371.

Cecil R. (1963). In *The Proteins* (H. Neurath, ed.) vol. 1, p. 379, Academic Press, New York.

http://rcin.org.pl

Davis B. J. (1964). *Ann. N. Y. Acad. Sci.* **121**, 404.
Davis N. C. & Smith E. L. (1957). *J. biol. Chem.* **224**, 261.
Drum D. E., Li T-K. & Vallee B. L. (1969). *Biochemistry* **8**, 3783.
Ellman G. L. (1959). *Archs. Biochem. Biophys.* **82**, 70.
Frieden C. (1958). *Biochim. biophys. Acta* **27**, 431.
Hill R. L., Spackmann D. H., Brown D. M. & Smith E. L. (1958). *Biochem. Prep.* **6**, 35.
Himmelhoch S. R. (1969). *Archs. Biochem. Biophys.* **134**, 597.
Himmelhoch S. R. & Peterson E. A. (1968). *Biochemistry* **7**, 2085.
Hopsu V. K., Makinen K. K. & Glenner G. G. (1966). *Archs. Biochem. Biophys.* **114**, 557.
Kagi J. H. R. & Vallee B. L. (1960). *J. biol. Chem.* **235**, 3188.
Kretschmer K. &. Hanson H. (1968). *Hoppe-Seyler's Z. physiol. Chem.* **349**, 831.
Lisowski J. &. Stein E. A. (1967). *4th FEBS Meeting, Oslo.* Abstr. of Commun. no. 598.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.
Malmstrom B. G. (1956). In *Methods of Biochemical Analysis* (D. Glick, ed.) vol. 3, p. 327. Interscience Publishers, New York.
Martin R. G. & Ames B. N. (1961). *J. biol. Chem.* **236**, 1372.
Moseley M. H. & Melius P. (1967). *Can. J. Biochem.* **45**, 1641.
Oppenheimer H. L., Green R. W. & McKay R. H. (1967). *Archs. Biochem. Biophys.* **119**, 552.
Prescott J. M. & Wilkes S. H. (1966). *Archs. Biochem. Biophys.* **117**, 328.
Schwabe C. (1969). *Biochemistry* **8**, 783.
Simpson R. T., Vallee B. L. & Tait G. H. (1968). *Biochemistry* **7**, 4336.
Smith E. L. & Spackmann D. H. (1955). *J. biol. Chem.* **212**, 271.
Spackmann D. H., Smith E. L. & Brown D. H. (1955). *J. biol. Chem.* **212**, 255.
Stein E. A. & Fischer E. H. (1960). *Biochim. biophys. Acta* **39**, 287.
Thiers R. E. (1956). In *Methods of Biochemical Analysis* (D. Glick, ed.) vol. 5, p. 273. Interscience Publishers, New York.
Vallee B. L. & Hoch F. L. (1955). *Proc. natn. Acad. Sci. U.S.A.* **41**, 327.
Vallee B. L. & Neurath H. (1954). *J. Am. Chem. Soc.* **76**, 5006.

ISTNIENIE TRWALE ZWIĄZANEGO CYNKU W LEUCYLOAMINOPEPTYDAZIE Z NERKI ŚWINI

Streszczenie

1. Otrzymano w formie jednorodnej leucyloaminopeptydazę (EC 3.4.1.1.) z nerki świni i wykazano, że zawiera ona do 5 gramo-atomów silnie związanego cynku na mol białka (cięż. cząsteczk. 300 000).

2. Dializa enzymu do roztworów substancji wiążących jony metali powoduje usunięcie cynku z białka. Otrzymany apo-enzym jest nieaktywny. Dodanie jonów cynku nie powodowało reaktywacji enzymu; pełną reaktywację obserwowano natomiast w obecności jonów Mn^{2+} i 2-merkaptoetanolu.

3. Rodzimy enzym zawiera 12 oznaczalnych grup -SH, natomiast apo-enzym zawiera 24 grupy. Inkubacja apo-enzymu z jonami cynku powoduje obniżenie ilości oznaczalnych grup -SH. Wskazuje to na możliwość udziału grup -SH w wiązaniu jonów cynku przez enzym.

4. Badania za pomocą filtracji w żelach wykazały, że usuwanie cynku z enzymu sprzyja tworzeniu agregatów o wyższym ciężarze cząsteczkowym. Agregaty te mogą być rozdysocjowane działaniem substancji sulfhydrylowych.

5. Uzyskane wyniki sugerują, że zarówno jony metali, jak i grupy -SH grają rolę w zjawisku agregacji i dysocjacji pozbawionej cynku leucyloaminopeptydazy i w ten sposób wpływają na jej aktywność enzymatyczną.

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INTERMEDIATES IN THE SYNTHESIS OF PURINES AND PTERIDINES: *N*-METHYLATED 6-CHLOROURACILS

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The N_3-methylated derivative of 6-chlorouracil may be prepared by treatment of 6-chlorouracil in strongly alkaline medium with dimethylsulphate. The hitherto unreported N_1-methyl-6-chlorouracil is readily obtained by reaction of 6-chlorouracil in anhydrous ethanol with diazomethane. The 1,3-dimethyl-6-chlorouracil may be obtained by treatment of 3-methyl-6-chlorouracil with dimethylsulphate in weakly alkaline medium, or by prolonged treatment of 6-chlorouracil in anhydrous ethanol with diazomethane. Dimethylated 6-chlorouracil exhibits the alkaline lability typical for a number of 1,3-dimethyl-2,4-diketo pyrimidines. The acid strengthening effect of the 6-halogeno substituent has been measured and compared with that for other 5- and 6-halogeno 2,4-diketopyrimidines.

The *N*-methylated derivatives of 6-chlorouracil are intermediates in the synthesis of methylated 6-aminouracils, purines and pteridines (e.g. Nübel & Pfleiderer, 1962; Pfleiderer & Bühler, 1966), and syntheses of 3-methyl-6-chlorouracil and 1,3-dimethyl-6-chlorouracil have already been described. The former may be prepared from 2-thiobarbituric acid by methylation with dimethylsulphate, chlorination of the appropriate *N*- and *S*-methyl derivatives, and acid hydrolysis of the appropriate chloro derivative (Daves, Robins & Cheng, 1962); or by chlorination with $POCl_3$ in aqueous medium of *N*-methylbarbituric acid (Nübel & Pfleiderer, 1962). The dimethylated derivative may be obtained by reacting 2,4-dimethoxy-6-chloropyrimidine with methyl iodide (Fisher & Johnson, 1932), as well as by chlorination of 1,3-dimethylbarbituric acid with $POCl_3$ containing 4% water (Pfleiderer & Schündehütte, 1958). As far as we are aware, the synthesis of 1-methyl-6-chlorouracil has not been reported.

We have now found that all three *N*-methylated derivatives of 6-chlorouracil can be prepared in a much simpler manner by the direct methylation of the readily accessible 6-chlorouracil (Cresswell & Wood, 1960), as illustrated in Scheme 1, the course of the reactions being followed by thin-layer and paper chromatography (Table 1).

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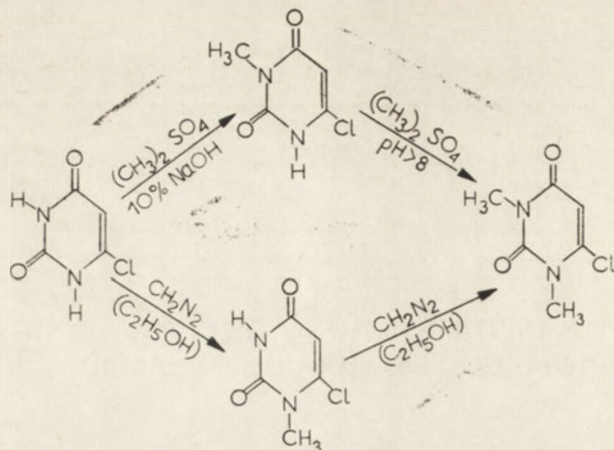

Scheme 1

Treatment of 6-chlorouracil with dimethylsulphate in strongly alkaline medium led to the isolation of 3-methyl-6-chlorouracil in better than 50% yield. The dimethyl derivative of 6-chlorouracil was the major product resulting from the treatment of 3-methyl-6-chlorouracil in 0.2 N-NaOH with dimethylsulphate, care being taken to maintain the pH of the reaction mixture between about 8 and 12; the resulting 1,3-dimethyl-6-chlorouracil, following recrystallization, is obtained in almost 70% yield. The necessity for pH control in this instance is due to the known alkaline lability of a variety of 1,3-dialkyl-2,4-diketopyrimidines (e.g. Shugar & Fox, 1952; Janion & Shugar, 1960). In effect, at pH 13 (0.1 N-NaOH) and 20° the decomposition of 1,3-dimethyl-6-chlorouracil, followed by the rate of disappearance of its characteristic ultraviolet absorption, proceeds with a rate constant of 2.5×10^{-2} min^{-1}. For a compound such as 1,3-dimethyl-5-bromouracil the rate-constant for decomposition, although not measured quantitatively, has been reported to be much higher under these conditions (Berens & Shugar, 1963).

Methylation of 6-chlorouracil in anhydrous ethanol with diazomethane, with termination of the reaction following the disappearance of the starting compound, led to isolation of the required 1-methyl-6-chlorouracil in better than 50% yield. The product gave an elementary analysis corresponding to that for a monomethylated 6-chlorouracil, but with a melting point (196 - 197°) much lower than that for 3-methyl-6-chlorouracil (276°). Furthermore its absorption spectrum (Fig. 1) in neutral and alkaline media is precisely that expected for an N_1-alkylated 2,4-diketopyrimidine (Shugar & Fox, 1952).

Spectral titration of 1-methyl-6-chlorouracil in alkaline medium (Fig. 1) gave a pK_a for dissociation of the N_3 proton at 20° of 9.05. Since the pK_a for the corresponding 1-methyluracil is 9.5 (Shugar & Fox, 1952), there is a moderate acid strengthening effect due to the presence of the halogen substituent *para* to the N_3 proton. When the halogeno substituent is *meta* to the N_3 proton, the acid strengthening effect is increased as in the case of 5-chlorouridine (an analogue of the unavailable 1-methyl-

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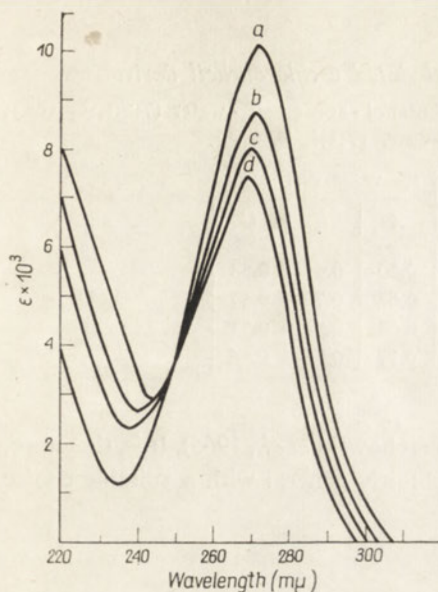

Fig. 1. Absorption spectrum of 1-methyl-6-chlorouracil at: *a*, pH 2-7; *b*, pH 8.95; *c*, pH 9.44; *d*, pH 12. Calculated pK_a at 20°, 9.05.

5-chlorouracil), pK_a 8.20 (Berens & Shugar, 1963). Even more striking is the case of an *ortho* substituent, as demonstrated by Wempen & Fox (1964) for 3-methyl-6-chlorouracil, the pK_a of which (for dissociation of the N_1 proton) is 5.87. The similarity of this latter value with that for 6-chlorouracil itself (5.64), together with a comparison of the absorption spectra of the monoanionic forms of the two compounds, demonstrated that the monoanionic form of 6-chlorouracil does not form an equilibrium mixture of two monoanionic species (Nakanishi, Suzuki & Jamazaki, 1961; Wempen & Fox, 1964; Wierzchowski, Litońska & Shugar, 1965), but only one corresponding to dissociation of the N_1 proton. If any further confirmation of this conclusion were needed, it is provided by both the absorption spectrum of the monoanionic form of 1-methyl-6-chlorouracil (Fig. 1) and the pK_a of the latter.

It remains to add that prolonged treatment of 6-chlorouracil in anhydrous ethanol with diazomethane leads to the formation in almost quantitative yield of 1,3-dimethyl-6-chlorouracil.

It requires only a cursory survey of the literature to demonstrate the possible extension of the above procedures to the preparation of all three *N*-methylated derivatives of 2,4-diketopyrimidine analogues in those instances where the parent pyrimidine is readily accessible. For example, it was long ago noted that treatment of 6-methyluracil with dimethylsulphate in moderately alkaline medium leads initially to methylation on N_3 (Scannell, Crestfield & Allen, 1959). Pfleiderer & Nübel (1961) prepared 1-methyl-6-methylaminouracil by reaction of 6-methylaminouracil with dimethylsulphate in moderately alkaline medium. More striking, perhaps, was the demonstration that carefully controlled treatment of 5-fluorouracil with dimethylsulphate in aqueous medium could be used for the isolation of both 1-methyl-

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Table 1

Paper chromatography (ascending) of methylated 6-chlorouracil derivatives

Solvent systems: *A*, water-saturated *n*-butanol; *B*, *n*-butanol - acetic acid - water (7:1:1, by vol.); *C*, *n*-propanol - conc. NH_4OH - water (7:2:1, by vol.).

Compound	R_F value in solvent		
	A	*B*	*C*
6-Chlorouracil	0.50	0.65	0.53
1-Methyl-6-chlorouracil	0.62	0.70	0.57
3-Methyl-6-chlorouracil	0.68	0.81	0.73
1,3-Dimethyl-6-chlorouracil	0.77	0.85	0.86

5-fluorouracil and 3-methyl-5-fluorouracil (Wierzchowski *et al.*, 1965). It is, therefore, clear that the procedures described above are fairly general with a suitable choice of solvent and methylating agent.

EXPERIMENTAL

The starting compound, 6-chlorouracil, was prepared by chlorination of barbituric acid, followed by hydrolysis, as described by Cresswell & Wood (1960). Melting points (uncorrected) were measured on a Boetius microscope hot stage. Absorption spectra in the UV were measured on a Zeiss VSU2-P instrument. All solutions were made up in glass-distilled water. Measurements of pH made use of a Radiometer Model PHM 4d instrument, with glass electrode. For extreme pH values, use was made of 0.01 N-HCl (pH 2) and 0.01 N-NaOH (pH 12). Elementary analyses were kindly performed by the Dept. of Organic Chemistry, University of Warsaw.

3-Methyl-6-chlorouracil: 4 g. of 6-chlorouracil was dissolved in 30 ml. of 10% NaOH and, with constant stirring, 6 ml. of dimethylsulphate was added dropwise over a period of 2.5 hr. at room temperature. The mixture was stirred for an additional 30 min., acidified with conc. HCl and left overnight in the cold room. The resulting crystalline precipitate was removed by filtration and recrystallized from water to give 2.2 g. (51%) with m.p. 275 - 276°, as compared to 276 - 277° reported by Nübel & Pfleiderer (1962).

1,3-Dimethyl-6-chlorouracil: (*a*) 1.5 g. of 3-methyl-6-chlorouracil was dissolved in 20 ml. of 0.2 N-NaOH and, with constant and vigorous stirring, 2.5 ml. dimethylsulphate was added over a period of 2 hr., the pH being maintained above 8 by repeated addition of 2 N-NaOH with a micropipette. Following completion of the reaction, as indicated by disappearance of the starting product by thin-layer chromatography on GF_{254} silica gel with chloroform as solvent, the solution was neutralized and extracted with 4 portions of 15 ml. chloroform. The chloroform extract was brought to dryness and the residue recrystallized from water to give 1.1 g. (68%) with m.p. 110 - 112°, as compared to 113° reported by Pfleiderer & Schündehütte (1958).

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(*b*) Prolonged treatment of 6-chlorouracil in anhydrous ethanol with diazomethane gave one product in almost quantitative yield.

1-Methyl-6-chlorouracil: 1 g. of 6-chlorouracil was dissolved in 150 ml. anhydrous ethanol, followed by the portionwise addition of 40 ml. of an ethereal solution of diazomethane (2.4 g./100 ml. ether). The reaction was allowed to proceed until the starting compound had disappeared, as shown by thin-layer chromatography on GF_{254} silica gel with chloroform as solvent; at 20°, this occurred within 40 min. The solution was then brought to dryness and the residue crystallized from 50% ethanol to give 570 mg. (52%) with m.p. 192 - 194°, and containing traces of 1,3-dimethyl-6-chlorouracil. Recrystallization from methanol raised the m.p. to 196 - 197° and gave a single spot on chromatography. For $C_5H_5N_2O_2Cl$: Calculated C, 37.4%: H, 3.11%; N, 17.5%; Observed C, 37.6%; H, 3.15%; N, 17.6%.

REFERENCES

Berens K. & Shugar D. (1963). *Acta Biochim. Polon.* **10**, 25.
Cresswell R. M. & Wood H. C. S. (1960). *J. Chem. Soc.* 4768.
Daves G. D., Jr, Robins R. K. & Cheng C. C. (1962). *J. Am. Chem. Soc.* **84**, 1724.
Fisher H. J. & Johnson T. B. (1932). *J. Am. Chem. Soc.* **54**, 727.
Janion C. & Shugar D. (1960). *Acta Biochim. Polon.* **7**, 309.
Nakanishi K., Suzuki N. & Jamazaki P. (1961). *Bull. Chem. Soc. Japan* **34**, 53.
Nübel G. & Pfleiderer W. (1962). *Chem. Ber.* **95**, 1605.
Pfleiderer W. & Bühler E. (1966). *Chem. Ber.* **99**, 3022.
Pfleiderer W. & Nübel G. (1961). *Liebigs Ann. Chem.* **647**, 155.
Pfleiderer W. & Schündehütte K. H. (1958). *Liebigs Ann. Chem.* **612**, 158.
Scannell J. P., Crestfield A. M. & Allen F. W. (1959). *Biochim. Biophys. Acta* **32**, 406.
Shugar D. & Fox J. J. (1952). *Biochim. Biophys. Acta* **9**, 199.
Wempen I. & Fox J. J. (1964). *J. Am. Chem. Soc.* **86**, 2474.
Wierzchowski K. L., Litońska E. & Shugar D. (1965). *J. Am. Chem. Soc.* **87**, 4621.

SUBSTRATY DO SYNTEZY PURYN I PTERYDYN: N-METYLOWANE 6-CHLOROURACYLE

Streszczenie

3-Metylo-6-chlorouracyl otrzymano działając na 6-chlorouracyl siarczanem dwumetylu w środowisku silnie alkalicznym, 1-metylo-6-chlorouracyl przez metylowanie 6-chlorouracylu dwuazometanem w bezwodnym alkoholu etylowym, 1,3-dwumetylo-6-chlorouracyl metylując 3-metylo-6-chlorouracyl siarczanem dwumetylu w środowisku słabo alkalicznym oraz poprzez wyczerpujące metylowanie 6-chlorouracylu dwuazometanem w alkoholu etylowym. 1,3-Dwumetylo-6-chlorouracyl jest nietrwały w środowisku alkalicznym podobnie jak i większość 1,3-dwumetylo-2,4-dwuketopirymidyn. Zmierzono i porównano z innymi 5- i 6-halogeno-2,4-dwuketopirymidynami wpływ chloru w pozycji 6 na charakter kwasowy związku.

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CARBOXYLATION OF PROPIONYL-CoA IN ERYTHROMYCIN BIOSYNTHESIS

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1. The time course of the activity of propionyl-CoA carboxylase (EC 6.4.1.3) and methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1) in *S. erythreus* indicates participation of the former enzyme in biosynthesis of erythronolide, a polypropionate lactone ring of erythromycin. 2. The activity of propionyl-CoA carboxylase is decreased by acetate and pyruvate and increased by citrate and propanol on incubation of mycelium; this effect was not observed with the cell-free extracts. The increase of the activity by citrate and propanol is prevented by actinomycin. 3. Citrate counteracts the effect of pyruvate but the effect of propanol is neither fully additive with citrate nor fully counteractive to pyruvate, probably due to a different permeability. 4. The positive and negative response of erythromycin biosynthesis to propanol in *S. erythreus* mutants is associated with corresponding changes in the activity of propionyl-CoA carboxylase. The role of propionyl-CoA carboxylase in erythromycin biosynthesis is discussed in relation to the role of acetyl-CoA carboxylase in lipogenesis.

A close analogy has been suggested between the mechanisms of fatty acid biosynthesis and formation of the lactone ring of macrolide antibiotics such as erythromycin: in both cases the energy for condensation of building units is similarly gained in the biotin mediated carboxylation reactions (Wawrzkiewicz & Lynen, 1964). In biogenesis of erythronolide, a polypropionate polycondensation product, methylmalonate instead of malonate is an intermediate, responsible for extension of the acyl chain. Six molecules of methylmalonyl-CoA and one molecule of propionyl-CoA, acting as an initiating unit, give rise to the C_{21} erythronolide lactone ring (Corcoran & Chick, 1966).

The present paper deals with the two carboxylation mechanisms in *Streptomyces erythreus*: propionyl-CoA carboxylase (propionyl-CoA:carbon dioxide ligase, EC 6.4.1.3) and methylmalonyl-CoA carboxyltransferase (methylmalonyl-CoA: pyruvate carboxyltransferase, EC 2.1.3.1) and the effect of metabolites and of propanol, a well known stimulant of erythromycin production (Hockenhull, 1963), on propionyl-CoA carboxylase.

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MATERIALS AND METHODS

Chemicals. Lactate dehydrogenase, NADH and reduced glutathione were products of Boehringer & Soehne (Mannheim, West Germany). ATP and methylmalonate were from Calbiochem (Los Angeles, Calif., U.S.A.) and CoA from Sigma (St. Louis, Mo., U.S.A.). Tris(hydroxymethylaminomethane) was obtained from Austranal (Loba-Chemie, Wien, Austria), β-mercaptoethanol from Koch-Light (Colnbrook, Bucks., England). Propionyl-CoA was synthesized by the method of Simon & Shemin (1953), the yield and stability of the product being determined with *p*-chloromercuribenzoate (Stadtman, 1957). Actinomycin was obtained at the Institute of Antibiotics, Warsaw. Other reagents were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

Strains and culture conditions. *S. erythreus* strain 64/575 I.A. and its two mutants A and B differing in the response to propanol, were grown in 500 ml. Erlenmayer flasks on the rotatory shaker (240 rev./min.) at 32° on the complex soluble medium of the following composition per liter: soya flour extract, 30 g.; glucose, 50 g.; NaCl, 5 g.; $(NH_4)_2SO_4$, 3 g.; $CaCO_3$, 6 g. In some experiments propanol (0.6%, v/v) was added to the growth medium. An inoculum of 5% was used to initiate the experiments. Under these conditions the maximum of growth (about 12 mg. dry wt./ml.) was reached in the 72 hr. cultures, except with mutant B, which continued to grow throughout the 120 hr. experimental period. The final yield of erythromycin was about 600 µg./ml., and about 250 µg./ml. in the case of mutant B.

Preparation of extracts. Every 24 hr. the contents of three fermentation flasks were pooled, the mycelium was spun down at 2000 rev./min. for 15 min. at 0°, washed twice with distilled water by centrifugation, suspended in 0.05 M-tris buffer, pH 8.0, containing 10 mM-β-mercaptoethanol, and disintegrated by the use of French press. The supernatant obtained on a 15 min. centrifugation at 12 000 rev./min., containing 4 - 6 mg. of protein, was used for enzyme assay.

Addition of metabolites and alcohols. Pyruvate, citrate, malonate, succinate, methylmalonate, oleate or amyl alcohol, propanol, propionate or erythromycin were alternatively added, separately or together with actinomycin, to the 72 hr. cultures. After the 2.5 hr. incubation under culture conditions no measurable increase in dry weight was noted; the mycelium was harvested, washed and extracted as above. Pyruvate, citrate and propanol in the same concentration were also added to the incubation mixture containing the cell-free extracts. In the experiments on combined action of pyruvate, propanol and citrate these compounds were added in concentrations from 10 to 160 mM.

Enzyme assay. The activity of propionyl-CoA carboxylase was determined after Tietz & Ochoa (1959) using the method of Ames, Garry & Werzenberger (1960) for determination of inorganic phosphate. The incubation mixture contained: tris buffer of pH 8.0, 50 µmoles; reduced glutathione, 1 µmole; $MgCl_2$, 2 µmoles; ATP, 1 µmole; $KHCO_3$, 25 µmoles; KCl, 50 µmoles; propionyl-CoA, 0.5 µmole and the enzymic protein 0.4 - 0.6 mg. in a total vol. of 0.5 ml. The mixture was incu-

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bated for 15 min. at 30°. The reaction was stopped by the addition of 0.5 ml. of 8% trichloroacetic acid, the precipitated protein was centrifuged off and 0.2 ml. samples of supernatant were used for phosphate estimation. The activity of carboxyl-transferase was measured according to Swick & Wood (1960) in a mixture containing in 1 ml.: triethanolamine buffer of pH 7.6, 100 µmoles; propionyl-CoA, 0.5 µmole; oxaloacetate, 2 µmoles; reduced glutathione, 2 µmoles and enzymic protein (1.6 - 2.4 mg.). The mixture was incubated for 15 min. at 30° and deproteinized as above. The increase in pyruvate was measured at 340 nm with lactate dehydrogenase. The control without propionyl-CoA was run parallelly in both assays. The rate of the reaction catalysed by each enzyme was proportional to the amount of added protein.

Protein and erythromycin determination. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) and erythromycin colorimetrically (Ford, Prescott, Hiuman & Caron, 1953) or microbiologically by the cylinder plate method (Grove & Randall, 1955).

RESULTS

Synthesis of methylmalonate by carboxylation and transcarboxylation. Carboxyl-transferase in *S. erythreus* is a less active enzyme than propionyl-CoA carboxylase (Fig. 1); it reached the maximum activity at the early logarithmic stage and decreased in the later hours of growth in contrast to propionyl-CoA carboxylase, which showed higher activity in the postexponential phase of growth, the period of intensive erythromycin synthesis. This time course of the activities of both carboxylation enzymes excludes participation of carboxyltransferase in the erythronolide biosynthesis and draws attention to the role of propionyl-CoA carboxylase.

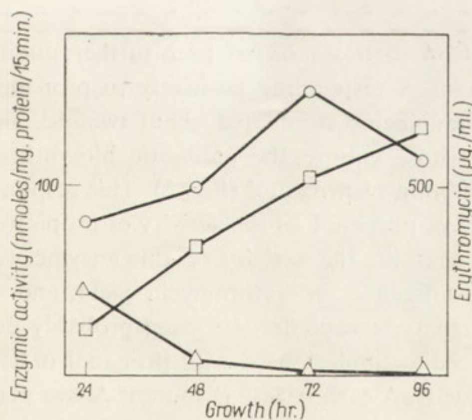

Fig. 1

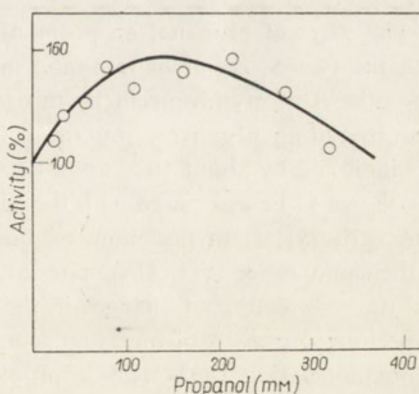

Fig. 2

Fig. 1. The activity of ○, propionyl-CoA carboxylase and △, methylmalonyl-CoA carboxyltransferase; and □, the content of erythromycin in *S. erythreus* grown on the complex soluble medium.

Fig. 2. The effect of propanol on the activity of propionyl-CoA carboxylase. The 72 hr. mycelium was incubated for 2.5 hr. with varying concentrations of propanol.

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Propionyl-CoA carboxylase in S. erythreus *mutants.* The activity of propionyl-CoA carboxylase was increased by citrate and decreased by pyruvate and acetate added to the 72 hr. cultures in 20 mM concentration and incubated for 2.5 hr. under culture conditions. The increase in the enzymic activity by citrate was abolished by actinomycin (5 µg./ml.) added simultaneously with citrate to the culture medium (Table 1). Actinomycin K, belonging to the group of actinomycin D (Korzybski, Kowszyk-Gindifer & Kuryłowicz, 1967) was used in our experiments. Its action on DNA and biosynthesis of RNA does not differ from that of actinomycin D (Grabowska, 1965, 1966) and in our experiments it prevented in an analogous way the stimulation of propionyl-CoA carboxylase. The addition of citrate to the incubation mixture containing the cell-free extract did not activate the enzyme and no effect was observed with acetate and pyruvate in this system.

Succinate, malonate, methylmalonate, oleate, propionate and glucose incubated with the intact mycelium under the same conditions as citrate, pyruvate and acetate, did not affect the activity of propionyl-CoA carboxylase. Repression by erythromycin, the end product of the antibiotic metabolic pathway, was not observed at the concentration of 8 mM found in the medium in the later hours of growth.

The activity of propionyl-CoA carboxylase was affected also by propanol, a well known stimulant of erythromycin biosynthesis in the large scale production of this antibiotic; the effect depended on propanol concentration, and during 2.5 hr. incubation of mycelium the maximum increase was observed with 80 - 200 mM-propanol (Fig. 2). The addition of amyl alcohol produced a similar, about 50%, increase in the activity of propionyl-CoA carboxylase when added in 20 mM concentration, whereas at 80 mM-amyl alcohol the cells were damaged. The addition of ethanol or butanol in this concentration did not induce any visible morphological changes in mycelium but resulted in a marked 50% decrease in the carboxylase activity (Table 2).

The effect of propanol on propionyl-CoA carboxylase has been further studied with the two *S. erythreus* mutants: mutant A responding positively to propanol, biosynthesis of erythromycin by this mutant being stimulated about twofold, and the responding negatively mutant B in whose culture the antibiotic biosynthesis was inhibited by about 16% upon the addition of propanol (Fig. 3). This response was shown to be associated with the effect of propanol on the activity of propionyl-CoA carboxylase. In mycelium of both mutants the activity of this enzyme was of the same order (Fig. 4) despite large differences in erythromycin yield, and so was the time course of changes in the activity, except the last stage probably due to prolonged growth of mutant B. On media supplemented with propanol (0.6%) before inoculation, the activity of propionyl-CoA carboxylase in mutant A was markedly elevated throughout 96 hr. of growth, and the enzyme in mutant B was significantly decreased parallelly to the decreased production of antibiotic. On a 2.5 hr. incubation with propanol the activity of propionyl-CoA carboxylase was induced in mutant A and repressed in mutant B. The stimulating effect of propanol, similarly to that of citrate, was abolished by actinomycin (Table 3).

The results presented in Fig. 5 show that the repression of propionyl-CoA carbo-

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Table 1

Effect of citrate, pyruvate and acetate on propionyl-CoA carboxylase in S. erythreus

Citrate, pyruvate and acetate (20 mM) were added to the 72 hr. cultures and incubated for 2.5 hr. Actinomycin (5 µg./ml.) was added where indicated; the activity was estimated as described in Methods.

Addition	Activity (nmoles/mg. protein/15 min.)
None	203
Citrate	300
Citrate+actinomycin	220
None	165
Pyruvate	79
None	153
Acetate	22

Table 2

Effect of alcohols on propionyl-CoA carboxylase in S. erythreus

A 72 hr. mycelium was incubated for 2.5 hr. with ethanol, butanol and propanol (80 mM) and amyl alcohol (20 mM) under culture conditions.

Alcohol	Activity (%)
None	100
Ethanol	49
Butanol	51
Propanol	151
Amyl alcohol	144

Table 3

Effect of propanol on propionyl-CoA carboxylase in S. erythreus *mutants*

Propanol (80 mM) was added separately or together with actinomycin (10 µg./ml.) to the 72 hr. cultures of mutants A and B, and incubated for 2.5 hr.

Addition	Activity (nmoles/mg. protein/15 min.)	
	Mutant A	Mutant B
None	97	120
Propanol	151	62
Propanol+actinomycin	102	—

xylase by pyruvate was counteracted by citrate added in equimolar concentration. In the presence of both compounds the activity was the same as in the control mycelium. The inhibitory effect of pyruvate was not totally balanced by the equivalent amount of propanol probably due to different permeability of the cell wall

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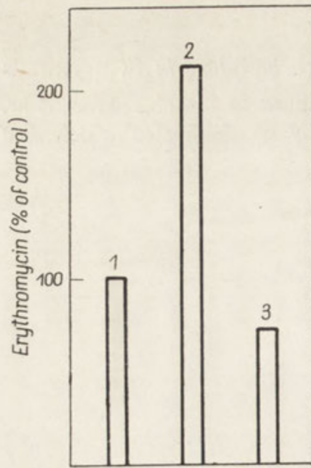

Fig. 3

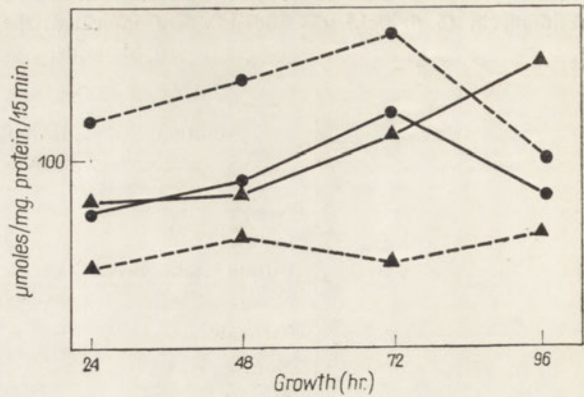

Fig. 4

Fig. 3. The effect of propanol on erythromycin biosynthesis by *S. erythreus* mutants. The yield of erythromycin in the 120 hr. of growth in the propanol-supplemented cultures of mutant A (*2*) and mutant B (*3*) is expressed as percentage of the control cultures of each mutant non-supplemented with propanol. Propanol (80 mM) was added to the medium prior to inoculation.

Fig. 4. The activity of propionyl-CoA carboxylase in *S. erythreus* mutants: ●, mutant A, and ▲, mutant B. The enzymic activity was measured in mutants grown (———), on the control medium, and (- - -), on the medium supplemented with 80 mM-propanol.

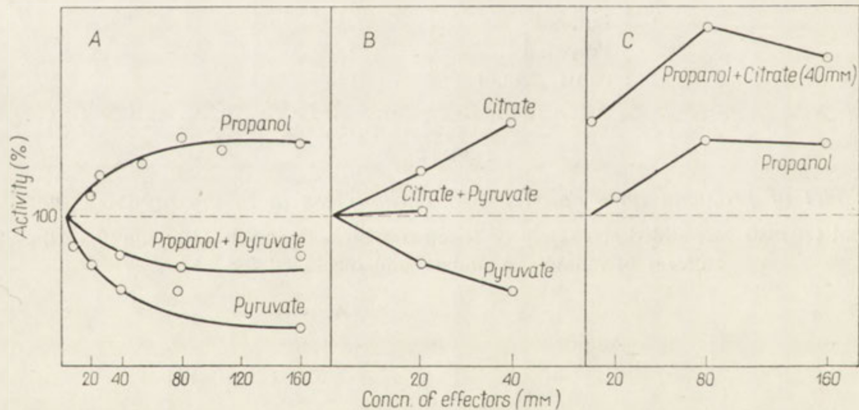

Fig. 5. Interaction of citrate, pyruvate and propanol on the activity of propionyl-CoA carboxylase in *S. erythreus*. The effectors were added to the 72 hr. mycelium, and incubated for 2.5 hr. under culture conditions. When two effectors were added together, in *A* and *B* each was applied at the concentrations indicated on the abscissa, and in *C*, citrate was applied at a concentration of 40 mM.

to both these compounds. This presumably explains the fact that the effects of propanol and citrate were not fully additive although an interaction of these compounds on the activity of propionyl-CoA carboxylase was clearly demonstrated.

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DISCUSSION

Physiological significance of biotin-dependent carboxylating enzymes, pyruvate carboxylase and acetyl-CoA carboxylase, is well established in regulation of gluconeogenesis and fatty acid synthesis (Weber, Singhall, Stamm, Fisher & Mentediek, 1964; Numa, Bortz & Lynen, 1965), and Lynen (1967) pointed to a general importance of these enzymes in biosynthesis. Carboxylation of propionyl-CoA could be considered as the first step in the biosynthetic sequence leading to erythronolide in *S. erythreus*. In this respect stimulation of propionyl-CoA carboxylase in this organism by citrate and propanol, and inhibition by pyruvate and acetate should be stressed. Synthesis of methylmalonate by the transcarboxylation mechanism seems to be of secondary importance in the metabolism of *S. erythreus* producing erythromycin.

The increase in the activity of propionyl-CoA carboxylase in *S. erythreus* by citrate is especially interesting in view of the activation of the animal and yeast acetyl-CoA carboxylase by this compound in the cell-free system (Ryder, Gregolin, Chang & Lane, 1967; Rasmussen & Klein, 1967): the role of acetyl-CoA carboxylase and that of propionyl-CoA carboxylase is analogous in biological polymerization of polyacyl chains. Citrate does not, however, activate propionyl-CoA carboxylase in the sonicates of *S. erythreus* similarly as in the case of acetyl-CoA carboxylase of wheat germ and *E. coli* (Burton & Stumpf, 1966; Alberts & Vagelos, 1968). Stimulation of the activity of propionyl-CoA carboxylase is observed only when the effectors, citrate or propanol, are incubated with the intact mycelium. The effects of citrate and propanol are additive and are counteracted by pyruvate. Since actinomycin abolishes the increase in the activity of propionyl-CoA carboxylase by citrate and propanol, it seems that these compounds affect directly or indirectly the biosynthesis of the enzymic protein of propionyl-CoA carboxylase in *S. erythreus*.

The effect of propanol and amyl alcohol on propionyl-CoA carboxylase both on short incubation of *S. erythreus* mycelium and in cultures on media supplemented with these alcohols is worth mentioning because of a marked about 50% increase in biosynthesis of erythromycin by these alcohols (Hockenhull, 1963; Ostrowska-Krysiak & Ruczaj, unpublished results). Ethanol and butanol decrease the activity of propionyl-CoA carboxylase under the same conditions. The relation between the effect of propanol on propionyl-CoA carboxylase and that on erythromycin biosynthesis has been confirmed in our studies with *S. erythreus* mutants. Stimulation of erythromycin biosynthesis by mutant A is associated with the induction of propionyl-CoA carboxylase, and inhibition of erythromycin production in mutant B with repression of this enzyme by propanol.

Stimulation of enzymes concerned with elongation of peptidyl chain by alcohols was observed by Monro, Staehelin, Celma & Vazquez (1969) and the increase in the activity of propionyl-CoA carboxylase by propanol and amyl alcohol in *S. erythreus* may constitute a part of a stimulatory mechanism of antibiotic biosynthesis by these alcohols.

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REFERENCES

Alberts A. W. & Vagelos P. R. (1968). *Proc. Natl. Acad. Sci.* **59**, 561.
Ames B. N., Garry B. & Werzenberger L. H. (1960). *J. Gen. Microbiol.* **22**, 369.
Burton D. & Stumpf P. K. (1966). *Arch. Biochem. Biophys.* **117**, 604.
Corcoran J. W. & Chick M. (1966). *Biochemistry of the Macrolide Antibiotics.* In *Biosynthesis of Antibiotics* (J. F. Snell, ed.) p. 159. Academic Press, New York, London.
Ford J. W., Prescott G. C., Hiuman J. W. & Caron E. L. (1953). *Analyt. Chem.* **25**, 1195.
Grabowska M. (1965). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **13**, 265.
Grabowska M. (1966). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **14**, 379.
Grove D. C. & Randall W. A. (1955). *Assay Methods in Antibiotics.* Medical Encyclopedia Inc., New York.
Hockenhull D. J. D. (1963). In *Biochemistry of Industrial Microorganisms* (W. C. Rabino & A. H. Rose, eds.) p. 159. Academic Press, London, New York.
Korzybski T., Kowszyk-Gindifer Z. & Kuryłowicz W. (1967). In *Antibiotics: Origin, Nature, Properties* vol. 1, p. 1025. Pergamon Press, Oxford; Polish Scientific Publishers, Warszawa.
Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265.
Lynen F. (1967). *Biochem. J.* **102**, 381.
Monro R. E., Staehelin T., Celma M. L. & Vazquez D. (1969). *Sixth Meeting of FEBS, Madrid. Abstr. of Commun.* no. 9.
Numa S., Bortz W. M. & Lynen F. (1965). *Advances in Enzyme Regulation* (G. Weber, ed.) vol. 5, p. 407. Pergamon Press, Oxford.
Rasmussen R. K. & Klein H. P. (1967). *Biochem. Biophys. Res. Commun.* **28**, 415.
Ryder E., Gregolin C., Chang H. C. & Lane M. D. (1967). *Proc. Natl. Acad. Sci.* **57**, 1455.
Simon E. J. & Shemin D. (1953). *J. Am. Chem. Soc.* **75**, 2520.
Stadtman E. R. (1957). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 938. Academic Press, New York.
Swick R. W. & Wood H. G. (1960). *Proc. Natl. Acad. Sci.* **46**, 28.
Tietz A. & Ochoa S. (1959). *J. Biol. Chem.* **234**, 1394.
Wawrzkiewicz E. & Lynen F. (1964). *Biochem. Z.* **340**, 213.
Weber G., Singhall R. L., Stamm N. B., Fisher E. A. & Mentediek M. A. (1964). *Advances in Enzyme Regulation* (G. Weber, ed.) vol. 2, p. 1. Pergamon Press, Oxford.

KARBOKSYLACJA PROPIONYLO-CoA W BIOSYNTEZIE ERYTROMYCYNY

Streszczenie

1. Przebieg aktywności karboksylotransferazy metylomalonylo CoA (EC 2.1.3.1) i karboksylazy propionylo CoA (EC 6.4.1.3) podczas wzrostu *S. erythreus* wskazuje na udział tego ostatniego enzymu w biosyntezie erytronolidu — polipropionianowego pierścienia laktonowego erytromycyny.

2. Czynność karboksylazy propionylo CoA jest hamowana przez octan i pirogronian, i stymulowana przez propanol i cytrynian; stymulacja czynności enzymatycznej jest znoszona przez aktynomycynę i obserwowana jest wyłącznie jako wynik inkubacji grzybni z badanymi związkami, nie stwierdza się jej natomiast w układzie bezkomórkowym.

3. Cytrynian zapobiega hamowaniu pod wpływem pirogronianu, natomiast wpływ propanolu i cytrynianu nie jest w pełni addytywny ani też nie obserwuje się całkowitego znoszenia przez propanol hamowania wywołanego przez pirogronian; różnice te wywołane są najprawdopodobniej inną przepuszczalnością tych związków przez ścianę komórkową.

4. Efekt stymulujący względnie hamowanie biosyntezy erytromycyny przez propanol u mutantów *S. erythreus* znajduje swoje odbicie w czynności karboksylazy propionylo CoA. Rolę karboksylazy propionylo CoA przedyskutowano w odniesieniu do roli karboksylazy acetylo CoA w lipogenezie.

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A C T A B I O C H I M I C A P O L O N I C A

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CONFORMATION OF ACID FORMS OF POLY C: TEMPERATURE AND IONIC STRENGTH DEPENDENCE OF PROTONATION OF CYTIDINE AND CYTIDINE-5′-PHOSPHATE

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1. The p*K* values for protonation of the ring N_3 nitrogen in cytidine-5′-phosphate have been measured over a temperature range of 10 - 80° and at ionic strengths of 0.1, 0.2 and 1.0. 2. The influence of a divalent cation, 10 mM-Mg^{2+}, has also been investigated. 3. The thermodynamic constants for the protonation reaction have been calculated. 4. The significance of the results in relation to the helix-coil transitions of the acid, twin-helical forms of various poly C analogues is discussed. 5. The absorption spectra of both the fully neutral and the fully protonated forms of cytidine have been shown to exhibit a small, but definite, intrinsic temperature dependence similar to that exhibited by other pyrimidines and purines. Although the origin of this temperature effect on an electronic absorption spectrum remains to be clarified, it is shown to be significant enough to be taken into account in some studies on helix-coil transitions in polynucleotides by UV absorption techniques.

The conformations of the so-called "acid" forms of poly rC, poly dC, and the corresponding poly 5MerC and poly 5MedC[1], have been the object of numerous investigations. In the case of poly rC the acid form has been shown by X-ray diffraction to consist of a twin-stranded helix in which two cytosine residues form a "base-pair" *via* hydrogen bonding between the C_2 carbonyls and the amino hydrogens, and the formation of an ionic bond by the sharing of a proton between the N_3 nitrogens, as illustrated in Scheme 1 (Langridge & Rich, 1963). Similar structures prevail in solution for poly rC (Akinrimisi, Sander & Ts'o, 1963), poly 5MerC (Szer & Shugar, 1966), poly dC (Inman, 1964) and poly 5MedC (Żmudzka, Bollum & Shugar, 1969). However, whereas the "acid" forms of the polyribonucleotides are formed only in acid medium, those of the polydeoxyribonucleotides begin to form in slightly alkaline medium (hence the use, above, of the term "so-called acid forms").

[1] The following abbreviations are used in this text: poly rC, polyribocytidylic acid; poly dC, polydeoxycytidylic acid; poly 5MerC, poly 5-methylribocytidylic acid; poly 5MedC, poly 5-methyl-deoxycytidylic acid; poly U, polyuridylic acid; poly A, polyadenylic acid; pK_a, apparent p*K* value; p*K*, thermodynamic value.

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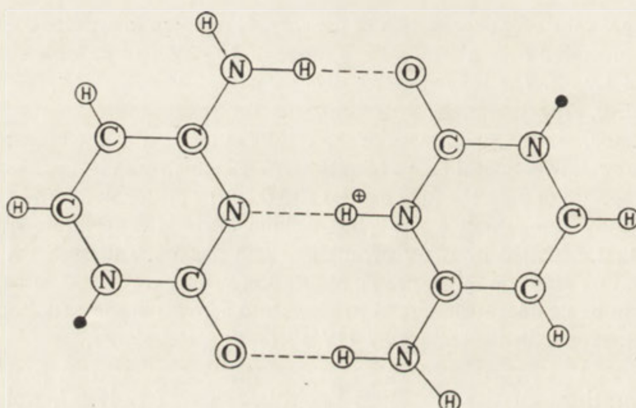
In most studies on the helix-coil transitions of the acid forms of the poly C analogues, the uptake or release of the proton shared between the N_3 nitrogens of two cytosine residues has been examined only at room temperature (Akinrimisi *et al.*, 1963; Hartmann & Rich, 1965; Inman, 1964). This is, however, quite unwarranted since the transitions occur at fairly elevated temperatures. Furthermore, at least in those instances where the helix-coil transition is studied in acid medium, at pH values not far removed from the p*K* for protonation of cytidine or deoxycytidine, i.e. pH 4.2, the coil form contains cytosine residues partially protonated on the N_3 nitrogen. Consequently a complete interpretation of the nature of the helix-coil transitions for the various poly C's, and the calculation of the thermodynamic parameters associated with these transitions, should take into account the dissociation constant for cytidine (or deoxycytidine) residues as a function of temperature and ionic strength of the medium (for review of this question, see Żmudzka *et al.*, 1969). An analogous situation exists in the case of the acid form of poly A, which has been studied in detail by Holcomb & Timasheff (1968).



Scheme 1

As far as we are aware, the only such measurements extant are those for cytidine over a limited temperature range, 20° - 50° (Lewin & Humphreys, 1966). The present paper presents more extensive data for cytidine and cytidine-5′-phosphate over a temperature range embracing the T_m values for the helix-coil transitions of the acid forms of the various poly C's at several values of ionic strength, obtained by means of spectral titration methods.

In view of the known role of magnesium on the structure of nucleic acids and, in particular, ribosomes, an examination was also made of the influence of physiological concentrations of Mg^{2+} (0.01 M) on the protonation of cytidine and cytidine-5′-phosphate. It is conceivable that specific interaction of magnesium ions with the charged phosphate groups, and the resulting modifications in structure of the solvent in the vicinity of the CMP molecule, might result in a change in the p*K* value for protonation.

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MATERIALS AND METHODS

Cytidine and cytidine-5′-phosphate were, respectively, Serva (London, England), and Sigma (St. Louis, Mo., U.S.A.) A-grade products, checked for purity by spectral and chromatographic methods.

Buffer materials were analytical grade and glass-distilled water was employed throughout. All pH measurements were made with a Radiometer Type PHM-4d compensating instrument with a G220B glass electrode and a K400 calomel electrode, using as standards a 0.05 M-potassium hydrogen phthalate solution (the pH of which, at room temperature, is in the neighbourhood of that corresponding to the p*K* of cytidine); a Radiometer standard pH 6.50 buffer; and 0.1 N-HCl.

The temperature dependence of the pH of the acetate buffer used was determined experimentally over a temperature range of 10 - 80° for a buffer with a pH of 4.0 at 10°, using as standard 0.05 M-phthalate, the pH-temperature dependence of which is known (King, 1965). The pH-temperature dependence of the pH 4.0 acetate buffer was found to be almost identical with that for the phthalate standard. It was assumed that the temperature dependence for the other acetate buffer solutions is similar, and this is supported by the fact that the pK_a values calculated from the UV spectra at different pH values exhibited a scatter which was within the limits of experimental error for a given pK_a. Differences in measured pH values prior to, and following completion of, a set of readings did not exceed 0.005 pH units.

Absorbance measurements were carried out on a Unicam SP700 instrument fitted with a jacketted cuvette compartment through which circulated an aqueous glycol mixture fed from a Hoeppler ultrathermostat. Temperatures were controlled and measured by means of a Cu/Cn thermocouple to an accuracy of 0.3°. The absorbance and wavelength scales of the instrument were checked at suitable intervals. Optical density measurements made use of teflon-stoppered matched Suprasil 10-mm. cells with the appropriate buffer solution in the blank cell. Cell corrections were checked at regular intervals but, in general, exhibited no changes with time or temperature.

Acetate buffers of various pH values were made up to a total acetate concentration of 0.08 M, and to each of these was added the appropriate amount of NaCl (or NaCl plus magnesium acetate when the influence of Mg^{2+} was to be studied) to give ionic strengths of 0.10, 0.20, and 1.00. Solutions of 0.1 N-HCl and 0.1 M-cacodylate buffer, pH 7.06, were employed to measure the spectra of the fully protonated and fully neutral forms, respectively; these solutions likewise contained NaCl (or NaCl and magnesium acetate) to give the required ionic strength.

A series of absorption spectra was obtained on a single solution at a given pH value by stepwise variation of the temperature over the appropriate range. Similar series were then run at other selected pH values. In this way it was possible, by checking the optical densities at the isosbestic point, to immediately eliminate any errors arising from errors in preparation of the solutions. Furthermore, following measurements at elevated temperatures, the cuvettes were weighed on an analytical balance to ensure that the teflon stoppers effectively prevented evaporation during the course of recording a spectrum.

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RESULTS AND DISCUSSION

Effect of temperature on spectra of cytidine and CMP. Measurements of optical densities of cytidine and cytidine-5′-phosphate at various temperatures were not corrected for thermal expansion of the solutions, since pK_a values were calculated from the absorption spectra for the fully neutral and protonated forms at each given temperature. However, during the course of these measurements, the following rather interesting observation emerged.

Figures 1A and 1B demonstrate that the absorption spectrum of cytidine, even at extreme pH values, 7.06 and 0.95, corresponding, respectively, to the neutral and fully protonated species exclusively, exhibits a temperature dependence for both species. In the absence of any corrections for thermal expansion, the λ_{max} optical density for each form at 80° should be about 3% lower than that at 10°. The actual decreases of the optical densities are 8% for the neutral molecule and 6% for the protonated form. The reality of this effect is further testified to by the fact that, whereas at λ_{max} the high temperature spectrum is *hypo*chromic with respect to that at 10°, it is *hyper*chromic at longer wavelengths.

The origin of this temperature-dependence of an electronic absorption spectrum is not clear. Such effects have been sporadically reported in the literature. But the suggestion of Morita & Nagakura (1968) that, in the case of cytidine, it is due to the influence of temperature on the distribution of the tautomeric forms (amino - tmino) is untenable in the light of the fact that the modifications in absorption ipectrum are exhibited by both the neutral and protonated species at pH values sar removed from the pK_a. Nor is this phenomenon confined to cytidine; a similar temperature-dependence of the absorption spectrum has been noted for the neutral form of adenosine, as well as for such pyrimidine derivatives as 1,3-dimethyluracil, 1,3-dimethylthymine, 1,3-dimethyl-5-ethyluracil, where the tautomeric forms are fixed (Shugar, 1969, unpublished results). Two possible factors which may be responsible for this effect are modifications of the degree of solvation of the dissolved molecules, or the decrease in dielectric constant of the aqueous medium at elevated temperatures. It is, however, of undoubted interest in this connection that there exists a small, but finite, intrinsic temperature dependence of the phosphorescence lifetimes for a variety of aromatic hydrocarbons (Jones & Siegel, 1969), although the source of this effect remains to be clarified. It is also of interest that a temperature-dependent decrease in extinction coefficient was noted by Adler, Grossman & Fasman (1967) for cytidine-5′-phosphate and deoxycytidine-5′-phosphate (see Table II of the foregoing publication), although this fact was not commented upon by the authors.

Attention is drawn to the foregoing because of the fact that, in accurate studies of helix-coil transitions of polynucleotides by optical methods, the temperature effect on the monomer absorption spectrum may have to be taken into account. A case in point is that involving transitions of multiple-stranded forms of helices of poly A and poly U at wavelengths in the neighbourhood of 285 mμ (see Barszcz & Shugar, 1968), where the hypochromicity of the helical form (relative to the coil

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form) is fairly small and could derive, at least in part, from the effect of temperature on the monomer residues.

Variation of extent of protonation with temperature. The temperature dependence for protonation of cytidine is illustrated in Fig. 2, which shows how the absorption spectrum of partially protonated cytidine varies with temperature at approximately constant pH and a given ionic strength. Note that, as the temperature increases, the absorption spectrum shifts towards that of the neutral form (Fig. 1A), clearly indicative of deprotonation. This demonstrates rather strikingly what a number of observers have previously referred to, viz. that the spectral changes occurring during the helix-coil transitions of the acid twin-helical forms of poly C's, at pH values

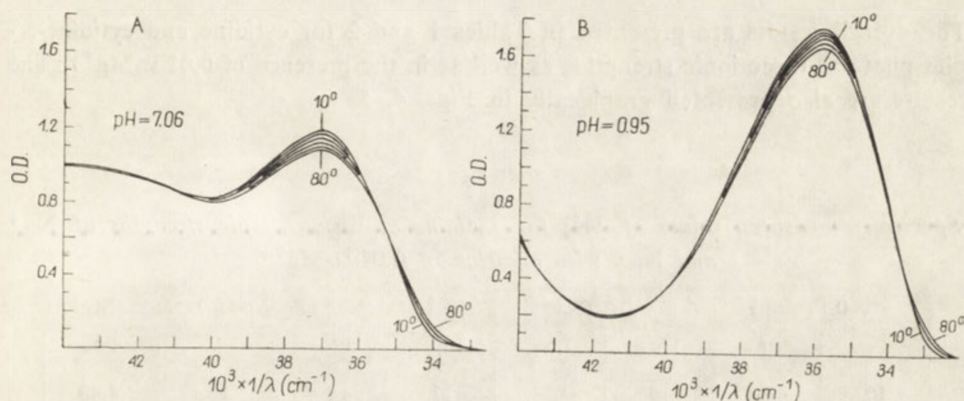

Fig. 1. Temperature dependence between 10° and 80° of absorption spectra of neutral and protonated species of cytidine at an ionic strength, μ, of 0.20: (*A*), Neutral form, 0.1 M-cacodylate buffer, pH 7.06. (*B*), Protonated form, pH 0.95 (0.1 N-HCl). *Note*: not corrected for thermal expansion of solutions (see text).

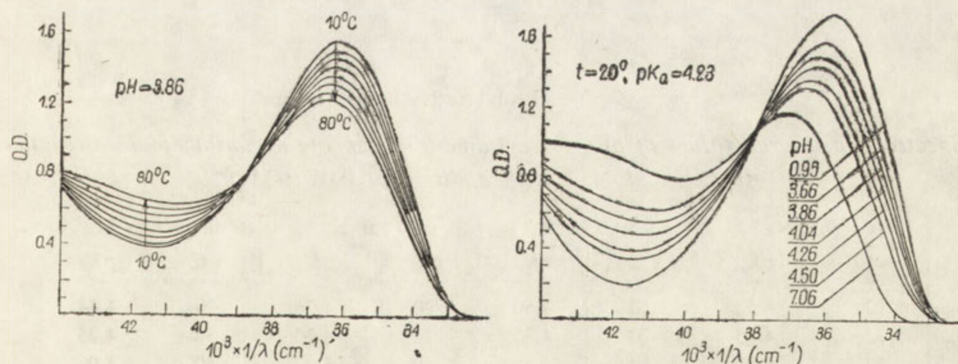

Fig. 2 Fig. 3

Fig. 2. Temperature dependence of absorption spectrum of cytidine at pH 3.86 (0.08 M-acetate buffer), ionic strength 0.20, over the temperature range 10° - 80°.

Fig. 3. Absorption spectrum of cytidine in 0.08 M-acetate buffer, μ = 0.20, at various pH values between those for neutral and protonated forms, at 20.0°. Calculated pK_a under these conditions, 4.23.

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not far removed from the pK for protonation of the cytosine residues, are very complex.

Calculations of pK_a *and* pK *values.* A typical set of absorption spectra for the calculation of pK_a is presented in Fig. 3 for cytidine at 20° and an ionic strength, μ, of 0.20. The apparent pK (pK_a) values were calculated for a given temperature and ionic strength from the optical densities at three wavelengths in the range 265 - 285 mμ of the neutral form (D_C), the fully protonated form (D_{CH^+}), and an intermediate form (D) at a measured pH value, from the formula,

$$pK_a = pH - \log \frac{D_{CH^+} - D}{D - D_C}.$$

The overall results are presented in Tables 1 and 2 for cytidine and cytidine-5′-phosphate at three ionic strengths, as well as in the presence of 0.01 M-Mg^{2+}. The results are also presented graphically in Figs. 4, 5, 6.

Table 1

Spectrally measured values of pK_a *for cytidine at various ionic strengths of* Na^+ *and* Na^+ *with addition of* 0.01 M-Mg^{2+}

μ = 0.1 (Na^+)		μ = 0.2 (Na^+)		μ = 1.0 (Na^+)		μ = 0.2 ($Na^+ + Mg^{2+}$)	
t°C	pK_a	t°C	pK_a	t°C	pK_a	t°C	pK_a
10	4.34	10	4.34	21	4.30	10	4.40
33	4.10	20	4.23	30	4.23	25	4.11
57	3.93	30	4.12	40	4.14	40	3.98
80	3.77	40	4.02	50	4.05	55	3.83
		50	3.93	60	3.95	70	3.69
		60	3.85	70	3.88	85	3.62
		70	3.79	80	3.86		
		80	3.75				

Table 2

Spectrally measured values of pK_a *for cytidine-5′-phosphate at various ionic strengths of* Na^+ *and* Na^+ *with addition of* 0.01 M-Mg^{2+}

μ = 0.1 (Na^+)		μ = 0.2 (Na^+)		μ = 1.0 (Na^+)		μ=0.2($Na^+ + Mg^{2+}$)	
t°C	pK_a	t°C	pK_a	t°C	pK_a	t°C	pK_a
10	4.52	10	4.50	20	4.36	20	4.44
33	4.25	20	4.37	35	4.20	35	4.25
57	4.00	30	4.29	50	4.07	50	4.09
80	3.87	40	4.18	65	3.95	65	3.98
20	4.42	50	4.10	80	3.90	80	3.88
35	4.24	60	3.99				
50	4.11	70	3.93				
65	3.98	80	3.89				
80	3.91						

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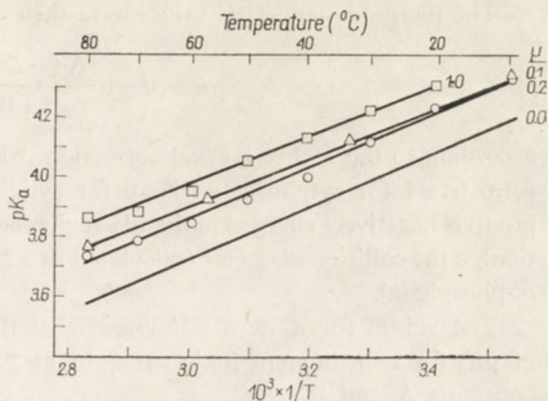

Fig. 4. Plots of pK_a *versus* 1/T for cytidine at various ionic strengths as indicated; and of pK ($\mu = 0$) *versus* 1/T.

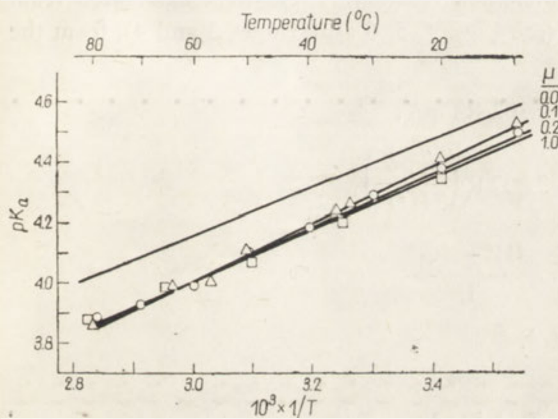

Fig. 5. Plots of pK_a *versus* 1/T for cytidine-5′-phosphate at various ionic strengths as indicated; and of pK ($\mu = 0$) *versus* 1/T.

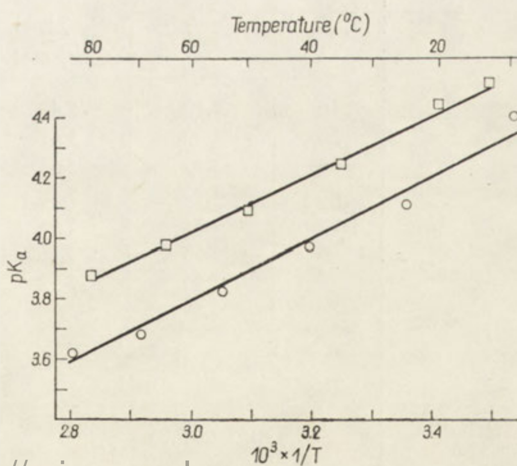

Fig. 6. Plots of pK_a *versus* 1/T for cytidine (○) and cytidine-5′-phosphate (□) at an ionic strength of 0.2 in the presence of 0.01 M-Mg^{2+}.

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The thermodynamic pK values were then obtained from the usual relationship,

$$pK = pK_a + \frac{A(-2Z+1)\sqrt{\mu}}{1+Ba\sqrt{\mu}},$$

according to the Debye-Hückel correction, where Z is the total charge of the acid form (Z=1 for cytidine, and Z=0 for cytidine-5′-phosphate since the phosphate group is negatively charged in the pH region embracing the pK for cytidine protonation), a the collision diameter (calculated as 3.2 Å for cytidine and 7.2 Å for cytidine-5′-phosphate).

The values for a were estimated from the experimentally determined values of pK_a for two different ionic strengths at 20°, using Bates (1954) figures for the constants A and B.

The pK values are shown graphically in Figs. 4, 5, 6 and are also included in Tables 3 and 4.

Thermodynamic constants for protonation reaction. These were calculated from the temperature dependence of pK (see Figs. 4, 5, 6 and Tables 3 and 4), from the following relationships,

$$\Delta G° = 2.302\ RT\ pK,$$

$$\Delta H° = 2.302\ R\left(\frac{\partial\, pK}{\partial(1/T)}\right),$$

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T}$$

and are presented in Tables 3 and 4.

Table 3

Thermodynamic constants for protonation of cytidine and cytidine-5′-phosphate

Temp. (°C)	Cytidine		Cytidine-5′-phosphate	
	pK	$\Delta G°\ \frac{kcal}{mol}$	pK	$\Delta G°\ \frac{kcal}{mol}$
10	4.19	5.42	4.58	5.93
20	4.09	5.48	4.48	6.01
30	3.99	5.53	4.39	6.09
40	3.89	5.58	4.31	6.17
50	3.81	5.63	4.23	6.25
60	3.73	5.68	4.16	6.33
70	3.65	5.72	4.09	6.42
80	3.58	5.78	4.02	6.50
	$\Delta H° = 4.01\ \frac{kcal}{mol}$		$\Delta H° = 3.64\ \frac{kcal}{mol}$	
	$\Delta S° = -5.0\ \frac{cal}{mol\ °K}$		$\Delta S° = -8.0\ \frac{cal}{mol\ °K}$	

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Table 4

Thermodynamic constants for protonation of cytidine and cytidine-5'-phosphate in the presence of 10^{-2} M-Mg^{2+}

Temp. (°C)	Cytidine		Cytidine-5'-phosphate	
	pK	$\Delta G^{\circ} \frac{kcal}{mol}$	pK	$\Delta G^{\circ} \frac{kcal}{mol}$
10	4.20	5.43	4.64	6.01
20	4.07	5.45	4.53	6.07
40	3.84	5.49	4.32	6.19
60	3.63	5.53	4.14	6.31
80	3.45	5.57	3.99	6.44
	$\Delta H^{\circ} = 4.88 \frac{kcal}{mol}$		$\Delta H^{\circ} = 4.29 \frac{kcal}{mol}$	
	$\Delta S^{\circ} = 1.9 \frac{cal}{mol\,°K}$		$\Delta S^{\circ} = 2.9 \frac{cal}{mol\,°K}$	

The estimated errors in pK_a, pK and ΔG° have been calculated as 0.01, 0.06 and 0.09, respectively, with a confidence level of 80%. The standard error of the values of ΔH° is calculated as 0.08.

CONCLUDING REMARKS

The values for the dissociation constants for cytidine, and associated thermodynamic parameters, obtained in this study are compared in Table 5 with those of other observers. In general the agreement is quite satisfactory, lending confidence to the pK_a and pK values reported herein for a fairly wide range of temperatures. One major discrepancy in the Table 5 is the value of Lewin & Humphreys (1966) for the pK of cytidine. Unfortunately we are not in a position to account for this difference, since the foregoing authors provide no details as to how the pK value was arrived at from their measurements.

Good evidence for the fact that protonated cytidine residues in poly C do not behave like protonated monomers is provided by Guschlbauer's (1967) spectra for the coil forms of acid twin-helical poly C at various pH values, at 90°. However, the use of these spectra by the author to establish the pK of the monomer units in the polymer is hardly warranted. It is undoubtedly coincidental that he obtains a pK_a for protonation of the coil form of poly C at 90° which is close to the pK_a of cytidine-5'-phosphate at 20°.

It is somewhat surprising that the presence of Mg^{2+} cations affects not only the pK_a of cytidine-5'-phosphate, but also that of cytidine, and this to an even greater extent. The observed differences between the solutions in the presence and absence of Mg^{2+} apply likewise to the temperature-dependence of the pK_a values. This is reflected in the differences in molar enthalpy, which amount to 0.87 kcal/mole for

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Table 5

Published p*K values and thermodynamic constants for protonation of cytidine*

p*K*	ΔH°	ΔS°	Temp. (°C)	μ	Method	Reference
4.22	—	—	25	—	titrimetric	Levene & Simms (1925)
4.1	—	—	—	—	spectral	Fox & Shugar (1952)
4.11 4.25	—	—	—	—	titrimetric spectral	Fox *et al.* (1953)
4.15	—	—	—	—	spectral	Cohn (1955)
4.11±0.05	—	—	—	—	spectral	Fox *et al.* (1959)
4.22	4.4 ±0.5	—5.0	25	0.1	titrimetric	Sukhorukov *et al.* (1964)
4.54±0.02 4.24±0.04	3.70±0.5	—8.66±1.2	20	0.0 0.2	titrimetric spectral	Lewin & Humphreys (1966)
4.44±0.02 4.16±0.04	3.70±0.5	—8.66±1.2	30	0.0 0.2	titrimetric spectral	
4.08±0.01	4.47±0.16	—3.9±0.6	25	0.0	titrimetric	Christensen *et al.* (1967)
4.17	—	—	—	0.0	spectral	Clauwaert & Stockx (1968)
4.09±0.06 4.23±0.01	4.01±0.08	—5.0±0.5	20	0.0 0.2	spectral spectral	This study

cytidine and 0.65 kcal/mole for cytidine-5′-phosphate, as well as for the other thermodynamic parameters (Tables 3 and 4). These findings do not lend themselves readily to interpretation, but they do suggest that the interaction of the bases or nucleosides with divalent cations is more complex than usually assumed. The foregoing results are somewhat at variance with those of Clauwaert & Stockx (1968) for the interaction of uracil derivatives with Ca^{2+} cations, the biological effects of which, in many instances, are analogous to those of Mg^{2+}.

It would obviously be desirable to obtain similar data for the protonation of adenosine and adenosine-5′-phosphate for possible applications to the helix-coil transitions in poly A's. However, in view of the small modifications in absorption spectrum accompanying protonation of adenosine, such measurements will undoubtedly require the use of titrimetric methods.

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REFERENCES

Adler A., Grossman L. & Fasman G. D. (1967). *Proc. Nat. Acad. Sci. (U.S.)* **57**, 423.
Akinrimisi E. O., Sander C. & Ts'o P. O. P. (1963). *Biochemistry* **2**, 340.
Barszcz D. & Shugar D. (1968). *Europ. J. Biochem.* **5**, 91.
Bates R. G. (1964). *Determination of pH.* J. Wiley & Sons, Inc., New York, London, Sydney.
Christensen J. J., Rytting J. H. & Izatt R. M. (1967). *J. Phys. Chem.* **71**, 2700.
Clauwaert J. & Stockx J. (1968). *Zeitschr. Naturforsch.* **23b**, 25.
Cohn W. E. (1955). In *The Nucleic Acids* (E. Chargaff & J. N. Davidson, eds.). vol. 1, p. 211. Academic Press, New York.
Fox J., Cavalieri L. & Chang N. (1953). *J. Am. Chem. Soc.* **75**, 4315.
Fox J. J., Pragg D. V., Wempen I., Doerr I. L., Cheong L., Knoll J. E., Eidenoff M. L., Bendich A. & Brown G. B. (1959). *J. Am. Chem. Soc.* **81**, 178.
Fox J. & Shugar D. (1952). *Biochim. Biophys. Acta* **9**, 369.
Guschlbauer W. (1967). *Proc. Nat. Acad. Sci. (U.S.)* **57**, 1441.
Hartmann K. A. & Rich A. (1965). *J. Am. Chem. Soc.* **87**, 2033.
Holcomb D. N. & Timasheff C. N. (1968). *Biopolymers* **6**, 513.
Inman R. B. (1964). *J. Mol. Biol.* **9**, 624.
Jones P. F. & Siegel S. (1969). *J. Chem. Phys.* **50**, 1134.
King E. J. (1965). *Acid-Base Equilibrium.* Pergamon Press, Oxford.
Langridge R. & Rich A. (1963). *Nature* **198**, 725.
Levene P. & Simms M. (1925). *J. Biol. Chem.* **65**, 519.
Lewin S. & Humphreys D. A. (1966). *J. Chem. Soc. (B)*, p. 210.
Morita M. & Nagakura S. (1968). *Theoret. Chem. Acta (Berl.)* **11**, 279.
Sukhorukov B. I., Poltev V. I. & Blumenfeld L. A. (1964). *Abhandl. Deut. Acad. Wiss. Berlin, Kl. Med.* 381.
Szer W. & Shugar D. (1966). *J. Mol. Biol.* **17**, 174.
Żmudzka B., Bollum J. F. & Shugar D. (1969). *Biochemistry* **8**, 3049.

KONFORMACJA KWAŚNEJ STRUKTURY POLI C: ZALEŻNOŚĆ PROTONACJI CYTYDYNY I 5'-FOSFORANU CYTYDYNOWEGO OD TEMPERATURY I SIŁY JONOWEJ

Streszczenie

1. Zmierzono wartości p*K* dla protonacji azotu N_3 pierścienia w cytydynie i 5'-fosforanu cytydynowego w zakresie od 10 - 80° i przy siłach jonowych 0.1, 0.2 i 1.0.

2. Badano wpływ na p*K* dwuwartościowego kationu Mg^{2+}.

3. Obliczono stałe termodynamiczne dla reakcji protonacji.

4. Przedyskutowano znaczenie wyników w odniesieniu do przejścia spirala-kłębek w środowisku kwaśnym dwuniciowej formy różnych analogów poli C.

5. Widma absorpcyjne formy obojętnej i uprotonowanej cytydyny wykazują małą, ale wyraźną, zależność od temperatury, podobną jak w innych pochodnych pirymidyn i puryn. Chociaż pochodzenie efektu na widmo elektronowe nie jest jasne, podkreślono jego znaczenie dla interpretacji przejść spirala-kłębek w polinukleotydach badanych techniką absorpcji w nadfiolecie.

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ELECTROREDUCTION OF HALOGENOPYRIMIDINES

ELECTROCHEMICAL REDUCTION OF 5-HALOGENODERIVATIVES OF URACIL, ITS NUCLEOSIDES AND OLIGONUCLEOTIDES

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1. The mechanism of the electrochemical reduction of 5-halogenoderivatives of uracil, 1-methyluracil, 1,3-dimethyluracil, uridine, and oligonucleotides was investigated by classical polarography. Macroscale electrolysis was done in order to identify products of the electrochemical reduction. The electrolysis products were investigated polarographically, spectrophotometrically and chromatographically. 2. All the investigated compounds were found to be reduced on the mercury dropping electrode. The electroreduction mechanism of I-, Br-, and Cl-derivatives consists in a two-electron carbon-halogen bond fission. The reduction products are, respectively, uracil or its glycoside and a halogen ion. 3. The electrochemical reduction of 5-F-uracil is a four-electron process which involves a fission of the carbon-fluorine as well as C=C bond.

In the course of a study on the electrochemical reduction of pyrimidine derivatives (Czochralska, 1970) our interest was directed to 5-halogenoderivatives of uracil. Uracil itself in aqueous medium is polarographically inactive, as indicated by previous investigations (Cavalieri & Lowy, 1952; Hamer, Waldron & Woodhouse, 1953). The polarographic activity of 5-halogenoderivatives of uracil may be due, therefore, to the reactivity of the carbon-halogen bond. This fact facilitates considerably the investigations of the reaction mechanism not only of the bases themselves but also their nucleotides and oligonucleotides. So far, the electroreduction properties of several nucleic acids have been studied by direct current polarography, alternating current (Paleček, 1969) polarography and oscillopolarography (Paleček, 1966; Janik & Paleček, 1966; Paleček & Vetterl, 1968). Electrochemical and polarographical properties of base, nucleosides and nucleotides have been comprehensively discussed by Elving & Janik (1968) and Smith & Elving (1962a,b). The importance of 5-halogenoderivatives of uracil from a biological point of view, as structural analogues of natural pyrimidine bases, substantiates our interest in the compounds. 5-F-uracil is an analogue of uracil and may be incorporated into RNA. The other 5-halogenoderivatives may be substituted for thymine (Lozeron & Gordon, 1964) in DNA. 5-Br-uracil is especially worthy of note as a strong mutagenic agent (Freese,

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1959). 5-F-uracil has been the subject of photochemical investigations (Fikus, Wierzchowski & Shugar, 1964, 1965) owing to its high reactivity to ionizing radiation and ultraviolet light.

The present work is devoted to investigations of the mechanism of electrochemical reduction of 5-substituted halogenouracils, passing from the bases through the nucleosides to the oligonucleotides. The influence of the structure and size of the molecule on its electrochemical properties was taken into account as done by Janik & Paleček (1964) for cytosine derivatives. In this manner derivatives 5-I-uracil, 5-Br-uracil, 5-Cl-uracil, and 5-F-uracil were investigated; unfortunately, sufficient amounts of the nucleotides and oligonucleotides were not always available.

MATERIALS AND METHODS

Reagents from the following sources were used: 5-I-uracil, 5-I-uridine, 5-Cl-uridine, 1-methyl-5-Br-uracil and 1,3-dimethyl-5-Cl-uracil as described elsewhere (Berens & Shugar, 1963). 5-Br-uracil from Reanal (Budapest, Hungary); 5-F-uracil from Hoffman La Roche, Switzerland.

5-Cl-uracil was synthesized by the Gershon method (1961). In 200 ml. of a 5% mixture of acetic anhydride and acetic acid 11.2 g. (0.1 mole) of uracil was dissolved and catalytic quantity of aluminium chloride was added, then 18.4 g. (0.11 mole) of sulfuryl chloride was added in small portions to the solution. Mixture was refluxed for an additional 2 hr. until no more hydrogen chloride came off. The material was cooled to room temperature and the solids filtered off. The yield was 12.5 g. The product, crystallized from ethanol, has m.p. 321° (decomposition).

1,3-Dimethyl-5-Br-uracil was obtained by treating 1,3-dimethyluracil (20 m-moles) with an equivalent amount of bromine (1.04 ml.) according to the procedure given by Wand (1959). The product crystallized from water has m.p. 179 - 180°.

5,6-Dihydro-5-Br-uracil was prepared by Dr. M. Fikus according to the procedure of Gearien & Binkley (1958).

5-I- and 5-Cl-oligonucleotides containing mixed 2′ and 3′ bonds (from pentanucleotide upwards) were kindly supplied by Dr. A. M. Michelson.

Purity of the reagents were checked spectrophotometrically and chromatographically.

The investigations were made in aqueous buffer solutions in the pH range 1 - 13 with exception of oligonucleotides. The insufficient amount of the last named compounds obliged us to investigate their polarographic reduction at pH 7.4 only.

The polarographic measurements were made with a Hungarian Radelskis OH-120 polarograph. Two dropping mercury electrodes of the following parameters were used: $h=65$ cm., $m_1=1.81$ mg./sec., $m_2=2.96$ mg./sec., $t_1=3.75$ sec., $t_2=2.7$ sec. in distilled water at open circuit. Oxygen was removed from the solution by bubbling nitrogen through it.

The solutions of the concentration 0.5 mM to be investigated polarographically were prepared by dilution with an appropriate buffer solution of a 10 mM ethanol stock solution. The buffer solutions used are given in Table 1. Ionic strength of

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the buffer solutions was kept constant, equal to 0.2 M, by addition of a calculated quantity of KCl. The linear dependence of limiting current on the square root of mercury column and the values of the temperature coefficients (see Table 2) for all investigated compounds, except 5-F-uracil, indicates that polarographical waves of these compounds are diffusion controlled. Limiting current is a linear function of the concentration of depolarizer in the concentration range 0.1 - 1 mM (except 5-F-uracil). The logarithmic analysis shows that all waves of investigated compounds were irreversible.

The pH of solutions was measured by using Radiometer pH-meter.

Macroscale electrolysis was done with a mercury electrode (surface area 12 $cm.^2$) vigorously stirred with a magnetic stirrer. For electrolysis, 10 mg. of the substance, dissolved in 20 ml. of buffer, was used. The potentials were measured against saturated calomel electrode (S.C.E.). To the electrolysis circuit was incorporated an additional coulometric system to determine the number of electrons involved in the reduction.

The electrolysis products were identified spectrophotometrically and by ascending paper chromatography on Whatman no. 1 and 3 papers. Uracil derivatives, which did not absorb UV light, were made visible on the chromatogram by the so-called Fink reaction (cf. Janion & Shugar, 1960). The following solvents were used: *I*, ethyl acetate - water - formic acid (60:28:12, by vol.); *II*, ethyl acetate - water - formic acid (70:5:25, by vol.); *III*, butanol - acetic acid - water (2:1:1, by vol.).

Spectrophotometric measurements in the region of 220 - 320 mμ were made with a Zeiss USU 2-P spectrophotometer.

Anions of iodine, bromine, and chlorine were detected with $AgNO_3$. Anions of fluorine were determined colorimetrically by measurement of the formation of a blue complex in the reaction of a fluoride ion with a Ce(III) salt and 1,2-dihydroxyanthraquinone-3-methylamino-*N*,*N*-diacetic acid (Belcher & Wast, 1961; Method Sheet no. 69, Unicam Instruments Ltd, 1962).

RESULTS

5-I-*derivatives of uracil, uridine, and uracil oligonucleotides*

Polarography. 5-I-uracil, 5-I-uridine and oligonucleotides give a single diffusion-controlled wave.

A polarographic maximum appears on the wave of the iodo-derivatives, whose height is pH-dependent but it is independent of the height of the mercury column and the depolarizer concentration. The maximum is due to strong interactions between iodine and mercury and to streaming phenomenon at the electrode (Feoktistov, 1966). The maximum can be suppressed easily by addition to the solution of a small amount of 0.1% gelatine solution. The reduction potential of I-uracil and I-uridine is pH-independent in the pH range corresponding to the reduction of neutral molecules, as indicated by the dependence of $E_{1/2}$ on pH (Fig. 1 and Table 2). $E_{1/2}$ becomes more negative with increasing pH for pH values corresponding to the reduction of anions. The slope of the plot $E_{1/2}$ *versus* pH corresponds to the

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Table 1

Buffer and background electrolyte solutions

pH	Composition
1 - 2	HCl+KCl
3 - 5	0.2 M-CH_3COOH+0.2 M-CH_3COONa+KCl
6 - 8	0.66 M-KH_2PO_4+0.66 M-Na_2HPO_4+KCl
9 - 11	0.2 M-NH_3+0.2 M-Na_2HPO_4+KCl
12 - 13	NaOH+KCl

Table 2

Polarographic data for the reduction of halogenated uracils and their glycosides

Compound	pH range	$-E_{1/2}$ V	pK_a^*	pK_a^{**}	Diffusion current I_d constant	The temperature coefficients (per cent/deg)
5-I-uracil	1.0-8.4	1.02	8.25	8.40	4.3±0.2	2.3
	8.4-13	1.02+0.1/pH				
5-I-uridine	1.0-8.9	0.92				
	8.9-13	0.92+0.1/pH	8.50	8.90	3.3±0.2	2.0
Oligonucleotides of 5-I-uracil	7.4	0.96	—	—		2.1
5-Br-uracil	6.1-8.6	1.62+0.02/pH				
	8.6-11	1.67+0.08/pH	8.05	8.60	4.7±0.1	1.7
1-Me-5-Br-uracil	6.2-8.4	1.60+0.02/pH	8.30	8.40	3.8±0.2	1.6
	8.4-11	1.65+0.07/pH				
1,3-DiMe-5-Br-uracil	6.1-13	1.60	—	—	2.9±0.2	1.5
5-Br-deoxyuridine	6.2-8.3	1.48+0.04/pH	8.10	8.30	3.1±0.2	1.8
	8.3-11	1.56+0.08/pH				
5,6-Dihydro-5-Br-uracil	3.5-11	—	—	—	—	—
5-Cl-uracil	6.0-7.9	1.75+0.01/pH	7.95	7.90	4.3±0.1	2.5
	7.9-10	1.77+0.05/pH				
1,3-DiMe-5-Cl-uracil	7.0-10	1.80	—	—	4.5±0.3	2.2
5-Cl-uridine	6.0-8.6	1.65+0.02/pH	8.50	8.60	3.3±0.4	1.1
	8.6-11	1.70+0.07/pH				
Oligonucleotides of 5-Cl-uracil	7.4	1.75	—	—	—	2.6
5-F-uracil	7.4	1.845	7.8	—	8.9±0.8	4.4

* Dissociation constant taken from literature (Berens & Shugar, 1963).

** Apparent polarographic dissociation constant.

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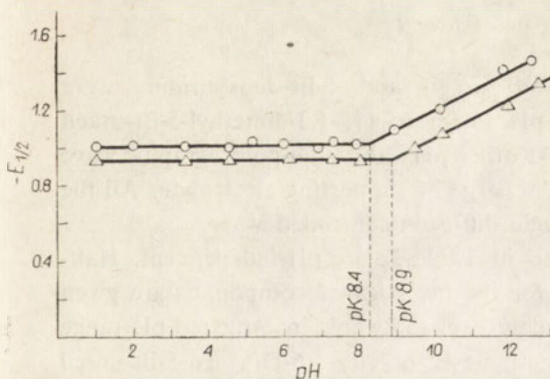


Fig. 1

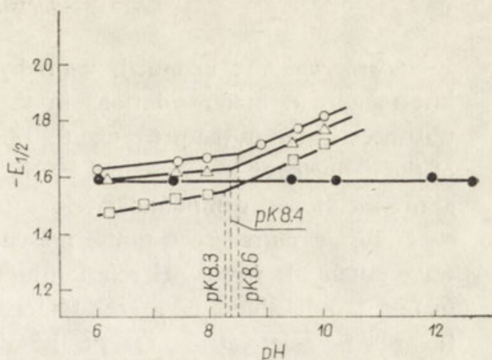


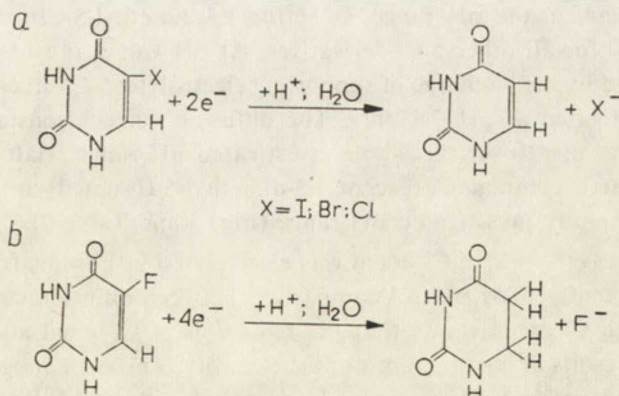
Fig. 2

Fig. 1. The dependence of $E_{1/2}$ on pH of: ○, 5-I-uracil; △, 5-I-uridine.

Fig. 2. The dependence of $E_{1/2}$ on pH of: ○, 5-Br-uracil; △, 1-Me-5-Br-uracil; ●, 1,3-DiMe-5-Br-uracil; □, 5-Br-deoxyuridine.

p*K* of the investigated compounds. The dependence of $E_{1/2}=f(pH)$ for 5-I-uracil oligonucleotides was not determined, since the compounds were not available in sufficient amounts. The results of polarographic analysis of the oligonucleotides are summarized in Table 2.

Macroscale electrolysis. 5-I-uracil was electrolysed at a constant, controlled potential of -1.25 V, which corresponded to the beginning of the limiting current of the wave in phosphate buffer pH 7.43. The coulometric investigations showed the reduction of 5-I-uracil to be a two-electron process. The results of investigations, given in Table 3, indicate the reduction product and uracil to be identical. Simultaneously, in the electrolysed solution iodides were detected. On the basis of these data it was concluded that the investigated polarographic wave corresponded to the reduction of the C-I bond in 5-I-uracil, according to the mechanism given in Scheme 1.



Scheme 1. Mechanism of electroreduction of 5-halogenoderivatives of uracil.

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5-Br-*derivatives of uracil*

Polarography. 5-Br-uracil, 1-methyl-5-Br-uracil and 5-Br-deoxyuridine were investigated in buffer solutions in the pH range 6 - 11, 1,3-dimethyl-5-Br-uracil was investigated in the pH range 6 - 13. At other pH values the polarographic wave of the depolarizer was obscured by the discharge of supporting electrolyte. All the above-mentioned compounds yield a single diffusion-controlled wave.

Diffusion current constants, presented in Table 2, are pH-independent. Half-wave potentials *versus* pH relationships for the investigated compounds are given in Fig. 2 and Table 2. $E_{1/2}$ are pH-dependent over the whole investigated pH range for all 5-Br-derivatives except 1,3-dimethyl-5-Br-uracil. 5,6-Dihydro-5-Br-uracil was found to be polarographically inactive.

Macroscale electrolysis. 5-Br-uracil and 1,3-dimethyl-5-Br-uracil were electrolysed at a potential of -1.75 V in phosphate buffer pH 7.4. Coulometric measurements show the reduction of these substances to be a two-electron process throughout the investigated pH range, since the diffusion current constant is pH-independent.

The results of investigations of electrolysis products are given in Table 3. These data indicate that the products of reduction of 5-Br-uracil and 1,3-dimethyl-5-Br-uracil are uracil and 1,3-dimethyluracil, respectively. At the same time, in both cases in the electrolysed solution, bromide ions were detected. Thus, it can be stated on the basis of these data, that the reduction of 5-Br-uracil and 1,3-dimethyl-5-Br-uracil involves a C-Br bond fission according to the mechanism given in Scheme 1.

Also, the reduction of the other 5-Br-derivatives, i.e. 1-methyl-5-Br-uracil and 5-Br-deoxyuridine proceeds according to the same mechanism. This is indicated by the analogous (as in the case of 5-Br-uracil) character of the waves and similarity in half-wave potentials and diffusion current constants (Table 2).

5-Cl-*derivatives of uracil, uridine and uracil oligonucleotides*

Polarography. 5-Cl-uracil, 1,3-dimethyl-5-Cl-uracil, 5-Cl-uridine and oligonucleotides of 5-Cl-uracil give a single diffusion wave in aqueous buffer solutions. The wave appears in the pH range 7 - 11 for 1,3-dimethyl-5-Cl-uracil and in the pH range 6 - 10 for all other 5-Cl-derivatives. At pH values outside this range the wave is obscured by the discharge of supporting electrolyte. 5-Cl-uracil oligonucleotides were investigated at pH 7.4 only. The diffusion current constants (see Table 2) are pH-independent over the whole investigated pH range. Half-wave potential of the investigated compounds (except 1,3-dimethyl-5-Cl-uracil) are pH-dependent throughout the entire investigated pH range (Fig. 3 and Table 2).

Macroscale electrolysis. 5-Cl-uracil was electrolysed in phosphate buffer pH 7.4 at a constant potential of -1.80 V. A comparison between diffusion current constants of Br-uracil and Cl-uracil shows that the reduction of Cl-uracil also involves two electrons. The results of investigations of the electrolysis product are given in Table 3. The investigated product was found to be identical with uracil. Simultaneously, in the electrolysed solutions chloride ions were detected. These data indicate that the

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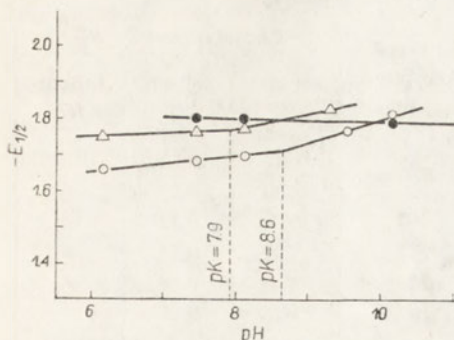

Fig. 3

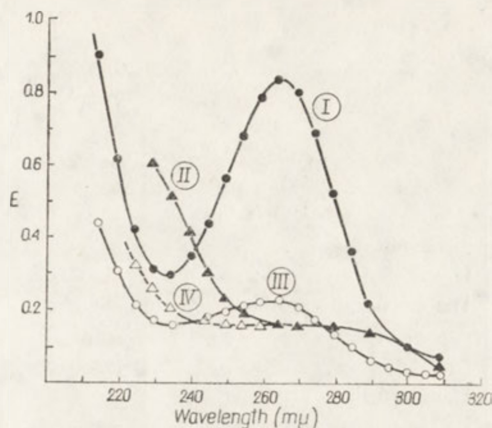

Fig. 4

Fig. 3. The dependence of $E_{1/2}$ on pH of: △, 5-Cl-uracil; ○, 5-Cl-uridine; ●, 1,3-DiMe-5-Cl-uracil.

Fig. 4. Absorption spectra of: *I*, 5-F-uracil at pH 7.43; *III*, electrolysis product of 5-F-uracil at pH 7.43; *II*, electrolysis product at pH 13; *IV*, electrolysis product at pH 13 after 1 hr.

polarographic wave of 5-Cl-uracil corresponds to the reduction of the C-Cl bond according to the mechanism given in Scheme 1.

Also, in the case of other 5-Cl-derivatives, i.e. 1,3-dimethyl-5-Cl-uracil, 5-Cl-uridine, and oligonucleotides of 5-Cl-uracil, the C-Cl bond is reduced, as indicated by analogous character of waves, similar half-wave potentials and diffusion current constants.

5-F-*uracil*

Polarography. 5-F-uracil gives in phosphate buffer pH 7.4 a single, irreversible polarographic wave with a half-wave potential of -1.84 V. In other buffer solutions the wave is obscured by the discharge of supporting electrolyte. The wave is twice as high as those of all the compounds described previously. This suggests therefore that the reduction of 5-F-uracil involves four electrons. The temperature coefficient is equal to 4.4 ± 0.1 per cent/deg, i.e. its value is larger than that of purely diffusion-controlled waves. At the same time, the dependence of the limiting current on the height of the mercury column and on concentration of the depolarizer shows the wave to have a kinetic character.

Macroscale electrolysis. Electrolysis of 5-F-uracil was done in phosphate buffer pH 7.4 at a constant controlled potential of -1.9 V.

The electrolysis product of 5-F-uracil is polarographically inactive and shows only a residual UV absorption (Fig. 4). The spectrum of the electrolysis product disappears rapidly on alkalization to pH 13. The electrolysis product, investigated by paper chromatography, yielded a yellow spot (made visible by the Fink method) having an R_F identical with that of dihydrouracil (see Table 3).

At the same time, fluorides were determined in the electrolysed solutions by

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Table 3

Identification of the electrolysis products

Compound	Polarography	UV absorption λ_{max}(mμ)	Chromatography R_F^*		
			solvent *I*	solvent *II*	solvent *III*
5-I-uracil	active	285	0.90	0.72	—
Uracil	inactive	260	0.78	0.59	—
The electrolysis product	inactive	260	0.78	0.59	—
5-Br-uracil	active	277	—	0.54	—
Uracil	inactive	260	—	0.38	—
The electrolysis product	inactive	260	—	0.38	—
1,3-DiMe-5-Br-uracil	active	283	—	—	0.87
1,3-DiMe-uracil	inactive	267	—	—	0.80
The electrolysis product	inactive	267	—	—	0.80
5-Cl-uracil	active	275	—	0.66	—
Uracil	inactive	260	—	0.60	—
The electrolysis product	inactive	260	—	0.60	—
5-F-uracil	active	268	—	—	—
5,6-Dihydrouracil	inactive	residual absorption	0.82	0.50	—
The electrolysis product	inactive	residual absorption	0.82	0.50	—

* The electroreduction product of 5-F-uracil and 5,6-dihydrouracil following ring opening, gave a yellow colour after detection according to the procedure described by Janion & Shugar (1960).

means of a colour reaction with alizarine complex. Since phosphate ions, present in the solution, interfered with the formation of the complex (decreasing the extinction at the analytical wavelength), the solution was diluted to 1/8 of its initial volume; the following amounts of fluoride ions were found for three samples analysed subsequently: 40, 40 and 36 μg./ml. The calculated theoretical amount of fluoride was 45 μg./ml. The data obtained show the electrolysis product of 5-F-uracil to be identical with 5,6-dihydrouracil; the reduction proceeds according to the mechanism given in Scheme 1.

CONCLUDING REMARKS

It was found on the basis of our investigations that all the compounds having halogens at the 5-position were polarographically active and formed a two-electron irreversible diffusion polarographic wave (except 5-F-uracil). This wave corresponded to the reduction of the C-X bond (X is a halogen). As a result of the reduction, halogen ions and uracil or its derivatives were formed according to the mechanism given in Scheme 1.

In the pH range below pK_a, where a neutral molecule is reduced, the half-wave potential is pH-independent only for iodo-derivatives, as indicated by the course

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of the $E_{1/2}$ *versus* pH plot. The acid dissociation constants pK_a, determined polarographically for the investigated compounds, differ only slightly from those determined spectrophotometrically (Table 2).

The ease of reduction of a halogen increases in the order F<Cl<Br<I. Substitution of uracil at the 1-position with ribose affects only slightly the half-wave potential shift towards more positive potentials, due to the mesomeric substituent effect, and does not change the reaction mechanism.

Oligonucleotides are reduced somewhat more easily, probably owing to the ordered state of the molecule and to the influence of the phosphate groups. Half-wave potentials of uracil chloro-derivatives may be arranged in the following order: base<oligonucleotide<nucleoside. A similar order of half-wave potential has been found by Janik & Paleček (1964) for cytosine derivatives.

Substitution of a 5-chlorouracil molecule by methyl at positions 1 and 3 affects only slightly the half-wave potential shift (Table 2) in relation to the corresponding 5-halogenouracils.

Half-wave potentials of 1,3-dimethyl-derivatives of 5-halogenouracil are pH-independent owing to the absence of any protons capable of dissociation in the molecule. It is interesting to note that in this case any ability of a halogen (bromine or chlorine) to react with a proton disappears. This might be evidence of the dependence of $E_{1/2}$ on pH, observed for 5-chlorouracil and 5-bromouracil, to be connected with the interaction of the halogen atom with a proton in the molecule.

Polarographic inactivity of 5,6-dihydro-5-bromouracil over the investigated pH range indicates that the saturation of the double bond at position 5,6 increases the resistance of the C-X bond to polarographic reduction. This represents the effect of a double bond on the reduction of a halogen substituted at α-position (Fukui, Morokuma, Kato & Yonezawa, 1963).

The electrochemical reduction of 5-F-uracil differs from that of the other halogeno-derivatives. 5-F-uracil gives an irreversible four-electron kinetic-diffusion wave. The wave is observed in buffer of pH 7.4 only. It has been stated, on the basis of the investigation of reduction products, that during the reduction a fission of both the C-X bond and the 5,6 double bond takes place simultaneously. The energy of the two bonds (107 and 100 kcal/mole for the C-F and the C=C bond, respectively) is comparable, which supports the conclusion concerning the simultaneous reduction of the two bonds.

The fact that the reduction of 5-F-uracil can be observed at pH 7.4 (pK_a=7.8) only, shows that the reaction limiting the kinetics of the electrode process is probably related with the dissociation of the molecule.

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REFERENCES

Belcher R. & Wast T. S. (1961). *Talanta* **8**, 853.
Berens K. & Shugar D. (1963). *Acta Biochim. Polon.* **10**, 25.
Cavalieri L. F. & Lowy B. A. (1952). *Arch. Biochem. Biophys.* **35**, 83.
Czochralska B. (1970). *Roczniki Chemii* **44**, 2207.
Elving P. J. & Janik B. (1968). *Chem. Rev.* **68**, 295.
Feoktistov L. G. (1966). In *Uspiechy electrochemii organiczeskich sojedinienij.* "Nauka", Moskwa.
Fikus M., Wierzchowski K. L. & Shugar D. (1964). *Biochem. Biophys. Res. Commun.* **16**, 478.
Fikus M., Wierzchowski K. L. & Shugar D. (1965). *Photochem. Photobiol.* **4**, 521.
Freese E. (1959). *J. Mol. Biol.* **1**, 87.
Fukui K., Morokuma K., Kato H. & Yonezawa T. (1963). *Bull. Chem. Soc. Japan* **36**, 217.
Gearien J. E. & Binkley S. B. (1958). *J. Org. Chem.* **23**, 491.
Gershon P. (1961). *J. Org. Chem.* **26**, 1874.
Hamer D., Waldron P. M. & Woodhouse D. L. (1953). *Arch. Biochem. Biophys.* **47**, 272.
Janik B. & Paleček E. (1964). *Arch. Biochem. Biophys.* **105**, 225.
Janik B. & Paleček E. (1966). *Z. Naturforschg.* **21b**, 1117.
Janion C. & Shugar D. (1960). *Acta Biochim. Polon.* **7**, 1.
Lozeron H. A. & Gordon M. P. (1964). *Biochemistry* **3**, 507.
Paleček E. (1966). *J. Mol. Biol.* **20**, 263.
Paleček E. (1969). *J. Electronal. Chem.* **22**, 347.
Paleček E & Vetterl V. (1968). *Biopolymers* **6**, 917.
Smith D. L. & Elving P. J. (1962a). *J. Am. Chem. Soc.* **84**, 2741.
Smith D. L. & Elving P. J. (1962b). *J. Am. Chem. Soc.* **84**, 1412.
Wand A. (1959). *J. Org. Chem.* **24**, 11.

ELEKTROREDUKCJA POCHODNYCH CHLOROWCOWYCH PIRYMIDYNY

ELEKTROCHEMICZNA REDUKCJA 5-CHLOROWCO POCHODNYCH URACYLU, JEGO NUKLEOZYDÓW I OLIGONUKLEOTYDÓW

Streszczenie

1. Badano mechanizm elektroredukcji 5-chlorowco pochodnych uracylu, 1-metylouracylu, 1,3-dwumetylouracylu, urydyny i oligonukleotydów na elektrodzie rtęciowej. W celu identyfikacji produktów elektroredukcji przeprowadzono elektrolizę preparatywną.

2. Proces elektroredukcji J-, Br- i Cl-pochodnych polega na dwuelektronowej redukcji wiązania C-X (węgiel-chlorowiec). Jako produkt tworzy się odpowiednio uracyl lub jego glikozydy oraz anion chlorowca.

3. Redukcja 5-F-uracylu jest procesem czteroelektronowym, podczas którego następuje przerwanie wiązania węgiel-fluor oraz podwójnego wiązania C=C w pierścieniu w pozycji 5,6. Produktem jest 5,6-dwuhydrouracyl.

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PHOTOCHEMISTRY OF 5-ETHYLURACIL AND ITS GLYCOSIDES

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1. Irradiation of neutral aqueous solutions of 5-ethyldeoxyuridine (or 5-ethyluridine) at 254 mμ leads to more than 80% conversion to deoxyuridine (or uridine) *via* an intermediate consisting of two isomers, one of which is labile in 0.1 N-NaOH. Both isomers, on irradiation at 254 mμ, either in neutral or alkaline media, undergo de-ethylation and conversion to deoxyuridine (or uridine) with a quantum yield of about 0.30. 2. The intermediate has been shown to be a rearrangement product of 5-ethyldeoxyuridine, in which the 5-ethyl substituent forms a cyclobutane ring with the pyrimidine 5,6 bond; the intermediate is therefore 5,6-dihydro-5,6-cyclobutanyluridine, a member of a new class of dihydropyrimidine nucleosides. 3. The photochemical conversion of 5-ethyldeoxyuridine (EtUdR) to deoxyuridine (UdR) at 254 mμ is essentially unaffected by the presence of oxygen or paramagnetic ions and probably proceeds *via* the first singlet excited state. 4. Under the foregoing conditions there occurs also formation of a minor photoproduct with a UV absorption at longer wavelengths; during the initial stages of the reaction, 10 - 15% of the photoproducts also include 5-ethyldeoxyuridine photodimers. 5. The rate of photochemical transformation of the *β*-anomer of EtUdR is twice that for the *α*-anomer. The *α*-anomer of UdR·H_2O prepared from *α*-UdR (obtained by photochemical de-ethylation of *α*-EtUdR) undergoes dehydration in acid medium at a rate 8-fold that for *β*-UdR·H_2O. 6. At wavelengths to the red of 265 mμ, 5-ethyldeoxyuridine undergoes primarily photodimerization. All four principal photodimer isomers are formed, with the *cis* isomers predominating. The quantum yield for photodimerization is somewhat higher than that for thymidine; the quantum yield for photodissociation of the dimers is about 0.60. 7. The photodimerization reaction is strongly inhibited by oxygen and paramagnetic ions (Mn^{2+}), indicating that it proceeds *via* an excited triplet state. This is further supported by the observation that photodimerization (but not the de-ethylation pathway with formation of deoxyuridine) is sensitized by acetone (with approximately the same yield as for thymidine), suggesting that the triplet energy level of 5-ethyldeoxyuridine is about the same as that for thymidine. 8. Irradiation of 5-ethyldeoxyuridine in an ice matrix at 254 mμ leads to photodimerization at approximately the same rate, and to almost the same photostationary state, as for thymidine. The same applies to 1-methyl-5-ethyluracil; whereas 5-ethyluracil itself is almost unaffected. By contrast, in aqueous medium, both 5-ethyluracil and 1-methyl-5-ethyluracil undergo the same photochemical transformations as the glycosides. 9. The mechanisms of the foregoing reactions, and their application to the photochemistry of DNA phages containing 5-ethyluracil residues in place of thymine, and their bearing on the photochemistry of thymine and its glycosides, are discussed.

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It has previously been shown that 5-ethyluracil, EtU[1], can replace thymine (5-methyluracil) in bacterial (Piechowska & Shugar, 1965) and bacteriophage (Pietrzykowska & Shugar, 1966, 1967) DNA. Attention was also directed to the fact that of all known base analogues, 5-ethyluracil is the first which possesses the same base-pairing properties as its parent base, in this case thymine (Świerkowski & Shugar, 1969, 1970). In accordance with this, its incorporation into phage DNA is non-mutagenic (Pietrzykowska & Shugar, 1967, Świerkowski & Shugar, 1969).

During the course of a study of the photochemistry of phage T_3, in which 65% of the thymine residues had been substituted by EtU (Pietrzykowska & Shugar, 1968a), it was observed that the radiation sensitivity, as well as the dark and photo reactivation, of this bacteriophage were practically identical with those for the normal, control, phage. This prompted the initiation of an investigation into the photochemistry of EtU and its glycosides, which forms the subject of the present paper. Some of the earlier results have been published in condensed form (Pietrzykowska & Shugar, 1968b).

One might also expect the photochemical behaviour of EtU to be of some relevance to the photochemistry of thymine itself, and this point will be elaborated upon in the Discussion, below.

MATERIALS AND METHODS

EtU, EtUdR, EtUR, 2′,3′-isopropylidene-EtUR, 1-methyl-EtU and 1,3-dimethyl-EtU were all synthesized as elsewhere described (Świerkowski & Shugar, 1969, 1970). UR, UdR and TdR were Calbiochem. (Los Angeles, Calif., U.S.A.) grade A products, checked spectrally and chromatographically. Glass doubly distilled water was used throughout and pH measurements were made with a Radiometer PM22 instrument equipped with a glass microelectrode. Spectral measurements made use of a Unicam SP-500 and Beckman DU instruments.

Irradiation sources were as follows:

A, A Phillips 6-watt germicidal lamp (254 mμ) surrounded by a 2-mm. layer of saturated sodium acetate to eliminate traces of radiation below 230 mμ.

B, A Phillips 40-watt germicidal lamp (254 mμ), the radiation of which was freed of wavelengths below 230 mμ by passing through a 5-mm. layer of glacial acetic acid in water (1:2, v/v). The emission of this source at 254 mμ one cm. from the lamp surface was 1.6×10^{17} quanta/cm^2/min.

C, Irradiation at wavelengths to the red of 265 mμ and 275 mμ was achieved by use of a 700-watt medium pressure mercury therapeutic lamp together with Zeiss (Jena) WG7 and WG6 filters, respectively.

Irradiation of solutions was conducted in 10-mm. spectrophotometer cuvettes at concentrations of 0.1 mM. For chromatography, or estimation of liberated ethanol, concentrations of 1 mM in 1-mm. cuvettes were employed. Spectral changes resulting from irradiation, or subsequent dark treatment, were followed by means of Unicam

[1] Abbreviations employed: EtU, 5-ethyluracil; EtUR, 5-ethyluridine; EtUdR, 5-ethyldeoxyuridine; UR, uridine; UdR, deoxyuridine; T, thymine; TdR, thymidine.

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SP-500 and Beckman DU instruments. Dimerization in frozen aqueous medium was followed by irradiation with sources *A* or *B* of a 1-mm. frozen layer of a 1 mM solution. The degree of photodimerization was evaluated by thawing the irradiated solution, bringing the pH to 13 with NaOH, irradiating with source *A*, or *B*, and following the appearance of monomer spectroscopically or by chromatography.

Irradiation in absence of oxygen was conducted with the use of 1-mm. and 10-mm. cuvettes with ground joints and fitted with either glass or teflon stoppers containing inlet and outlet tubes of glass or nylon, respectively. The solutions in the cuvettes were freed of oxygen by flushing for 20 - 40 min., prior to irradiation, with argon which had been passed through a train of three wash-bottles of pyrogallol. Flushing with argon was usually continued during irradiation; for longer irradiation periods the teflon stoppers with nylon inlet and outlet tubes were employed, and the latter sealed off in a match flame after removal of oxygen.

Ethanol estimations on irradiated solutions were carried out enzymically by the method of Bonischen (1963). For this purpose solutions were irradiated at 1 mM concn. in 1-mm. cuvettes and the liquid phase collected by cryoscopy to eliminate interference from other photoproducts. Gas chromatography was also employed using a Pye Model 64 instrument with a Carbowax 20M on 10% Celite column.

Chromatography. Photoproducts were separated and/or isolated by ascending paper and thin-layer chromatography, in one or two dimensions, using Whatman no. 1 paper, MN 300 cellulose (Macher & Nagel, Düren, West Germany) and silica gel GF_{254} (Merck, Darmstadt, West Germany), as well as Eastman (Rochester, N.Y., U.S.A.) 6065 TLC plates, with the following solvent systems (all proportions by vol.):

A. Water-saturated butanol
B. Butanol - acetic acid - water (80:12:30)
C. Butanol - acetic acid - water (74:19:50)
D. Butanol - acetic acid - water (5:2:1)
E. Butanol - water - conc. NH_4OH (86:8:5)
F. Butanol - glacial acetic acid - water (2:2:1, upper phase)
G. Butanol - formic acid - water (80:12:30)
H. Butanol - propanol - water (1:1:1)
I. Water-saturated *sec.*butanol
J. *Tert.*butanol - methyl-ethyl-ketone - water - conc. NH_4OH (40:30:20:10)
K. Isopropanol - water (3:1)
L. Isopropanol - conc. HCl - water (68:15.5:16.5)
M. Isopropanol - conc. NH_4OH - water (7:1:2)
N. *n*-Propanol - water (7:3)
O. *n*-Propanol - water (10:3)
P. Methanol - ethanol - conc. HCl - water (50:25:6:19)
Q. Sat. $(NH_4)_2SO_4$ - 1 M-sodium acetate - isopropanol (40:9:1)
R. Formic acid - *tert.*butanol - methyl-ethyl-ketone - water (15:40:30:15)
S. Water-saturated phenol
T. Ethyl acetate - formic acid - water (70:20:10)
U. 1% $(NH_4)_2SO_4$ - isopropanol (1:2)
V. Chloroform - methanol (85:15)
W. Isobutyric acid - acetic acid - water (100:1:50).

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RESULTS

Irradiation of a 0.1 mM neutral aqueous solution of EtU at 254 mμ leads to the stepwise disappearance of the absorption maximum at 267 mμ at an appreciable rate, as compared to the relative resistance of thymine under the same conditions. This is illustrated in Fig. 1, which also exhibits the relative rates of photolysis of

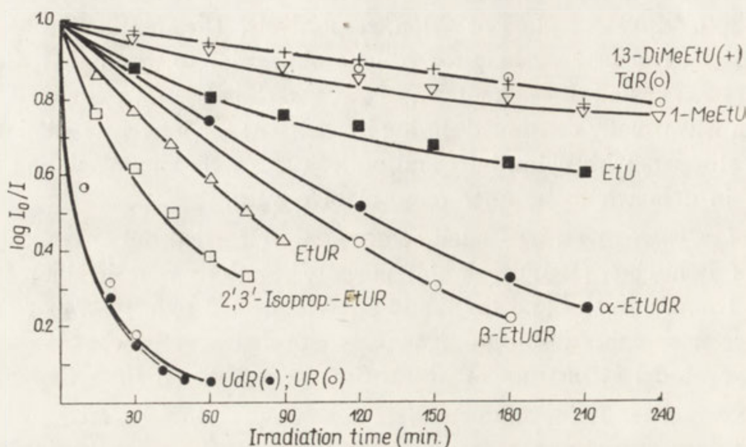

Fig. 1. Course of photochemical transformation at 254 mμ with source *B* (incident intensity 1.6 × 10^{17} quanta/cm^2/min.) of 0.1 mM neutral aqueous solutions of various uracil and 5-ethyluracil derivatives, as measured by decrease in optical density at absorption maximum. Abbreviations as in Table 1.

several EtU analogues and their ribosides, as well as of UR and UdR for comparison purposes. It will be observed that, whereas 1-methyl-EtU and 1,3-dimethyl-EtU are more resistant than EtU itself, the nucleosides of EtU are considerably more susceptible. Table 1 presents the initial quantum yields for photochemical transformation of the various compounds relative to that for UdR. Note that the quantum yield for disappearance of EtUdR is only one-sixth that for UdR.

For 0.1 mM-UdR in neutral aqueous medium, the photochemical transformation is known to involve addition of a water molecule to the 5,6 bond of the pyrimidine ring to give 5-hydro-6-hydroxy-UdR. The water molecule can be eliminated in the dark, by what is essentially an acid-base catalysed reaction, either in acid or alkaline medium, or at elevated temperature at neutral pH, to regenerate UdR (Shugar, 1960).

A 0.1 mM solution of EtUdR was irradiated to the point where the decrease in the height of the absorption maximum was about 75%. A sample of the irradiated solution was then heated in a sealed ampoule at 100°. Additional samples at room temperature were brought to pH 1 (0.1 N-HCl) or pH 12 (0.01 N-NaOH, or addition of conc. NH_4OH to give pH 11.8), and changes in absorption spectra followed as a function of time (Fig. 2). All of these treatments led to appreciable regeneration of the absorption spectrum, i.e. to dark reversal. The degree of "reversal" varied

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Table 1

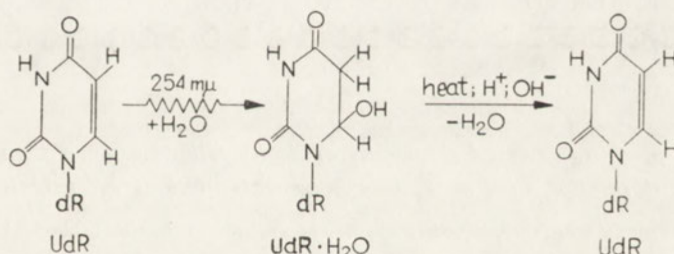
Initial quantum yields for photochemical transformation at 254 mμ of 0.1 mM solutions at neutral pH *of 5-ethyluracil derivatives and 5-ethyluracil glycosides, as compared to uridine and deoxyuridine*

Decrease in optical density at λ_{max} for each compound measured the extent of photochemical transformation.

Compound	$\varnothing \times 10^{-3}$	$\frac{\varnothing UdR}{\varnothing}$
Uridine (UR)	17.0	1.3
Deoxyuridine (UdR)	21.6	1.0
5-Ethyluridine (EtUR)	4.3	5.0
2′,3′-*O*-Isopropylidene-5-ethyluridine	6.5	3.3
α-5-Ethyldeoxyuridine (*α*-EtUdR)	2.5	8.5
β-5-Ethyldeoxyuridine (*β*-EtUdR)	4.3	5.0
α,β-5-Ethyldeoxyuridine (*α,β*-EtUdR)	3.6	6.0
5-Ethyluracil (EtU)	2.2	9.5
1-Methyl-5-ethyluracil (1-MeEtU)	1.0	22.0
1,3-Dimethyl-5-ethyluracil (1,3-DiMeEtU)	0.6	38.0

from 70% to 85%, being maximal on heating at neutral pH for 120 min. or in 1 N-HCl for 10 - 15 min.

The foregoing behaviour is analogous to that for uracil glycosides, referred to above, and which can be depicted as in Scheme 1.



Scheme 1

Two facts argue against this reaction sequence for EtUdR. First, the dark-regenerated absorption spectrum possesses a maximum at 262 mμ at neutral pH, as compared to 267 mμ for the initial EtUdR. Furthermore, when the dark-regenerated solution was again irradiated at 254 mμ, it underwent transformation at a rate 4- to 5-fold greater than that for the initial EtUdR, i.e. its radiation sensitivity was now approximately that for UdR and UR (Fig. 1). It is clear that the photochemical transformation of EtUdR at 254 mμ, and its subsequent dark "reversal" are more complex than in the case of UR or UdR.

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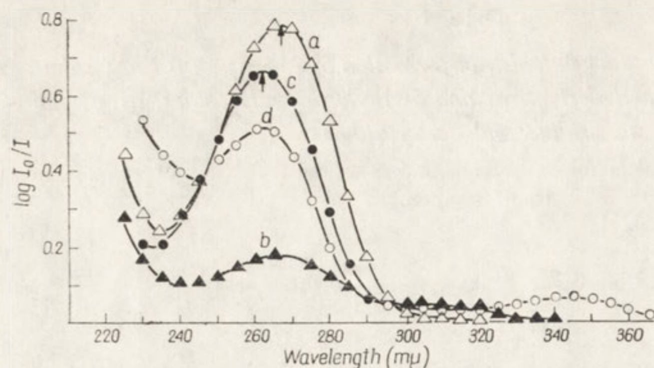

Fig. 2

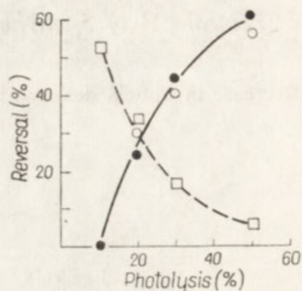

Fig. 3

Fig. 2. Photochemical transformation at 254 mμ, and subsequent transformation in the dark, of 0.1 mM-EtUdR in neutral aqueous medium: (*a*), absorption spectrum prior to irradiation, and (*b*), following 180 min. irradiation; (*c*), following heating of irradiated solution for 2 hr. at 100° (*d*), spectrum of heated irradiated solution at pH 12. Note in particular the shifts in λ_{max}.

Fig. 3. Relative contents, in a solution of EtUdR at neutral pH irradiated at 254 mμ, of photoproducts which subsequently revert to UdR by (●), exposure to 1 N-HCl; (○), exposure to 0.01 N-NaOH; (□) irradiation at 254 mμ in 0.01 N-NaOH. The ordinate scale refers to % regeneration of absorption of UdR at λ_{max} 262 mμ (see page 368).

Photolysis products of EtUdR. The photolysis, and subsequent dark reversal, products of EtUdR were studied by means of thin-layer and paper chromatography with the aid of solvent systems *A*, *B*, *K*, *L*, *M*, *N* (Table 2) and *S*, *T*, *U*, *V*.

An aqueous 1 mM solution of EtUdR was irradiated in a 1-mm. cuvette until the optical density had decreased by 90% at λ_{max}. The irradiated solution was divided into two portions. One of these was not subjected to further treatment, the other was

Table 2

R_F values of products detected chromatographically following more than 80% photochemical transformation of 1 mM aqueous neutral solutions of EtUdR at 254 mμ

Chromatography with various indicated solvent systems on Eastman 6065 TLC plates.

Solvent system	R_F values					
	Standards		Identified products			
	EtUdR	UdR	EtUdR	Photoprod.$_{345}$*	UdR	UdR·H_2O*
A	0.70	0.39	0.70	0.54	0.39	0.30
B	0.73	0.55	0.73	0.62	0.55	0.48
K	0.86	0.70	0.85	0.78	0.69	0.61
L	0.95	0.75	0.95	—	0.75	0.64
M	0.82	0.60	0.82	0.73	0.60	—
N	0.83	0.70	0.83	0.78	0.70	0.63

* The spots corresponding to Photoprod.$_{345}$ (photoproduct with absorption at 345 mμ) and UdR·H_2O were revealed on the chromatograms following exposure to moist ammonia vapour.

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heated in a sealed ampoule for 2 hr. at 100°. Both samples were then deposited on a chromatogram (MN 300 cellulose plate) along with controls of UdR and EtUdR. With solvent *A* the irradiated sample exhibited two spots under a dark UV-lamp, one with R_F 0.77 (EtUdR), the second with R_F 0.48 (UdR); the irradiated-heated sample exhibited one spot at R_F 0.77 with the same intensity as in the irradiated sample, and a second spot with R_F 0.48 about 3 - 5 times as intense as for the irradiated sample. Exposure of the developed chromatogram to ammonia vapour for 30 min. at room temperature, *or* heating to 110° for 2 hr., led to the appearance of a third spot with R_F 0.27 in the irradiated sample. Furthermore, following elution of the spots with R_F 0.27 and 0.48, their absorption spectra at acid, neutral and alkaline pH corresponded to those for UdR; this was further confirmed by rechromatography with several solvent systems (Table 2), and by the demonstration that their sensitivity to irradiation and subsequent alkaline reversal (pH 12) were identical to that of UdR.

Further examination demonstrated that the spot with R_F 0.27, which appears in the chromatogram of irradiated non-heated EtUdR only after heating, or exposure to ammonia fumes, of the chromatogram, is UdR·H_2O. This was confirmed by running a comparison chromatogram of irradiated UdR, the major photoproduct of which is UdR·H_2O; as well as by elution of the area with R_F 0.27 of irradiated non-heated EtUdR, and showing that the kinetics for reversal of this product to UdR under various conditions is identical with that for authentic UdR·H_2O.

Quantitative elution of the spots from the above chromatograms demonstrated that irradiation under these conditions led to photochemical transformation of 80% of the EtUdR to a mixture of UdR·H_2O and UdR in the ratio about 3:1. Since UdR·H_2O is known to be stable to irradiation at 254 mμ, its appearance must have been preceded by that of UdR. It follows that UdR must have been formed by photochemical de-ethylation of EtUdR. It is consequently pertinent to ask whether this latter reaction is a direct one, or proceeds *via* some intermediate (we shall come back, below, to the nature of the photoproducts of the remaining 20% EtUdR not accounted for above).

Mechanism of de-ethylation of EtUdR. On the basis of the observation that the photochemical transformation of EtUdR at 254 mμ is accompanied by the liberation of ethanol, the following scheme (Scheme 2) for this reaction was previously proposed (Pietrzykowska & Shugar, 1968b), and the products III, IV, and V are readily identified by chromatography.

Initial attempts to detect the presumed hydrated intermediate of EtUdR, i.e EtUdR·H_2O, by two-dimensional chromatography (with attempts at dehydration by heating or exposure of the chromatogram to NH_3 fumes before running solvent in the second direction) were unsuccessful. It was inferred that this might be due to the radiation sensitivity of the presumed intermediate (see below). The search for the intermediate was therefore concentrated on the initial stages of photolysis.

Following irradiation of solutions of EtUdR with increasing doses, an examination was made of the degree of regeneration of UV absorption in the neighbourhood of 267 mμ by acidification or alkalization of the solutions. It was found in

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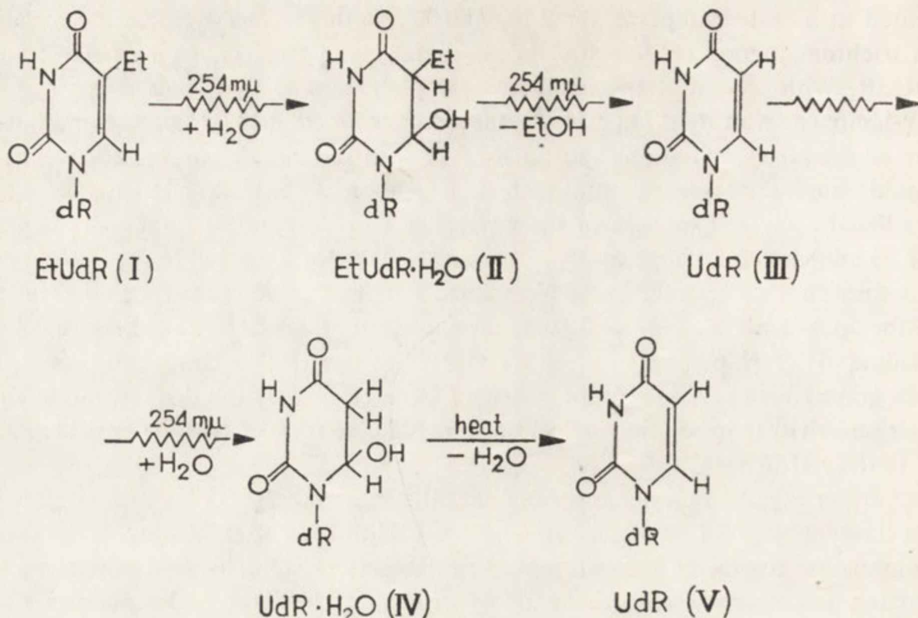

Scheme 2

this way that, the lower the degree of photochemical transformation of EtUdR, the lower the extent of regeneration of UV absorption by acid or alkali. Hence the initial stage of photolysis of EtUdR is accompanied by formation of a photoproduct which exhibits no absorption maximum in the quartz ultraviolet and which does not regenerate such a maximum under conditions leading to dehydration of UdR·H_2O. It was at first suspected that this product (or products) might be photodimers. Attempts were therefore made to regenerate the pyrimidine spectrum by irradiation in alkaline medium (Shugar, 1960). Solutions of EtUdR were irradiated to 10, 20, 30, 50 and 90% photolysis. Each solution was then brought to 0.01 N-NaOH and left for 15 - 20 min., which was sufficient to regenerate UdR from UdR·H_2O, and then irradiated at 254 mμ for several minutes and observing any changes in the optical density at the absorption maximum. In this way the presence was established of a photoproduct which, on irradiation in alkaline medium at 254 mμ, was converted to a product with absorption at 262 mμ. The quantity of this photoproduct detectable in the irradiation medium decreased with increasing photolysis of EtUdR. The relationship between the extent of photolysis of EtUdR and the amount of photoproduct which in 0.01 N-NaOH reverts to UdR (i.e. UdR·H_2O), and that which regenerates absorption at 262 mμ on irradiation in 0.01 N-NaOH, is illlustrated in Fig. 3.

Following 10% photolysis of EtUdR, 70% of the photoproducts formed was subsequently transformed by irradiation in alkaline medium to a product with absorption at 262 mμ, while 30% reverted in alkaline or acid medium in the dark. With increasing degrees of photolysis, the relative amount of the former product decreased, while that of the latter (UdR·H_2O) increased.

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In order to isolate the photoproduct which regenerates an absorption maximum at 262 mμ on irradiation in alkaline medium, 1 mM solutions of EtUdR were irradiated to increasing degrees of photochemical transformation and then chromatographed. In this way it was found that, following low degrees of photolysis and development with solvent *A*, the spot corresponding to UdR (R_F 0.42) contained an additional product which regenerated the 262 mμ absorption maximum on irradiation at 254 mμ in alkaline medium. On the basis of chromatographic evidence, and absorption at various pH values, the regenerated product was identified as UdR. Consequently the (partially) isolated photoproduct appears to be the desired intermediate which undergoes photochemical de-ethylation to give UdR. The optimum ratio of this photoproduct (with respect to UdR), obtained following 15% photolysis, was 7:3 in this case for the β-anomer of EtUdR; for the α-anomer of EtUdR the optimum ratio was 5:5 following 10% photolysis.

Isolation and properties of EtUdR intermediate. Isolation of the intermediate was achieved in the following manner. A 1 mM neutral solution of EtUdR was subjected to 15% photolysis and then developed on Whatman no. 1 paper with solvent *A* (a neutral solvent was used because of the presence of UdR·H_2O). The UV-absorbing UdR spot with R_F 0.42 was eluted and then run on an Eastman TLC 6065 plate with solvent *M* to give a good separation of the intermediate (R_F 0.81) and UdR (R_F 0.66). The absorption spectrum of the intermediate is shown in Fig. 4. At neutral pH it exhibits only end absorption in the quartz ultraviolet; at pH 12 it shows a maximum at 240 mμ, as expected for a 5,6-dihydropyrimidine nucleoside (Janion & Shugar, 1960). Irradiation of this photoproduct at 254 mμ in alkaline medium (pH 12) results in the disappearance of the 240 mμ maximum and the concomitant appearance of a maximum at 262 mμ. The same reaction occurs in neutral medium, but at an *apparently* lower rate, for two reasons: first, because of the lower absorption of the intermediate at neutral pH (Fig. 4) and, second, because the UdR formed from the intermediate undergoes photochemical hydration with concomitant loss of absorption.

The intermediate was fully stable in 0.01 N-NaOH at room temperature. In 0.1 N-NaOH, on the other hand, it appeared to be labile, the absorption decreasing by 70% to a plateau level at 30% with a $t_{1/2}$ of 30 min. at 20°. However, the remaining 30% still reverts to UdR on irradiation at 254 mμ. There is very little doubt but that the intermediate consists of a mixture of two isomers, one of which is relatively stable in 0.1 N-NaOH (Fig. 5).

The chromatographic properties of the intermediate are exhibited in Table 3. Its stability in 0.01 N-NaOH, its 240 mμ absorption maximum under these conditions, and its conversion to UdR on irradiation in neutral and alkaline media (Shugar, 1960) pointed to the possibility of its being a pyrimidine nucleoside dimer, either of EtUdR or of UdR. The first possibility was excluded since irradiation of the intermediate leads to formation of UdR, whereas the photodimer of EtUdR reverts photochemically to EtUdR (see below). The possibility that the intermediate might be UdR-photodimer was eliminated on the basis of its chromatographic behaviour (Table 3). In solvent *M* the intermediate has a higher R_F (0.81) than

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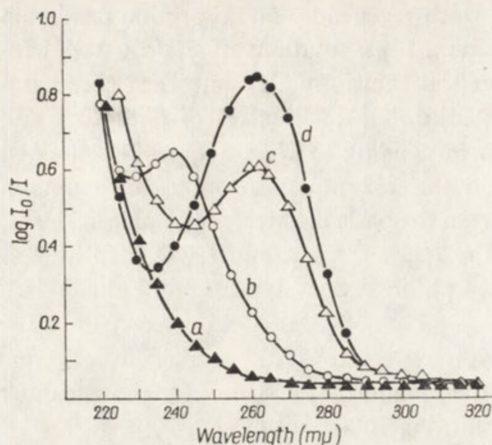

Fig. 4

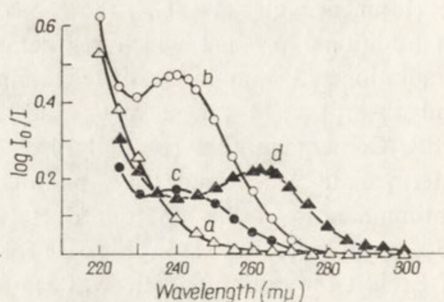

Fig. 5

Fig. 4. Absorption spectra of intermediate photoproduct formed in irradiated EtUdR at 254 mμ, and photochemical transformation of this intermediate to UdR: (*a*), Absorption spectrum of intermediate at neutral pH. (*b*), Spectrum of intermediate at pH 12. (*c*), Spectrum resulting from 254 mμ irradiation of intermediate at pH 12. (*d*), Spectrum resulting from neutralization of foregoing solution. Note that (*c*) and (*d*) correspond to the alkaline and neutral absorption spectra of UdR, also confirmed chromatographically.

Fig. 5. Alkaline lability of intermediate, formed from EtUdR by irradiation at 254 mμ, and which on irradiation undergoes de-ethylation and conversion to UdR: (*a*), Absorption spectrum of intermediate at neutral pH. (*b*), Absorption spectrum of intermediate at pH 13, recorded immediately following addition of alkali. (*c*), Absorption spectrum of intermediate following exposure to alkali for 4 hr. at room temperature. Curve (*c*) is stable to alkali. (*d*), Absorption spectrum resulting from irradiation of solution corresponding to (*c*). This is spectrum of UdR. It follows that (*b*) corresponds to a mixture of two isomeric intermediates, each of which undergoes photochemical de-ethylation to give UdR, and one of which is labile in 0.1 N-NaOH.

UdR (R_F 0.66); the converse would be true if the intermediate were the dimer of UdR, the R_F for which is lower than 0.5 with this solvent system. Furthermore, in the experiments described above on the relationship between the extent of photolysis of EtUdR and the subsequent degree of reversal, it was noted that when the extent of photochemical transformation of EtUdR was small, very little UdR·H_2O was formed; under analogous conditions it is easy to demonstrate that irradiation of UdR leads to formation only of the hydrate, i.e. low degrees of photolysis of 0.1 mM-UdR do not lead to formation of UdR-photodimer. It is therefore unlikely that UdR photodimers would be formed at a concentration of about 0.01 mM which might be expected in a 0.1 mM-EtUdR solution following 15% photolysis.

It is therefore reasonable to conclude that the intermediate described is that which, on irradiation, undergoes de-ethylation to give UdR. Attempts to measure the quantum yield for transformation of the intermediate to UdR at neutral pH were unsuccessful, because of the low absorption of the intermediate at 254 mμ. However, at alkaline pH the absorption at 254 mμ is appreciable (Fig. 4) and easily

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Table 3

R_F values of UdR, EtUdR, and EtUdR photo-intermediate which undergoes photochemical conversion to UdR

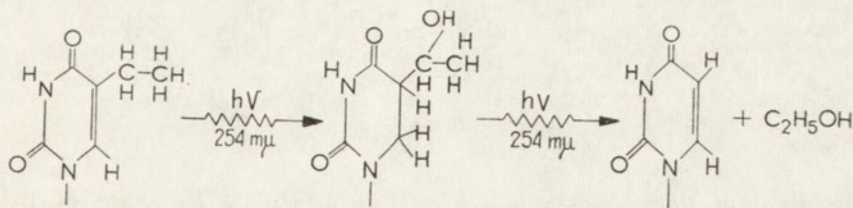
Chromatography on Eastman TLC 6065 plates.

Compound	Solvent		
	A	*M*	*N*
UdR	0.39	0.66	0.72
EtUdR	0.70	0.82	0.83
ErUdR photointermediate	0.39	0.81	0.75

measured; irradiation with source *A* gave a quantum yield of 0.3 for conversion of the intermediate to UdR. There is very little doubt but that the same value for the quantum yield applies at neutral pH (cf. Sztumpf & Shugar, 1962).

Fate of 5-ethyl substituent in irradiated EtUdR. De-ethylation of EtUdR by irradiation at 254 mμ suggested the possibility of formation of ethanol, as indicated in Scheme 2. Another possible pathway is indicated in Scheme 3. Initial attempts to detect ethanol in irradiated samples were based on the micromethod of Bonischen (1963) with the aid of the alcohol dehydrogenase system. It was, in fact, found that irradiated samples of EtUdR gave positive, but somewhat erratic, results for ethanol. Hence, following 90% photolysis of 1 mM-EtUdR solutions in 1-mm. cuvettes, the liquid phase was collected by cryoscopy (to eliminate possible interference from traces of other photoproducts), and then used for estimations of ethanol. The results were again consistently positive, but not always quantitative. Even in those instances where ethanol estimations agreed with that expected from the extent of de-ethylation, gas chromatography estimations were only one-half those obtained enzymically.

Subsequent analyses of irradiated solutions by both enzymic and gas chromatography procedures demonstrated that the volatile products resulting from photolysis of EtUdR were appreciably dependent on the storage time of the irradiated solutions.



Scheme 3

It was then found that freshly irradiated solutions subjected to gas chromatography initially contained ethylene (or ethane, the retention value of which is very close to that of ethylene). Irradiated samples kept for several days exhibited varying quantities of acetaldehyde, followed by ethanol. The overall results suggested that acetaldehyde and ethanol were secondary products and that these originated from the reaction of liberated ethylene with water.

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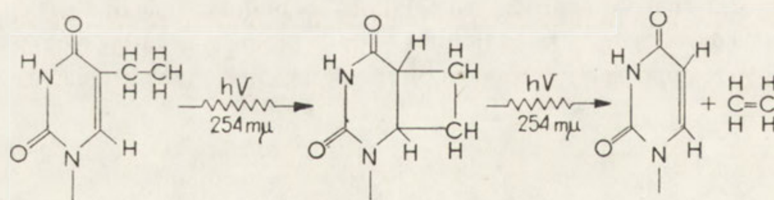
Consequently, in attempts to establish the primary product resulting from de-ethylation, a 1 mM solution of EtUdR was irradiated in a 2-mm. cuvette connected by means of a ground joint to an inverted small test tube filled with water, and the liberated gas collected. Following 80% photolysis, the cuvette was warmed to 50° to drive all the gas into the tube. The gas phase was then examined by gas chromatography. Only *one* very pronounced peak corresponding to ethylene appeared, and this finding was confirmed several times, using a column of 20% Ucon LB 550× on 60 - 80 mesh Celite 545. The sample was then first passed through a column of mercuric perchlorate on 60 - 80 mesh Celite 545, which completely adsorbs unsaturated hydrocarbons. Under these conditions the second column gave negative results, confirming the identity of the observed peak with ethylene.

It now became pertinent to inquire whether the EtUdR photointermediate described above (and which is transformed to UdR on further irradiation) still contains the ethyl group, as implied in Schemes 2 and 3. A 1 mM solution of EtUdR photointermediate was therefore irradiated at neutral pH at 254 mμ and the gas was collected as above and subjected to gas chromatography. Again the *only* product, with a pronounced peak, was ethylene.

Mechanism of photochemical de-ethylation of EtUdR. With the knowledge that ethylene and UdR are the products resulting from irradiation of either EtUdR or EtUdR photointermediate, and that acetaldehyde and ethanol are only secondary products (obviously due to reaction of ethylene with water), it now became possible to delineate with reasonable certainty the mechanism of de-ethylation (Scheme 4).

It will be seen that, with this scheme, the EtUdR molecule does not undergo initial hydration (as implied in Schemes 2 and 3) but an intramolecular rearrangement leading to formation of a cyclobutane ring on the 5,6 bond of the pyrimidine ring.



Scheme 4

Photochemical transformation of EtUdR anomers. The *β*-anomer of EtUdR is more susceptible to photochemical transformation at 254 mμ than the *α*-anomer (Fig. 1, Table 1), the quantum yield being twice as high for the former. The two anomeric photoproducts also exhibit differences in behaviour in acid medium; in 1 N-HCl at room temperature, the initial rate of "reversal" for *α*-EtUdR is 8-10 times that for *β*-EtUdR. Since, however, irradiation of EtUdR at 254 mμ leads to formation of UdR which, in turn, undergoes photohydration, the "reversal" of irradiated EtUdR in acid medium actually involves the dehydration of $UdR{\cdot}H_2O$ to UdR. Consequently the differences in rates of "reversal" of irradiated *α*- and *β*-

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anomers of EtUdR in acid medium must be due to differences in rates of dehydration of the *α*- and *β*- anomers of UdR·H_2O. The correctness of this assumption was demonstrated directly as follows:

α-UdR was prepared by photochemical cleavage of the ethyl group of *α*-EtUdR. A 1 mM solution of *α*-EtUdR was irradiated to the point where 90% of the absorption disappeared and the resulting *α*-UdR·H_2O dehydrated in ammoniacal medium at pH 11.8 (Fikus & Shugar, 1966), and then isolated by chromatography three times in the same direction on a silica gel plate with solvent system *V* (R_F 0.43). The *α*- and *β*- anomers of UdR, each at a concentration of 0.1 mM, were then photohydrated to the extent of 90% and the kinetics of dehydration examined in 1 N-HCl and in alkaline medium. The rates of photohydration of the two anomers were identical. But, in acid medium, *α*-UdR·H_2O underwent dehydration at 8 - 10 times the rate for *β*-UdR·H_2O. In alkaline medium (NH_4OH, pH 11.8) the rate for the *alpha* anomer was only twice that for the *beta*.

The two anomers of EtUdR also exhibit differences in behaviour as regards the intermediate which undergoes photochemical conversion to UdR. The intermediate *α*-anomer is the more sensitive, as shown by direct irradiation of the isolated intermediates. This was further confirmed by the fact that following 15% photolysis of *β*-EtUdR, the proportion of intermediate to (UdR+UdR·H_2O) was 6:4; whereas for *α*-EtUdR the corresponding value was about 1:9. However, as shown above, the proportion of *α*-anomer intermediate could be increased by decreasing the extent of photolysis, e.g. following 10% photochemical transformation of *α*-EtUdR, the ratio of intermediate to (UdR+UdR·H_2O) was 5:5.

Of additional interest was the alkaline lability of the intermediate formed from *α*-EtUdR. In 0.1 N-NaOH, the absorption maximum of the intermediate at 240 mμ decreased with time to a plateau level of about 65%; at 20° the $t_{1/2}$ for this 35% loss in absorption was about 60 min. It appears, therefore, that, as for the *β*-EtUdR intermediate (see above), the *α*-EtUdR intermediate photoproduct likewise consists of two isomers in the ratio 1:2 (as compared to approximately 2:1 for *β*-EtUdR), the alkali-labile isomer being more stable than the corresponding one from *β*-EtUdR.

Additional photoproducts from EtUdR. Following extensive photolysis of EtUdR at 254 mμ, up to 80 - 85% of the photolysis product(s), on heating in acid medium, is converted to UdR. Consequently about 15% of EtUdR is photochemically transformed by some other pathway. An additional photoproduct was, in fact, isolated chromatographically; in several solvent systems it exhibited a mobility slightly lower than that for EtUdR and, on Eastman plates containing fluorescein, was readily discerned following treatment with ammonia. This product exhibited only residual absorption in the quartz ultraviolet, with a low maximum at 305 mμ at neutral pH, and somewhat higher at 320 mμ in 1 N-HCl. At pH 12 it exhibited a very pronounced maximum at 345 mμ, which increased in optical density for 30 min. at room temperature, following which it slowly decreased (Fig. 6). The yield of this photoproduct, estimated from its maximum absorption at 345 mμ in alkaline medium, increased 2.5-fold when the extent of photochemical trans-

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formation of EtUdR was increased from 25% to 75%. The product was stable in 1 N-HCl at room temperature. On irradiation in alkaline medium at either 254 mμ or 366 mμ (Wood's lamp), the 345 mμ maximum disappeared with the simultaneous appearance of a weak band at about 265 mμ. Further characterization of this product proved difficult in the absence of adequate amounts of material, but its spectral behaviour is reminiscent in some respects of the thymine-thymine adducts described by Varghese & Wang (1968).

Photodimer formation with 254 mμ irradiation. During the course of attempts to separate chromatographically the isomers of the EtUdR photointermediate on silica gel with solvent *V*, the intermediate (R_F 0.34) was found to be contaminated with another spot, R_F 0.14, identified as EtUdR photodimer. The photodimer made up about 15% of the total (measured in terms of monomer). In all other solvent systems tested, the intermediate and EtUdR photodimer had identical R_F values. In none of the solvent systems used was it possible to achieve separation of EtUdR intermediate into isomers.

Behaviour of N-*methylated ethyluracils at 254 m*μ. An examination of 1-methyl-5-ethyluracil and 1,3-dimethyl-5-ethyluracil demonstrated that both of these compounds underwent at 254 mμ photochemical transformations similar to that for EtUdR, but with lower quantum yields (see Table 1 and Fig. 1). The principal final products were 1-methyluracil and 1,3-dimethyluracil, respectively, in yields ranging from 70 to 85%. On the other hand, neither of these compounds gave rise to a photoproduct with an alkaline absorption peak to the red of 300 mμ, as in the case of EtUdR (Fig. 6).

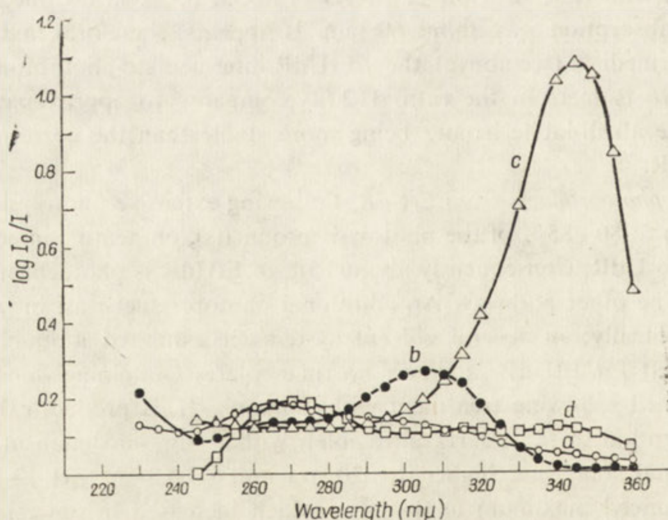

Fig. 6. Spectral properties of minor photoproduct of EtUdR irradiated at 254 mμ: (*a*), spectrum at neutral pH; (*b*), spectrum in 1 N-HCl; (*c*), spectrum in 0.01 N-NaOH; (*d*), spectrum following irradiation at pH 12 with 254 mμ source or 366 mμ (Wood's lamp).

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Photochemical transformation of EtUdR at $\lambda > 265$ mμ. Attention was then directed to the effects of longer irradiation wavelengths, using the sources described in Materials and Methods.

Irradiation of a 0.1 mM neutral aqueous solution of EtUdR with source *C* ($\lambda > 265$ mμ) led to a slow decrease in the height of the absorption maximum. Subsequent treatment of the irradiated solution with acid, base, or heating to 100° at neutral pH, was without appreciable effect, suggesting that the photoproduct(s) in this case differed from those formed at shorter wavelengths.

The principal photoproduct formed under the above conditions was isolated by thin-layer chromatography (MN 300 cellulose, R_F 0.34 with solvent system *A*). Its eluate (slightly contaminated with a product absorbing at about 265 mμ) exhibited end absorption in the quartz ultraviolet at neutral pH. In alkaline medium a clearly defined absorption band, with a λ_{max} about 240 mμ, made its appearance. Irradiation of an aqueous solution of this product at 254 mμ, either at neutral or alkaline pH, led to the formation of a new spectrum with a λ_{max} at 267 mμ, characteristic of that for EtUdR (Fig. 7). The course of this reaction, both at neutral and alkaline pH, is exhibited in Fig. 8, which shows that it is considerably more rapid in alkaline medium.

It therefore appears that photochemical transformation of EtUdR at longer wavelengths leads to a product which is, in turn, converted by irradiation at 254

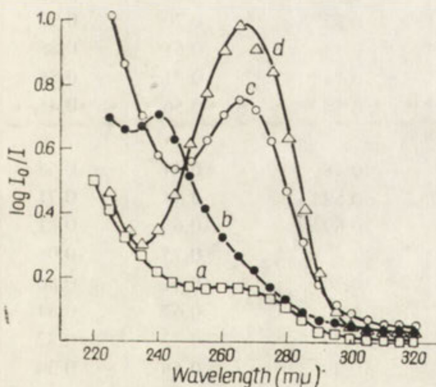

Fig. 7

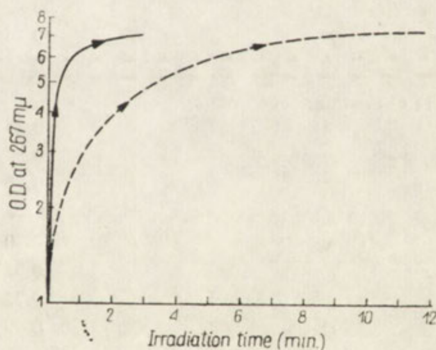

Fig. 8

Fig. 7. Absorption spectrum of EtUdR photodimer at neutral and alkaline pH and its photochemical conversion to EtUdR: (*a*) Absorption spectrum of EtUdR dimer at neutral pH, contaminated with some EtUdR (as seen from absorption in 270 mμ region). (*b*) Absorption spectrum of photodimer at pH 12. (*c*) Absorption spectrum resulting from irradiation of photodimer at 254 mμ at pH 12. (*d*) Absorption spectrum resulting from neutralization of solution *c*. Curves *d* and *c* are, respectively, the neutral and monoanionic forms of EtUdR.

Fig. 8. Kinetics of photodissociation of EtUdR photodimer, 0.1 mM in aqueous medium at neutral pH (- - -) and at pH 12 (———). Irradiation at 254 mμ. Conversion to monomer followed by rate of appearance of absorption maximum at 267 mμ (ordinate scale).

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mμ to the parent substance, EtUdR. The identity of this product with EtUdR was, in fact, established on the basis of its absorption spectrum at various pH values, its radiation sensitivity at 254 mμ, and its chromatographic behaviour.

It appeared reasonable to conclude from the foregoing that irradiation of EtUdR at longer wavelengths leads largely to photodimerization. The properties of the 265 mμ photoproduct were therefore compared with the EtUdR photodimer isolated from an irradiated frozen aqueous solution (see below). The 265 mμ photoproduct and the ice dimer were found to exhibit similar properties; both, to a certain extent, exhibited some stability in 2 N-KOH; in 70% $HClO_4$ for 1 hr. at 100°, both were *partially* converted to pyrimidine monomers (i.e. 5-ethyluracil) like the *cis-anti* and *trans-anti* photodimers of thymine (Weinblum & Johns, 1966); and they both exhibited the same R_F values with eight solvent systems on Whatman no. 1 paper, Eastman 6065 TLC plates and on silica gel plates (see Table 4).

Table 4

R_F values for UdR, EtUdR, EtUdR ice photodimer, and for major photoproduct resulting from irradiation of EtUdR in aqueous medium at wavelengths to red of 265 mμ (Photoprod.$_{265}$)

Solvent	EtUdR in ice dimer	Photoprod.$_{265}$	UdR	EtUdR
Whatman paper no. 1				
A	0.34	0.34	0.38	0.70
B	0.63	0.63	0.64	0.82
L	0.82	0.82	0.70	0.93
M	0.82	0.82	0.63	0.88
P	0.74	0.74	0.71	0.86
Q	0.56	0.55	0.56	0.45
TLC-Eastman 6065 plates				
A	0.45	0.48	0.44	0.68
B	0.57	0.59	0.54	0.71
K	0.70	0.69	0.68	0.81
L	—	—	0.75	0.96
M	0.70	0.70	0.61	0.80
O	0.71	0.70	0.67	0.84
P	0.73	0.73	0.72	0.82
Q	0.49	0.49	0.56	0.34
TLC-silica gel				
A	0.43	0.44	—	0.65
M	0.59	0.59	—	0.74

However, under the foregoing conditions it did not prove feasible to isolate the 265 mμ photoproduct in purified form, since its R_F was identical in several solvent systems with some other product absorbing in the neighbourhood of 265 mμ. This absorbing contaminant was found also in EtUdR ice photodimer (see below), and is readily placed in evidence by the slight absorption maximum at about 265 mμ in the spectrum (cf. Fig. 8). The nature of this additional photoproduct

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has not been established but, as shown below, it could be eliminated by irradiating EtUdR in solution at higher concentrations. The contaminant photoproduct was initially suspected to be UdR, since it exhibits the same R_F value with a number of solvent systems (Table 4), but this is excluded by the fact that no UdR·H_2O could be detected in the irradiated solution.

Oxygen and concentration effects on irradiation at $\lambda > 265$ mμ. Since the principal photoproduct of EtUdR on irradiation at longer wavelengths appears to be the photodimer, and photodimerization of orotic acid (Sztumpf-Kulikowska, Shugar & Boag, 1967), thymine, uracil and their glycosides (Greenstock & Johns, 1968) is inhibited by oxygen and paramagnetic ions, which may effectively quench triplet states, the photolysis of EtUdR at longer wavelengths was examined in the absence of oxygen. Oxygen-free solutions were obtained by bubbling with argon which had been freed from traces of oxygen with the aid of a train of three pyrogallol washing chambers. The photochemical transformation of EtUdR at longer wavelengths was, in fact, found to be extremely sensitive to traces of oxygen, much more so than that of orotic acid (Sztumpf-Kulikowska *et al.*, 1967). Figure 9 exhibits the course of photochemical transformation at longer wavelengths in the presence and absence of oxygen; it will be seen that the initial reaction rate is about 5-fold greater in the absence of oxygen. The subsequent decrease in rate of photolysis of the anaerobic sample is most likely due to the slow entry of atmospheric oxygen during the long irradiation times employed.

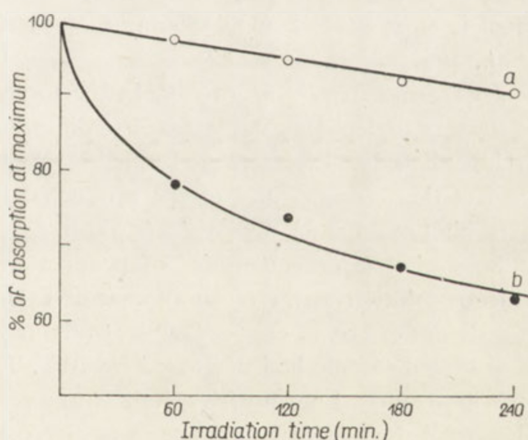

Fig. 9. Course of photochemical transformation of EtUdR, 0.1 mM in aqueous medium at neutral pH, irradiated at wavelengths to red of 265 mμ: (*a*), in presence of air, (*b*), in presence of argon. Reaction followed by decrease in optical density of λ_{max} at 267 mμ.

The influence of a paramagnetic ion was next examined. Mn^{2+} was selected for this purpose, since this ion had been shown by Kitler & Löber (1969) to be the most effective of a number of paramagnetic ions to inhibit the photochemical transformation of a variety of azauracils at 254 mμ. In the absence of oxygen, 2 mM-Mn^{2+} was found to very effectively inhibit the photolysis of EtUdR, the rate of photochemical transformation being then almost identical to that in the presence of air.

Both in the presence and absence of oxygen, the rate of photochemical transformation of EtUdR at $\lambda > 265$ mμ was relatively independent of its concentration over

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a range of 1 mM to 0.1 mM, and the presence of small quantities of the product with absorption about 265 mμ was observed (but see below for effect of higher concentrations).

Various criteria, including stability in acid and alkali, absorption spectra, chromatography, demonstrated that the principal photoproduct formed in the absence of oxygen was identical with that formed in its presence. The quenching effect of oxygen, and paramagnetic ions, may therefore be regarded as additional evidence for photodimerization of EtUdR in aqueous medium at wavelengths to the red of 265 mμ, most likely *via* a triplet state of EtUdR.

Photosensitized dimerization of EtUdR. It has been well established that a variety of 2,4-diketopyrimidines will undergo dimerization *via* triplet-triplet transfer in the presence of a suitable sensitizer, such as acetone or acetophenone amongst others (Elad, Krüger & Schmidt, 1967; Von Wilucki, Mathäus & Krauch, 1967). A solution of EtUdR, at a concentration of 1.6 mM, in 92% acetone was irradiated for 7 hr., using source *C* with a Zeiss WG3 filter cutting off at 321 mμ. The solution was then taken to dryness, the residue dissolved in water at a concentration of 0.1 mM, and the absorption spectrum examined at neutral and alkaline pH, followed by irradiation to dissociate to monomers. It was established in this way that 77% of the photoproduct(s) consisted of photodimer, thus constituting additional evidence for photodimerization by direct irradiation *via* the triplet state. This experiment also indicates that the triplet level of EtUdR must be reasonably close to that of TdR. No attempt was made to identify the remaining 23% of the photoproducts, but it is most likely that these consist of oxetane addition products (Von Wilucki *et al.*, 1967).

Photodimer isomers of EtUdR. In the foregoing experiments, the small amount of starting material available made it difficult to determine which of the possible EtUdR photodimer isomers are formed on irradiation at longer wavelengths, beyond the fact that susceptibility of the product(s) to acid and base suggested that the main photoproduct(s) were the *cis* dimers.

Somewhat larger quantities of product were isolated by irradiation of 0.1 M aqueous solution of the *alpha* anomer of EtUdR, with constant stirring by means of a stream of argon, at wavelengths to the red of 265 mμ, until the optical density of a diluted sample had decreased by 70%. The irradiation mixture was then run on Whatman no. 1 paper with solvent *A* to separate the monomer from photodimers. The area below EtUdR monomer was eluted and rechromatographed with solvent system *A* to give four clearly separated spots (revealed by irradiation of the chromatogram exposed to an atmosphere of ammonia) with R_F values of 0.30, 0.40, 0.50 and 0.73, which were eluted with water. All four exhibited only end absorption in the quartz ultraviolet at neutral pH, and absorption maxima at about 240 mμ at alkaline pH. It should also be emphasized that, at this high concentration, 0.1 M, no product with absorption about 265 mμ was detected amongst the photoproducts.

Irradiation of each of the isomers at alkaline or neutral pH led to photodissociation to EtUdR, identified by chromatography and spectral analysis. Attempts to

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measure the quantum yields for photodissociation at neutral pH were unsuccessful because of the low absorption at 254 mμ. However, at alkaline pH the absorption of the dimers was appreciable, and easily measured. The resulting quantum yield for dimer photodissociation was estimated in alkaline medium at 0.6 (cf. Sztumpf & Shugar, 1962), which is approximately that prevailing for photodissociation of other pyrimidine dimers.

The relative stability of the four isomers at alkaline pH was examined, and their probable assignments made on the basis of a comparison with those established for thymine dimer isomers by Herbert, Le Blanck, Weinblum & Johns (1969), as indicated in Table 5.

Photodimerization of 5-ethyluracil and EtUdR in ice. Irradiation at 254 mμ of thin layers of 1 mM frozen solutions of 5-ethyluracil and thymine demonstrated marked differences in photochemical transformation. With an irradiation dose leading to 82% photodimerization of thymine residues, only 10% of the ethyluracil was transformed to dimer. Under these conditions, subsequent irradiation of the melted aqueous solutions at alkaline pH (cf. Śmietanowska & Shugar, 1961) led to quantitative photodissociation to monomers. But a 4-fold increase in irradiation dose of the frozen solution gave only a 30% decrease in concentration of ethyluracil monomer, of which only 65% could be reverted to monomer.

Analogous experiments with the nucleosides showed that, under conditions where TdR underwent 39% dimerization in an ice matrix, the extent of photodimerization for EtUdR was 33%. Here again prolonged irradiation led to formation of photoproducts other than the dimers, both for EtUdR and TdR. For example, following a 50% decrease in absorption of a frozen solution of EtUdR, about one-fourth of this turned out to be a photoproduct other than a cyclobutane dimer. Of interest was the fact that the rate and extent of photochemical transformation in an ice matrix was the same for the *alpha* and *beta* anomers of EtUdR. No attempt was made to isolate the isomers of these photodimers.

The relatively high efficiency of photodimerization of EtUdR in frozen medium suggests that these molecules are stacked in an ice matrix almost as well as TdR. One might therefore reasonably expect appreciable photodimerization of neighbouring EtUdR residues in polynucleotide chains, the more so in that an examination

Table 5

R_F values, stability in alkali, and probable assignment of EtUdR photodimer isomers

Chromatography on Whatman no. 1 paper with solvent *A*.

R_F of dimer	Stability in		%	Assignment
	0.1 N-NaOH	1 N-NaOH		
0.30	stable	stable	30	*cis-syn*
0.40	stable	unstable	50	*cis-anti*
0.50	unstable	—	15	*trans-anti*
0.73	stable	stable	5	*trans-syn*

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of molecular models of the A and B forms of DNA shows that replacement of TdR by EtUdR residues does not result in any appreciable distortion of the helices.

The low extent of photodimerization of ethyluracil may reflect a lower degree of base stacking for these molecules in an ice matrix. However, when 1-methyl-5-ethyluracil was irradiated, 30% photodimerization was easily observed and chromatography permitted of the separation of three isomers with solvent *V* on GF_{254} silica gel. The R_F values for the three isomers were 0.46, 0.49 and 0.59 (R_F of starting compound 0.59). The isomers were not identified, but each underwent photodissociation to 1-methyl-5-ethyluracil in alkaline medium.

DISCUSSION

Of the two major photochemical transformations undergone by 5-ethyluracil and its glycosides in aqueous medium, it is the de-ethylation reaction at 254 mμ which is of special interest. The demonstration that the ethanol liberated as a result of de-ethylation (Pietrzykowska & Shugar, 1968b) is a secondary product, and that the primary product is ethylene produced *via* an intermediate is consistent with Scheme 4 for the de-ethylation reaction. The evidence for this pathway is mainly indirect and may be summarized as follows:

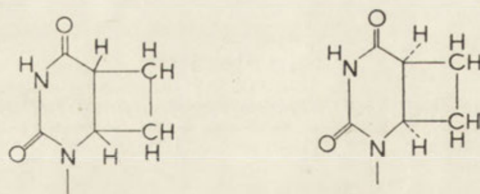
(a) The photointermediate is the species which photodissociates to UdR and ethylene. It exhibits the spectral properties characteristic of a pyrimidine with a saturated 5,6 bond.

(b) The relative stability of the photointermediate argues against (but does not prove) its being a hydrate, as implied in Schemes 2 and 3.

(c) The 6-hydroxy intermediate postulated in Scheme 2 is excluded by the observation that ethanol is a secondary product of the de-ethylation reaction.

(d) The demonstration that UdR and ethylene are the primary products is consistent with Scheme 4. This is further supported by the subsequent appearance in the irradiated solution of *both* acetaldehyde and ethanol, clearly resulting from the reaction of ethylene with water.

(e) The evidence suggesting the existence of two isomers of the intermediate is likewise consistent with Scheme 4. The two possible isomers are illustrated in Scheme 5.



Scheme 5

(f) The photointermediate exhibits the Fink reaction (with *p*-dimethylaminobenzaldehyde in alkaline medium), pointing to opening of the 3,4 bond in the alkali-labile isomer, as expected for dihydropyrimidines and pyrimidine dimers.

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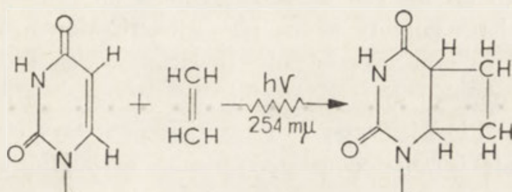
(g) The quantum yield for photo-deethylation of the photo-intermediate is 0.3, i.e. one-half that for photodissociation of EtUdR-photodimer (0.6). This is precisely what one would expect since the photodimer contains two pyrimidine rings.

In view of the indirect nature of the above evidence, it would obviously be desirable to isolate a sufficient quantity of the intermediate to permit of its characterization by elementary analysis, nuclear magnetic resonance etc. Such attempts are under way, but the task is quite an arduous one, because of the photosensitivity of the intermediate. It was consequently decided to attempt the preparation of the intermediate by a possible alternative route, viz. irradiation of UdR in the presence of ethylene in aqueous medium. If the photointermediate is as postulated in Scheme 4 (and Scheme 5), then one might anticipate its formation by addition of ethylene to the 5,6 double bond of the pyrimidine ring, as shown in Scheme 6. The following preliminary results have been obtained.



Scheme 6

A 1 mM solution of UdR was saturated with ethylene and irradiated at 254 mμ with continuous stirring by means of a stream of ethylene. Under these conditions the photochemical transformation of UdR was appreciably faster than in the absence of ethylene. Following 55% photochemical transformation of UdR (as estimated from the decrease in optical density at λ_{max}), the solution was found chromatographically to contain some hydrated UdR and UdR-photodimer, one unidentified photoproduct, and a spot consisting of the major photoproduct with characteristics strikingly similar to those of EtUdR photointermediate. This latter product was fully stable to 0.01 N-NaOH; whereas in 0.1 N-NaOH it was partially labile, 70% of the optical density of the absorption maximum at about 240 mμ disappearing at room temperature with a $t_{1/2}$ of about 30 min., as for EtUdR photointermediate. Irradiation of the isolated photoproduct at 254 mμ led to the appearance of UdR and ethylene. In solvent *M* (the only one which resolves EtUdR photointermediate from UdR, see Table 3), its R_F (0.78) was virtually identical with that for EtUdR photointermediate (0.80).

The evidence is consequently reasonably good that the EtUdR photointermediate is that depicted in Schemes 5 and 6. Attempts are now under way to isolate a sufficient quantity of the photoproduct to permit of its characterization by chemical and physicochemical techniques.

Wavelength dependence of photoproducts, and excited states. The wavelength dependence for photochemical transformation of EtUdR in aqueous medium is

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at first sight rather striking, and undoubtedly merits more detailed investigation over a more extensive range of wavelengths. However, this may well require the use of a high intensity monochromator (Johns & Rauth, 1965). A variety of photochemical reactions is known for which photoproduct formation apparently varies with the irradiation wavelength, each of the (generally two) reactions being associated with a different excited state (for review, see Ullman, 1968).

In the case of EtUdR irradiated at 254 mμ, the *total* absence of an oxygen or paramagnetic ion effect on the rate of de-ethylation points to the initial reactive species being an excited singlet state. But the observation of simultaneous photodimer formation at this wavelength, albeit low, implies some intersystem crossing to the triplet level. Furthermore the lifetime of this triplet state (or the rate of intersystem crossing, or both) in solution must be relatively high to compete at such a low concentration (0.1 mM) with photointermediate formation, which involves an *intra*molecular rearrangement (Scheme 5). An additional factor which may be responsible in part for the low observed yield of photodimerization at 254 mμ is the photochemical reversibility of the photodimerization reaction at this wavelength as compared to non-reversible de-ethylation which follows conversion of EtUdR to the photointermediate.

Analogous competition between two reaction pathways (each proceeding *via* a different excited state) is well documented for other pyrimidine analogues irradiated in aqueous medium at 254 mμ. For example, a 0.1 mM aqueous solution of uridine undergoes almost exclusively photohydration of the 5,6 bond *via* an excited singlet. But an increase in concentration to 1 mM leads to the appearance of 5 - 6% uridine photodimer (Fikus & Shugar, 1966), which is known to be derived from the triplet level. It would be of interest to examine the relative rates of formation of hydrates and dimers for either uridine or uracil as a function of wavelength, and at different concentrations. The only such investigation hitherto reported utilized oligonucleotides and polynucleotides of Up (Pearson, Whillans, Le Blanck & Johns, 1966); these, of course, do not reflect the behaviour of the monomers themselves in fluid medium.

Reverting now to the behaviour of EtUdR, the very pronounced oxygen and paramagnetic ion effects at wavelengths to the red of 265 mμ, taken in conjunction with the observation that the major photoproducts are the four photodimer isomers, constitutes fairly good evidence for the identity of the excited state with that of the triplet. It is indeed remarkable that the dimerization reaction proceeds with such efficiency at a concentration of 0.1 mM (at least three times the rate for thymidine), for which stacking of pyrimidines is normally not observable (cf. Lisewski & Wierzchowski, 1969). It appears reasonable to assume that this is due to the long lifetime of the EtUdR triplet.

EtUdR photodimerization *via* an excited triplet is supported by the acetone-sensitization experiments. Although dimer formation under these circumstances was relatively low (due perhaps to formation of oxetane adducts), there was no evidence for formation of the EtUdR photointermediate accompanying de-ethylation. This problem is being subjected to further study.

Implications for photochemistry of EtU in polynucleotides. From the foregoing it

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may be inferred that the predominant reaction of neighbouring ethyluracil residues in polynucleotide chains will be photodimerization. There is clearly no doubt about this when the irradiation wavelength is to the red of 265 mμ. But, bearing in mind that irradiation of EtUdR in an ice matrix, where the pyrimidine rings are favourably orientated, proceeds with the same efficiency as for thymidine, even with an irradiation wavelength of 254 mμ, it seems reasonable to conclude that in a polynucleotide chain irradiated at 254 mμ one may likewise anticipate substantial photodimerization of neighbouring EtU residues as compared to de-ethylation. The observed photochemical, and subsequent photo- and dark reactivation, behaviour of phage T3, in which thymine residues are replaced by EtU, which is identical with that for normal phage at 254 mμ (Pietrzykowska & Shugar, 1968a), is in agreement with this. But it would obviously be more conclusive to examine the photochemical behavioui at wavelengths to the red of 265 mμ.

We are indebted to Dr. J. Sobkowski for arranging for the gas chromatography analyses, to Dr. K. L. Wierzchowski for suggesting the irradiation of UdR in the presence of ethylene, and to Mrs. Maja Żylonis and Mrs. Krystyna Myszkowska for technical assistance. This investigation has profited from the support of the World Health Organization, The Wellcome Trust, and the Agricultural Research Service, USDA (UR-E21-(32)-30).

REFERENCES

Bonischen R. (1963). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) p. 285, Academic Press, New York.
Elad D., Krüger C. & Schmidt G. J. M. (1967). *Photochem. Photobiol.* **6**, 495.
Fikus M. & Shugar D. (1966). *Acta Biochim. Polon.* **13**, 39.
Greenstock C. L. & Johns H. E. (1968). *Biochem. Biophys. Res. Commun.* **30**, 21.
Herbert M. A., Le Blanck J. C., Weinblum D. & Johns H. E. (1969). *Photochem. Photobiol.* **9**, 33.
Janion C. & Shugar D. (1960). *Acta Biochim. Polon.* **7**, 309.
Johns H. E. & Rauth A. M. (1965). *Photochem. Photobiol.* **4**, 673.
Kitler L & Löber G. (1969). *Photochem. Photobiol.* **10**, 35.
Lisewski R. & Wierzchowski K. L. (1969). *Chem. Commun.* p. 348.
Pearson M., Whillans D. W., Le Blanck J. C. & Johns H. E. (1966). *J. Mol. Biol.* **20**, 245.
Piechowska M. & Shugar D. (1965). *Biochem. Biophys. Res. Commun.* **20**, 768.
Pietrzykowska I. & Shugar D. (1966). *Biochem. Biophys. Res. Commun.* **25**, 567.
Pietrzykowska I. & Shugar D. (1967). *Acta Biochim. Polon.* **14**, 169.
Pietrzykowska I. & Shugar D. (1968a). *Vth FEBS Meeting, Prague. Abstr. Commun.* p. 70.
Pietrzykowska I. & Shugar D. (1968b). *Science* **161**, 1284.
Shugar D. (1960). In *The Nucleic Acids* (E. Chargaff & J. N. Davidson, eds.) vol. 3, p. 39. Academic Press, New York.
Śmietanowska A. & Shugar D. (1961). *Bull. Acad. Polon. Sci. Cl. II.* **11**, 375.
Świerkowski M. & Shugar D. (1969). *J. Med. Chem.* **12**, 533.
Świerkowski M. & Shugar D. (1970). *J. Mol. Biol.* **47**, 57.
Sztumpf E. & Shugar D. (1962). *Biochim. Biophys. Acta* **61**, 555.

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Sztumpf-Kulikowska E., Shugar D. & Boag J. W. (1967). *Photochem. Photobiol.* **6**, 41.
Ullman F. E. (1968). *Accounts Chem. Res.* **1**, 353.
Varghese A. J. & Wang S. Y. (1968). *Science* **160**, 186.
Von Wilucki I., Mathäus H. & Krauch C. H. (1967). *Photochem. Photobiol.* **6**, 497.
Weinblum D. & Johns H. E. (1966). *Biochim. Biophys. Acta* **114**, 450.

FOTOCHEMIA 5-ETYLOURACYLU I JEGO GLIKOZYDÓW

Streszczenie

1. Naświetlanie wodnych roztworów 5-etylodezoksyurydyny (lub 5-etylourydyny) promieniowaniem o długości fali 254 mμ prowadzi do przekształcenia więcej niż 80% EtUdR w dezoksyurydynę (lub urydynę). Reakcja ta jest poprzedzona utworzeniem fotoproduktu pośredniego. Fotoprodukt pośredni tworzy 2 izomery, z których jeden jest niestabilny w 0.1 N-NaOH. Oba izomery podczas naświetlania promieniami o dł. fali 254 mμ w środowisku obojętnym i alkalicznym ulegają deetylacji z utworzeniem dezoksyurydyny (lub urydyny), z wydajnością kwantową około 0.30.

2. Wykazano, że fotoprodukt pośredni jest wynikiem przekształceń wewnątrzcząsteczkowych 5-etylodezoksyurydyny. Podstawnik etylowy tworzy pierścień cyklobutanowy z wiązaniem 5,6-pirymidyny, fotoprodukt pośredni jest zatem 5,6-dwuhydro-5,6-cyklobutanylodezoksyurydyną, a więc nową pochodną nukleozydów dwuhydropirymidyn.

3. Reakcja fotochemicznego przekształcenia 5-etylodezoksyurydyny w dezoksyurydynę pod wpływem promieni UV_{254} nie jest hamowana przez tlen ani przez jony paramagnetyczne i prawdopodobnie zachodzi w singletowym stanie wzbudzenia cząsteczki.

4. W tych warunkach naświetlania powstają także inne fotoprodukty: fotoprodukt bliżej niezidentyfikowany, który posiada absorpcję w dłuższych falach UV, oraz fotodimery 5-etylodezoksyurydyny, które w początkowych stadiach fotolizy stanowią 10 - 15% ilości fotoproduktu pośredniego.

5. Szybkość fotochemicznych przekształceń anomeru *β*-EtUdR jest dwukrotnie większa niż anomeru *α*. Anomer *α*-UdR·H_2O (uzyskany przez fotochemiczną deetylację *α*-EtUdR) ulega odwodnieniu w środowisku kwaśnym z szybkością ośmiokrotnie większą niż *β*-UdR·H_2O.

6. Pod wpływem promieniowania UV o długości fali 265 mμ, 5-etylodezoksyurydyna w roztworze wodnym ulega reakcji fotodimeryzacji. Powstają 4 izomery fotodimerów z przewagą izomerów *cis*. Wydajność kwantowa reakcji fotodimeryzacji jest nieco wyższa w porównaniu z tymidyną w tych samych warunkach. Wydajność kwantowa fotodysocjacji dimerów wynosi 0.60.

7. Reakcja fotodimeryzacji jest silnie hamowana przez tlen i jony paramagnetyczne (Mn^{2+}), co wskazuje, że reakcja ta zachodzi w tripletowym stanie wzbudzenia cząsteczki. Potwierdza to dodatkowo fakt, że fotodimeryzacja zachodzić może na drodze sensybilizacji poprzez aceton z wydajnością kwantową zbliżoną do wydajności dla tymidyny. Świadczy to, że poziomy energetyczne tripletu 5-etylodezoksyurydyny i tymidyny są zbliżone.

8. Podczas naświetlania 5-etylodezoksyurydyny lub 1-metylo-5-etylouracylu promieniami UV o długości fali 254 mμ w lodzie powstają dimery z szybkością podobną jak dla tymidyny. Sam 5-etylouracyl w tych warunkach jest dość oporny. W środowisku wodnym natomiast 5-etylouracyl i 1-metylo-5-etylouracyl ulega tym samym przekształceniom fotochemicznym co glikozydy (fotodeetylacja).

9. Dyskutuje się mechanizmy powyższych reakcji i ich zastosowanie do fotochemii DNA lub fagów zawierających 5-etylouracyl zamiast tyminy, a także odnośnie fotochemii tyminy i jej glikozydów.

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REGULATION OF LACTOSE UTILIZATION IN *ASPERGILLUS NIDULANS*

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1. The uptake of lactose by mycelia of *Aspergillus nidulans* grown in media containing various sugar sources was compared with the rate of induction of β-galactosidase. The induction of lactose-permease system was not time-coordinated with the induction of β-galactosidase. Some properties of β-galactosidase of *Aspergillus nidulans* are described.

Utilization of lactose by microorganisms requires both the activity of a protein or protein complex responsible for the penetration of the sugar molecule into the cell and that of an enzyme, β-galactosidase (EC 3.2.1.23). The first activity will be termed here a "permease". It has been demonstrated that in bacteria such as *Escherichia coli* (see review by Jacob & Monod, 1961) and *Staphylococcus aureus* (McClatchy & Rosenblum, 1963) both enzymes show time-coordinated induction in the presence of β-galactosides in the medium. The two β-galactosidases described in *Neurospora crassa* are also inducible (Bates & Woodward, 1964; Lester & Byers, 1965; Bates, Hedman & Woodward, 1967).

This work describes an inductive response of lactose-permease and β-galactosidase in *Aspergillus nidulans* to the presence of various sugars in the medium.

MATERIALS AND METHODS

Chemicals. D-Glucose, D-galactose, D-lactose and glycerol were obtained from Polskie Odczynniki Chemiczne (Gliwice, Poland); D-xylose from Fluka AG. (Buchs SG, Switzerland); *o*-nitrophenylgalactopyranoside (ONPG[1]) from Sigma Chemical Company (St. Louis, Mo., U.S.A.); Sephadex G-200 from Pharmacia Ltd. (Uppsala, Sweden); human albumin (mol. wt. 69 000) and human γ-globulin (mol. wt. 150 000) were from Warsaw Serum and Vaccine Plant (Warszawa, Poland). [1-^{14}C]Lactose (4 mc/m-mole) was from the Radiochemical Centre (Amersham, Bucks., England). All inorganic chemicals were reagent grade.

[1] Abbreviations used: ONP, *o*-nitrophenol; ONPG, *o*-nitrophenylgalactopyranoside.

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Biological material. The biotine requiring strain of *Aspergillus nidulans* (*bi 1*) which behaves like the wild type with respect to carbohydrate utilization, was obtained from the Department of Genetics, University of Glasgow.

Culture conditions. Mycelia were grown in 250 ml. Erlenmeyer flasks containing 100 ml. of minimal medium (Cove, 1966), supplemented with the appropriate carbon source to a final concentration of 2%. Flasks were inoculated with 2 - 3 ml. of a heavy conidial suspension in water and incubated at 31 - 32° on a rotary shaker.

Preparation of cell-free extracts. Mycelia were harvested by filtration on surgical gauze and washed with water. Blotted mycelial pads were mixed with glass powder and ground in a chilled mortar with 7 - 8 volumes of 0.1 M-potassium phosphate buffer, pH 7.6 (except where indicated). The resulting slurry was then centrifuged at 15 000 *g* for 15 min. at 2°, and the supernatant (2.5 - 4 mg. protein/ml.) was used for enzyme assays. Extracts prepared from fresh mycelia were used throughout this work.

Enzyme assays. Determination of *β*-galactosidase activity was based on the method described by Wallenfels (1962). Two procedures were used: (1) The reaction mixture (final volume, 2.5 ml.) contained: *o*-nitrophenylgalactopyranoside, 8 μmoles; NaCl, 125 μmoles; potassium phosphate buffer, pH 7.6, 200 μmoles, and an appropriate volume of cell-free extract. The reaction was started by the addition of enzyme extract, and its rate was followed by measuring the increase in extinction at 405 nm at 1 min. intervals, using a Unicam SP-500 spectrophotometer. The reference cell contained ONPG in the NaCl-buffer.

(2) The reaction mixture was the same as above, and after addition of enzyme extract, the tubes were incubated at room temperature for 5 or 8 min. Then the reaction was stopped by placing the tubes in boiling water for 3 min. The precipitated protein was removed by centrifugation for 5 min. at 15 000 *g*. The extinction at 405 nm was measured using as reference the control which contained boiled enzyme extract. A molar extinction coefficient for *o*-nitrophenol of 3.1×10 (Wallenfels, 1962) was used for calculation of enzyme activity.

The protein content of the extracts was determined by the method of Lowry *et al.* (1951).

Gel filtration of the extracts was performed on a 1.6×65 cm. column of Sephadex G-200 using a 0.145 M-NaCl solution containing 0.005 M-tris-HCl buffer, pH 7.6.

Growth measurements. Two litre flasks containing 500 ml. of minimal medium supplemented with the appropriate carbon cource were inoculated with 6 ml. of conidial suspension (2×10^8 conidia/ml.). The weight of mycelia in collected samples was determined by filtration on weighed glass fiber filters, followed by washing with distilled water and drying to constant weight at 80° (Romano & Kornberg, 1969).

Transfer experiments. Mycelia grown for 20 hr. in a glucose-containing medium were collected by filtration, washed with 50 mM-potassium phosphate buffer, pH 7.0, and resuspended in a fresh medium containing 2% lactose or galactose.

Uptake experiments. The procedure was based on that used by Romano & Kornberg (1969). Mycelia grown for 18 - 20 hr. on a medium containing glucose or galactose, or for 30 - 40 hr. on a medium containing lactose, were collected by

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filtration, washed, and resuspended in 50 mM-potassium phosphate buffer, pH 7.0. Mycelia from 10 - 15 ml. of this suspension were collected and their weight determined as above. To 12.5 ml. of the same suspension, 0.25 ml. of 50 mM-[1-^{14}C]lactose were added (1.07×10^7 counts/min./m-mole). Samples of 2 ml. were withdrawn at appropriate time intervals, filtered through glass fiber filters and washed three times with phosphate buffer. The filters together with the residue were then dried at 80° for 25 min. and transferred to scintillation vials. The assays of radioactivity were made using Bray's scintillation fluid (Bray, 1960), with a Packard Liquid Scintillation Spectrometer.

RESULTS

The wild type strain of *Aspergillus nidulans* is able to grow on lactose, but glucose and galactose are better carbon sources for this organism (Fig. 1). Mycelia grown on glucose or xylose do not have any detectable β-galactosidase activity. This activity was found in lactose- and galactose-grown mycelia (Table 1), where it was about the same. However, in galactose-grown mycelia this activity appeared later, almost none being observed in mycelia grown for only 18 hr. The reaction rate was linear with protein concentration from 0.75 to 3.75 mg. of protein/sample. The dependence of the reaction rate on pH is shown in Fig. 2A, and on temperature in Fig. 2B. As the activity determined in crude extracts was identical over a wide range of temperature, it was possible to perform standard enzyme assays at room temperature.

Gel filtration of the extract of lactose-grown mycelia on Sephadex G-200 (Fig. 3) indicated that one molecular type of β-galactosidase is present in *Aspergillus*

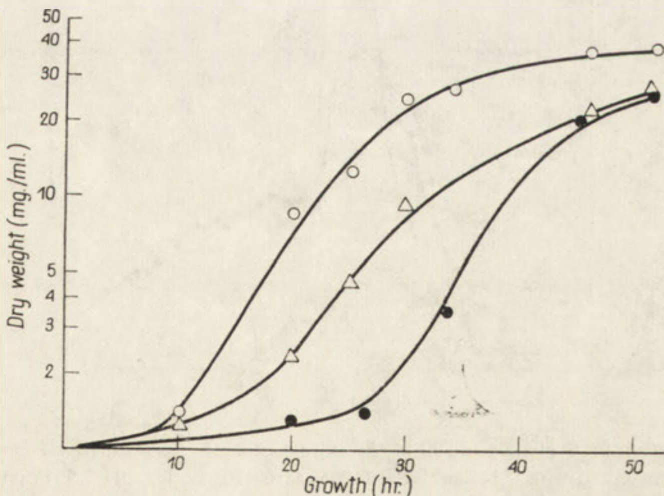

Fig. 1. Continuous growth of *Aspergillus nidulans* cultures with ○, glucose, △, galactose and ●, lactose as sole carbon source.

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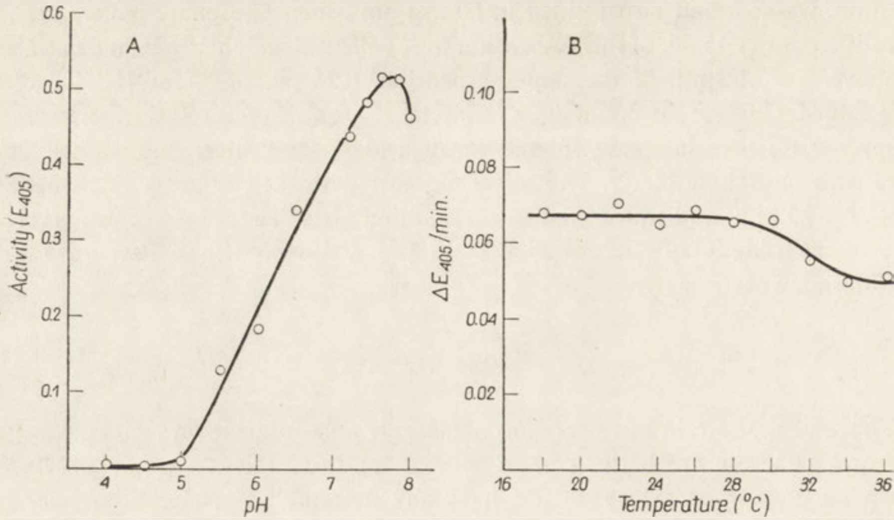

Fig. 2. Dependence of β-galactosidase activity on *A*, pH, and *B*, temperature. The assays were performed by procedure 2. In Expt. *A*, for pH 4 - 5, 80 mM-acetate buffer, and for pH 6 - 8, 80 mM-phosphate buffer was used. Protein concentration, 3 mg./sample, substrate 3.2 mM.

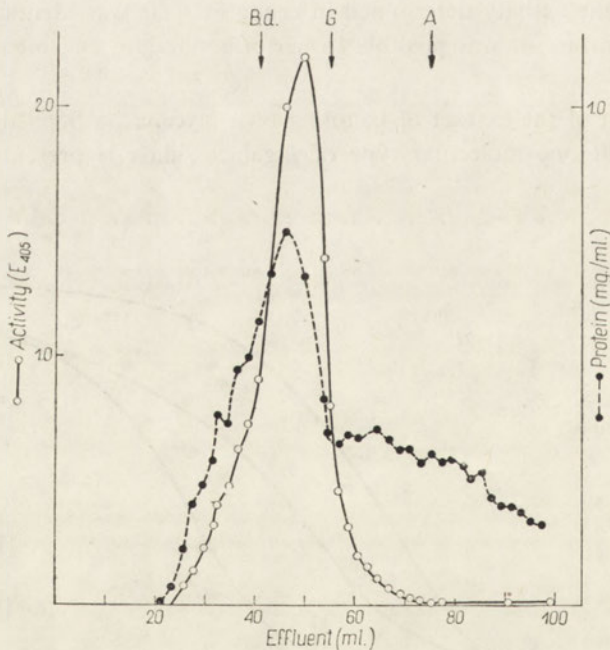

Fig. 3. Gel filtration on Sephadex G-200 of the extract of lactose-grown *A. nidulans*. The column (1.6×65 cm.) was eluted with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5. Fractions of 1.8 ml. were collected and assayed ○, for β-galactosidase activity using procedure 2 and ●, for protein. The elution volumes of Blue dextran (*B.d.*), human γ-globulin (*G*) and albumin (*A*) are marked with arrows.

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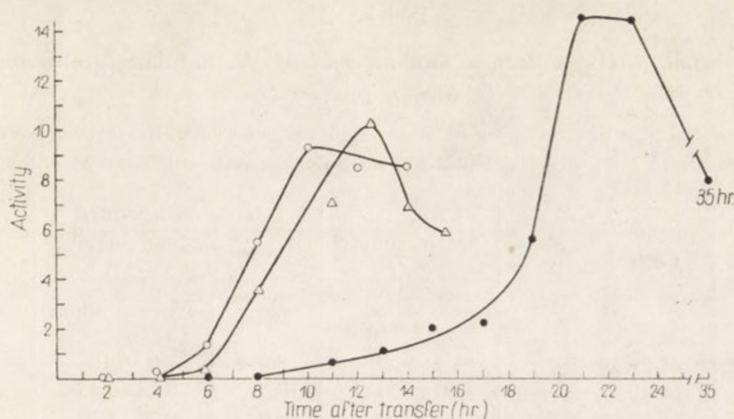

Fig. 4. Induction of *β*-galactosidase in *A. nidulans* after the transfer of mycelia from non-inductive to inductive media. Mycelia grown for 20 hr. on glucose, or for 30 hr. on glycerol were collected, washed with water and transferred to the fresh medium containing the appropriate carbon source. △, Transfer from glucose to lactose; ○, transfer from glycerol to lactose; ●, transfer from glucose to galactose. Samples were withdrawn at indicated time intervals and the activity of *β*-galactosidase determined by procedure 1. Specific activity is expressed in nmoles of ONP liberated/min./mg. protein.

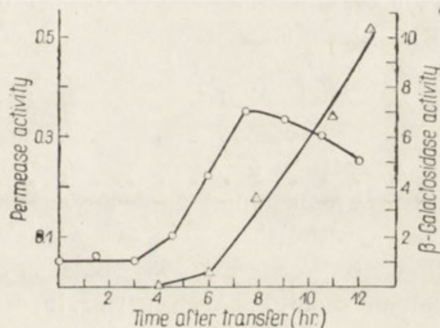

Fig. 5. Comparison of the inductive responses of △, *β*-galactosidase and ○, lactose-permease to transfer from glucose-containing medium to lactose-containing medium. The activity of the enzymes is expressed, respectively, as nmoles of *o*-nitrophenol liberated or lactose incorporated per 1 min. per 1 mg. protein

Table 1

β-Galactosidase activity in A. nidulans *grown on different carbon sources for 30 - 40 hr.*

The activity was assayed in cell-free extracts by procedure 2. For each sugar, 3 - 5 experiments were performed.

Carbon source	*o*-Nitrophenol liberated (nmoles/min./mg. protein)	
	mean	extreme values
D-Glucose	not detectable	
D-Galactose	55.7	36.5 - 75.0
D-Lactose	60.0	40.0 - 86.0
D-Xylose	not detectable	

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Table 2

The incorporation of [1-^{14}C]*lactose into mycelia of* A. nidulans *grown on different carbon sources*

After the indicated time of growth on the appropriate carbon source, the mycelia were harvested and incubated for 15 min. with [1-^{14}C]lactose, and samples were withdrawn at 4, 8 and 15 min.

Carbon source	Age of culture (hr.)	Lactose incorporated (nmoles/min./mg. of dry weight)	
		mean	extreme values
D-Glucose	18 - 20	0.075	0 - 0.10
D-Galactose	18 - 20	0.37	0.25 - 0.45
D-Lactose	30 - 40	0.93	0.70 - 1.30

nidulans. A single peak of the activity of this enzyme was found close to the elution volume of standard human γ-globulin (mol. wt. 150 000).

Transfer experiments indicated that lactose induces β-galactosidase activity earlier than does galactose (Fig. 4). The uptake of lactose was very low in glucose-grown mycelia, but it rose upon induction by lactose or galactose (Fig. 5 and Table 2). The induction of "permease", following the transfer of mycelia from a glucose-containing medium to a lactose-containing medium, preceded the induction of β-galactosidase.

DISCUSSION

Adaptation of *Aspergillus nidulans* to growth on lactose as a carbon source involves induction of β-galactosidase and a "permease". In contrast to *Neurospora crassa*, where there exist two β-galactosidases with pH optima at 4.2 and 7.5 (Bates & Woodward, 1964; Bates *et al.*, 1967; Lester & Byers, 1965), *A. nidulans* contained only one such enzyme. It corresponded to the "7.5" enzyme of *Neurospora*. There was no detectable activity below pH 5.0. The experiments with gel filtration also point to the presence of only one β-galactosidase in *Aspergillus nidulans*. D-Xylose, which induces the "4.2" enzyme in *Neurospora*, had no effect in *A. nidulans*. In this connection it is interesting to note that β-galactosidase found in *Aspergillus niger* (Bahl & Agrawal, 1969) corresponds to the "4.2" enzyme in *Neurospora*.

Galactose seems to be as good an inducer of β-galactosidase activity as lactose, but this is not so in the case of lactose-permease. The maximum rate of lactose uptake in galactose-grown mycelia was about one third of that observed in lactose-grown mycelia. "Permease" activity was less dependent than β-galactosidase activity on the age of the galactose-grown mycelia. Slower induction of β-galactosidase was observed after transfer to galactose-containing medium than after transfer to lactose-containing medium. This may be attributed to the utilization of galactose, so that its inductive concentration is reached later than in the case of lactose. The above suggestion may be supported by the fact that the rate of induction by galactose

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in the *Aspergillus* mutant which is unable to utilize this sugar because of the lack of hexose-1-phosphate uridylyl transferase (EC 2.7.7.12) (Chojnacki, Paszewski & Sawicka, 1969) was the same as the rate of induction by lactose in the wild type.

The results presented here seem to indicate a lack of time-coordinated induction of *β*-galactosidase and lactose-permease in *Aspergillus nidulans* or at least a different kind of coordination to that found in *Escherichia coli.*

REFERENCES

Bahl P. & Agrawal K. M. L. (1969). *J. Biol. Chem.* **244**, 2970.

Bates W. K. & Woodward D. O. (1964). *Science* **146**, 777.

Bates W. K., Hedman S. C. & Woodward D. O. (1967). *J. Bacteriol,* **93**, 1631.

Bray G. A. (1960). *Analyt. Biochem.* **1**, 279.

Chojnacki T., Paszewski A. & Sawicka T. (1969). *Acta Biochim. Polon.* **16**, 185.

Cove D. J. (1966). *Biochim. Biophys. Acta* **113**, 51.

Jacob F. & Monod J. (1961). *J. Mol. Biol.* **3**, 318.

Lester G. & Byers A. (1965). *Biochem. Biophys. Res. Commun.* **18**, 725.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265.

McClatchy J. K. & Rosenblum E. D. (1963). *J. Bacteriol.* **86**, 1211.

Romano A. H. & Kornberg H. L. (1969). *Proc. Roy. Soc. B.* **173**, 475.

Wallenfels K. (1962). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 5, p. 212. Academic Press, New York, London.

REGULACJA WYKORZYSTANIA LAKTOZY U *ASPERGILLUS NIDULANS*

Streszczenie

Porównano natężenie pobierania laktozy przez grzybnię *A. nidulans* ze stopniem indukcji *β*-galaktozydazy. Stwierdzono, iż indukcja systemu permeazy laktozy nie jest skoordynowana z indukcją *β*-galaktozydazy u badanego organizmu. Opisano niektóre właściwości *β*-galaktozydazy występującej u *A. nidulans.*

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RECENZJE KSIĄŻEK

PROTIDES OF THE BIOLOGICAL FLUIDS. Proceedings of the Sixteenth Colloquium, Bruges, 1968 (H. Peeters, ed.) Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1969; str. 773, cena Ł 8.

Kolejne 16 Sympozjum na temat białek płynów ustrojowych, które odbyło się w Bruges (Belgia) w r. 1968, poświęcone było trzem wiodącym problemom: 1) konformacji i strukturze cząsteczek białkowych, 2) charakterystyce białek wydzielanych przez poszczególne narządy, oraz 3) nowym metodom służącym oczyszczaniu, identyfikacji i oznaczaniu białek.

Biologiczna funkcja białek może być zarówno statyczna, jak i dynamiczna, przy czym ta pierwsza polega na zachowaniu właściwej dla danego białka konfiguracji przestrzennej, podczas gdy funkcja dynamiczna polega na zdolności do przechodzenia od jednej do drugiej konfiguracji w wyniku pękania lub tworzenia się nowych wiązań wodorowych, elektrostatycznych i innych, pozwalając w ten sposób na przejście białka w formę aktywną lub nieaktywną, lub na łączenie się nieaktywnych podjednostek białkowych w aktywnie czynne białka enzymatyczne. Stosując jako modele syntetyczne polipeptydy o określonej strukturze trzeciorzędowej, badano jej wpływ na fizyko-chemiczne i immunologiczne właściwości białek. Na przykładzie lizozymu przedstawiono znaczenie struktury trzeciorzędowej w mechanizmie działania tego enzymu, jak również sposób wiązania się tego białka z inhibitorami. W innych licznych pracach zawartych w pierwszej części książki opisano zmiany własności optycznych i dielektrycznych białek, zachodzące w roztworach o różnych stężeniach jonów wodorowych, mocznika lub elektrolitów. W pracach dotyczących struktury i funkcji immunoglobulin przedstawiono wzajemną zależność tych dwóch cech, przy czym nawet stosunkowo niewielkie zmiany strukturalne powodują bardzo istotne zmiany aktywności tych białek.

Druga część książki przedstawia wyniki badań dotyczących białek syntetyzowanych przez różne narządy. Wiele uwagi poświęcono wit. B_{12} i jej wpływowi na wydzielanie „intrinsic factor", przy czym, wbrew ogólnie dotychczas przyjętemu poglądowi, nie obserwowano korelacji między ilością podanej wit. B_{12} a wydzielaniem tego czynnika, jeżeli ilość wit. B_{12} przekroczyła określoną granicę. W licznych pracach przedstawiono znaczenie immunoglobulin wydzielanych przez śluzówki jelitowe i nosowe w zwalczaniu infekcji bakteryjnych.

W trzeciej części książki zebrane są liczne prace dotyczące nowych metod pozwalających oczyszczać, identyfikować i ilościowo oznaczać poszczególne białka. Jedną z nich jest metoda „electro-focusing" pozwalająca na rozdzielanie mieszaniny białek na frakcje różniące się punktem izoelektrycznym rzędu 0,02 jednostek pH. Pozwala to na oczyszczanie niektórych białek, np. gonadotropin, w stopniu dotychczas nieosiągalnym.

Książkę otwiera wykład jednego z pionierów immunologii, prof. P. Grabara, który w skrócie dokonuje przeglądu dotychczasowych wiadomości dotyczących immunologii białek.

Książka jest wydana bardzo starannie, ukazała się w krótkim czasie po terminie sympozjum, może więc być kopalnią informacji dla tych wszystkich, których interesuje biochemia białek.

Marek Ombach

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M. T. Beck, CHEMISTRY OF COMPLEX EQUILIBRIA. Akademiai Kiadó, Budapest; Van Nostrand Reinhold Comp. Ltd., 1970; str. 285.

Chemia związków kompleksowych w minionym ćwierćwieczu rozwinęła się w jedną z ważniejszych dziedzin chemii nieorganicznej. Przyczyniły się do tego stwierdzana powszechność występowania jonów metali wielowartościowych w postaci związanej (koordynacyjnie) z różnego typu ligandami nieorganicznymi i organicznymi oraz znaczenie tej klasy związków w wielu ważnych dziedzinach chemii stosowanej (np. kataliza), analitycznej i biochemii. Istotnej roli połączeń kompleksowych w tej ostatniej dziedzinie czytelnikom Acta Biochimica Polonica nie ma potrzeby w tym miejscu udowadniać. Piśmiennictwo przedmiotu jest bardzo obszerne, wydanych zostało wiele monografii i książek poświęconych różnym aspektom chemii związków kompleksowych. Do podstawowych zagadnień należą niewątpliwie problemy związane z ilościowym traktowaniem z reguły wielostopniowych równowag kompleksotwórczych w roztworach i ustalaniem budowy związków kompleksowych w nich uczestniczących. Klasyczne opracowanie w tym zakresie stanowi znana książka F. J. C. i H. Rossotti'ch pt. „*The Determination of Stability Constants*" zawierająca sformalizowany opis równowag i metod wyznaczania odpowiadających im stałych równowagi.

Beck w swojej książce podejmuje w zasadzie tę samą problematykę, nadaje jej jednak charakter unikalny przedstawiając metodykę stosowaną przy wyznaczaniu stałych równowagi w bliskim powiązaniu z chemią szeregu złożonych, lecz często realizujących się, i stąd ważnych, klas związków koordynacyjnych. Mimo braku formalnego podziału książka dzieli się wyraźnie na dwie części. W pierwszej części kreśli autor historię rozwoju badań w dziedzinie związków kompleksowych (rozdz. 1), wprowadza podstawowe pojęcia dotyczące nomenklatury i klasyfikacji związków kompleksowych oraz równowag kompleksotwórczych (rozdz. 2), omawia szczegółowo funkcję opisującą powstawanie kompleksów, tzw. funkcję Bjerruma (rozdz. 3) oraz numeryczne i graficzne metody stosowane przy obliczaniu stałych trwałości kompleksów w oparciu o tę funkcję i szereg innych funkcji tego typu; wreszcie w obszernym rozdziale (5), zajmującym niemal czwartą część objętości tekstu, daje przegląd doświadczalnych metod służących wyznaczaniu stałych trwałości. Rozdziały teoretyczne napisane są zwięźle, lecz w sposób umożliwiający korzystanie czytelnikowi z tekstu bez konieczności odwoływania się do licznie cytowanej literatury źródłowej. W rozdziale poświęconym metodom doświadczalnym szczegółowo przedstawione są tylko bardziej rozpowszechnione metody oparte o pomiary widm elektronowej absorpcji, dystrybucję składników między dwie fazy ciekłe, pomiary rozpuszczalności, wymianę jonową na żywicach jonowych oraz o pomiary potencjometryczne i polarograficzne. Inne omówione są skrótowo z odesłaniem czytelnika do oryginalnych opracowań. Dzięki pominięciu omówienia samych technik eksperymentalnych oraz podstaw teoretycznych rozdział ma charakter metodyczny i przeglądowy (czytelnik znajdzie w nim odsyłacze do 346 oryginalnych prac).

Pozostałe siedem rozdziałów książki stanowi jej drugą część, poświęconą równowagom w roztworach wybranych klas związków kompleksowych (rozdziały 6-10), dyskusji czynników decydujących o trwałości połączeń kompleksowych oraz wpływających na położenie równowagi kompleksotwórczej (rozdz. 11) oraz rozważaniom kierunków rozwoju chemii związków kompleksowych (rozdz. 12). Wybór klas omawianych związków kompleksowych podyktowany jest względami praktycznymi. Najczęściej omawianą klasą związków kompleksowych są kompleksy dwuskładnikowe, realizujące się zazwyczaj w układach modelowych. Beck natomiast zajmuje się bardziej złożonymi równowagami kompleksotwórczymi realizującymi się w praktycznie spotykanych układach chemicznych i biochemicznych. I tak rozdz. 6 poświęcony jest kompleksom, których ligandy uczestniczą w reakcjach protolitycznych, rozdz. 7 hydratacji jonów, rozdz. 8 kompleksom zawierającym różne ligandy w sferze koordynacyjnej; kolejne rozdziały zawierają omówienie kompleksów z zewnętrzną sferą utworzoną przez luźniejsze wiązanie anionów lub neutralnych ligandów (rozdz. 9) i kompleksom wielocentrowym (rozdz. 10). Autor nie ogranicza się do przedstawienia właściwości tych klas związków kompleksowych, zwracając uwagę na ich analityczne zastosowania oraz znaczenie dla kinetyki reakcji chemicznych, w których uczestniczą jony metali i wiążące się z nimi koordynacyjnie ligandy. Uwadze czytelników Acta Biochimica Polonica należy polecić zwłaszcza

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rozdział poświęcony kompleksom zawierającym różne ligandy ze względu na podstawowe znaczenie tej klasy związków koordynacyjnych w wielu reakcjach enzymatycznych.

Oceniając ogólne walory teoretycznej i metodycznej części książki oraz jej wartość informacyjną w części poświęconej chemii związków kompleksowych należy ją polecić biochemikom zainteresowanym analitycznymi zastosowaniami związków kompleksowych oraz podstawowymi aspektami roli związków kompleksowych w układach biologicznych.

K. L. Wierzchowski

H. Netter, THEORETICAL BIOCHEMISTRY. PHYSICO-CHEMICAL PRINCIPLES OF VITAL PROCESSES. Oliver & Boyd, Edinburgh 1969; str. 928, cena £ 15.0.0.

Wydane obecnie angielskie tłumaczenie znanej książki profesora Hansa Nettera jest właściwie drugim jej, rozszerzonym, uzupełnionym i unowocześnionym wydaniem. Objętość książki znacznie wzrosła w porównaniu z niemieckim wydaniem z 1959 r., co mogłoby świadczyć o tym, że biochemia nie wyszła jeszcze z okresu gromadzenia faktów i że może za wcześnie jest na to, ażeby wiadomości współczesnej nauki o chemicznym funkcjonowaniu żywych organizmów można było uogólnić w postaci krótkich teoretycznych prawideł. W każdej bowiem gałęzi nauk przyrodniczych po długim zazwyczaj okresie nagromadzania faktów doświadczalnych lub wynikających z obserwacji, następuje ujmowanie tych faktów w teoretyczne zależności dające się wyrazić językiem matematycznym. Biochemia wciąż jeszcze jest na etapie gromadzenia faktów pomimo istnienia pięknych uogólnień małych fragmentów wiedzy biochemicznej, jak np. model matematyczny przemian cyklu Krebsa (o którym to modelu nie ma mowy w ocenianej książce). Dlatego też książka H. Nettera nie jest — bo nie może być — teoretycznym uogólnieniem wiedzy o chemii życia, jest jednak najbardziej ścisłym spośród znanych mi podręczników biochemii ogólnej.

Książka jest podzielona na dwie części: statyka i dynamika. Możnaby powiedzieć, że część pierwsza to wprowadzenie do wybranych zagadnień fizyko-chemicznych ilustrowane przykładami zjawisk biochemicznych, natomiast w drugiej części autor wychodzi od zjawisk biologicznych (biochemicznych), aby zinterpretować je — tam gdzie to jest możliwe — przy pomocy równań i zależności fizyko-chemicznych. Szczególnie cenne i interesujące w omawianej książce jest to, że autor w wielu miejscach poświęcił dużo uwagi regulacjom procesów biochemicznych, a spośród trzynastu rozdziałów zawartych w książce, dwa końcowe: „Kontrola szybkości w reakcjach biochemicznych" oraz „Dynamiczne i strukturalne jednostki czynnościowe", dotyczą niemal w całości regulacyjnych procesów metabolicznych.

Obecne angielskie wydanie ocenianej książki jest owocem współpracy autora z tłumaczami i redaktorami tej wersji językowej. Jakkolwiek ta współpraca dała dzieło bardzo interesujące i cenne, to jednak tłumacze nie uchronili się od pewnych, drobnych zresztą, błędów. Np. na stronie 704 w wierszu 4-7 zdanie „It follows from the general law of cross-sections that the stationary concentration of an intermediate will be reduced if the enzyme removing it from the system is present in large amounts and has a large turnover number N, and will be increased if the enzyme producing it is present in small amounts", jest błędne w swojej drugiej części. Błąd wynika stąd, że zamiast słowa „removing" użyto słowa „producing", co oczywiście zmienia istotę wyrażonej myśli. Byłoby może także lepiej, gdyby w przytoczonym zdaniu, jak i w całej książce, użyto terminu zalecanego przez Komisję Enzymową IUB: „aktywność molekularna" lub „aktywność katalitycznego centrum", zamiast starego terminu „liczba obrotów" (turnover number). Opracowanie zarówno redaktorskie, jak i korektorskie omawianej książki jest bardzo staranne. Przedostały się tylko bardzo nieliczne błędy drukarskie (np. „orthithine" zamiast „ornithine" na str. 709 w wierszu 8).

Szczególnego waloru dodaje książce drobiazgowe opracowanie indeksów. Poza wykazem cytowanego piśmiennictwa, na końcu książki podany jest alfabetyczny spis autorów, indeks symboli, oraz dwa alfabetyczne indeksy rzeczowe: indeks chemikalii i indeks ogólny. Niewątpliwie ułatwi to korzystanie z książki jako ze źródła, do którego zaglądać się będzie wielokrotnie w poszukiwaniu szczegółów fizyko-chemicznego spojrzenia na zjawisko życia.

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Książka będzie przedmiotem zainteresowania ogółu biochemików, zwłaszcza tych, którzy nauczają w szkołach wyższych; może być także doskonałą lekturą uzupełniającą dla studiujących biochemię, dla biologów, chemików i wszystkich tych, którzy dążą do zrozumienia życia jako zjawiska dającego się opisać w ścisły liczbowy sposób.

Mariusz Żydowo

A. B. Roy and P. A. Trudinger, THE BIOCHEMISTRY OF INORGANIC COMPOUNDS OF SULPHUR, Cambridge University Press, Cambridge 1970; str. XVI+400, cena Ł 6.

Książka ta zawiera interesujące omówienie postępów biochemii nieorganicznych związków siarki w ciągu ostatnich kilkunastu lat, o czym można wnioskować choćby na podstawie zebranych sumiennie przez autorów danych z piśmiennictwa obejmującego ponad 1400 pozycji. Pracujący w Australii autorzy są znanymi specjalistami w zakresie badań nad arylosulfatazą oraz metabolizmem tiosiarczanu u samożywnych bakterii siarkowych i dzięki temu napisana przez nich monografia stanowi nie tylko prezentację wyników z literatury, ale zawiera krytyczną analizę materiału i wiele oryginalnych poglądów.

Na wstępie autorzy zestawiają listę enzymów biorących udział w metabolizmie nieorganicznych związków siarki oraz podają zasady nomenklatury biologicznie ważnych związków tej grupy, co jest pożyteczne przy chaosie panującym w zakresie nazewnictwa. Treść książki podzielona jest na 13 rozdziałów, z których pierwsze cztery dotyczą własności chemicznych, metod syntezy i analizy wybranych związków siarkowych. W dalszej kolejności omówione są metody i wyniki badań enzymatycznych, szczególnie na temat sulfataz oraz sulfotransferaz. Rozdziały 9 - 11 poświęcone są przemianom metabolicznym związków siarki w drobnoustrojach, roślinach i organizmach zwierzęcych. W wielu przypadkach rozważane są także przemiany organicznych połączeń siarki, np. utlenianie cysteiny do siarczanów. Na zakończenie autorzy omawiają interesujące a mało znane aspekty kliniczne i ekonomiczne biochemii nieorganicznych związków siarki. Książkę uzupełnia szczegółowy i starannie opracowany indeks rzeczowy oraz skorowidz nazwisk autorów.

Wydaje się, że monografia Roya i Trudingera zainteresuje nie tylko wąską grupę badaczy związanych bezpośrednio z metabolizmem siarki, ale może stanowić także cenne źródło informacji w zakresie przemiany aminokwasów i sterydów, a nawet patogenezy pewnych chorób układu nerwowego czy też zagadnień żyzności gleby.

Aleksander Koj

COMPENSATORY RENAL HYPERTROPHY (W. W. Nowiński & R. J. Goss, eds.) Academic Press, New York & London, 1969; str. 332.

W sto lat po pierwszej z powodzeniem wykonanej przez G. Simona operacji usunięcia jednej nerki u człowieka, odbyła się w Galvestonie, Teksas, międzynarodowa konferencja poświęcona zagadnieniom wyrównawczego przyrostu nerki po nefrektomii. W rok później, w 1969 r., ukazała się książka p.t. ,,*Compensatory renal hypertrophy*" będąca zbiorem wygłoszonych w czasie sympozjum referatów i związanych z nimi dyskusji. Organizatorami sympozjum, jak również redaktorami książki są: amerykański uczony polskiego pochodzenia W. W. Nowiński oraz R. J. Goss.

Książka zawiera 17 artykułów. Tematycznie można podzielić je na 4 grupy: ,,Mechanizmy wzrostu w warunkach normalnych", ,,Biochemia i fizjologia wyrównawczego przerostu nerki", ,,Doświadczalne i patologiczne aspekty wzrostu nerki" i ,,Wpływ wieku na czynność nerki", oraz dwa artykuły, których nie można zakwalifikować do żadnej z tych grup, W. W. Nowińskiego pt. ,,Historia nefrektomii "oraz R. J. Gossa pt. ,,Przyszłość nefrektomii". Naczelnym zagadnieniem poruszanym prawie we wszystkich artykułach i związanych z nimi dyskusjach jest znalezienie odpowiedzi na pytanie, co powoduje, że nerka pozostawiona w organizmie po operacyjnym usunięciu drugiej przerasta w takim stopniu, że w krótkim czasie jest w stanie przejąć funkcję obu

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nerek, zabezpieczając w ten sposób organizm przed powikłaniami. Według koncepcji przedstawionej przez Johnsona nerka powiększa się w wyniku wzmożonej pracy, podobnie jak mięśnie szkieletowe czy mięsień sercowy. Według Nowińskiego za przerost nerki odpowiedzialny jest jakiś czynnik humoralny, aktywator, który pojawia się w surowicy po usunięciu jednej nerki. Roels podaje natomiast, że wzrost nerki może być spowodowany nie tyle wzrostem ilości aktywatora, ile raczej zmniejszoną ilością inhibitora, który znajduje się w obu nerkach. Halliburton przedstawia wyniki, które mogą przemawiać za koncepcją Johnsona, ponieważ pozostawienie zwierząt na diecie wysokobiałkowej powoduje przerost nerki, nie stwierdza się jednak podobnego przerostu u zwierząt karmionych pokarmem zawierającym duże ilości mocznika. Gottschalk przedstawia wyniki wskazujące, że zmiany morfologiczne nerki zaczynają się wcześniej, niż można zaobserwować pierwsze oznaki wzmożonej czynności nerki i tym samym sugeruje, że przerost nerki nie musi być spowodowany jej wzmożonym obciążeniem dodatkową pracą. Malt omawia bardzo wczesne zmiany w syntezie kwasów nukleinowych i w polisomach, a Threlfall opisuje doświadczenia, w których podawanie operowanym zwierzętom kwasu foliowego wzmagało i tak już podwyższoną syntezę kwasów nukleinowych i białek. Szkoda, że omawiana książka nie zawiera ani jednego artykułu poświęconego wpływowi nefrektomii na organizm ludzki.

Bardzo duże doświadczenie naukowe i wydawnicze W. W. Nowińskiego i R. J. Gossa spowodowało, że książka jest wydana niezwykle starannie, czytelnie i w pięknej szacie graficznej, co powinno być dodatkowym bodźcem do jej przeczytania dla tych wszystkich, którzy interesują się procesami wzrostu i jego mechanizmami regulującymi.

Marek Ombach

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