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# SYNTHESIS AND PROPERTIES OF ANALOGUES OF COENZYME $B_{12}$ METHYLATED IN THE ADENOSYL GROUP

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Numerous analogues of coenzyme $B_{12}$ have been synthesized by substituting other groups for 5′-deoxyadenosyl. All these analogues proved to be inhibitors of the enzymic reaction dependent on coenzyme $B_{12}$, in which diols are transformed into deoxyaldehydes and glycerol into β-hydroxypropionic aldehyde [2, 9, 17, 14, 22]. It should be added that cyanocobalamin and hydroxycobalamin also inhibited competitively this reaction.

On the other hand, it is known that the analogues, at least some of them, are just as active as cyanocobalamin in promoting the growth of chicks [4]. This may be explained by the ease with which the analogues are converted in the animal body into coenzyme $B_{12}$ or some other active form. Such transformations have been observed in micro-organisms [14].

In spite of this discrepancy, it seems that certain analogues with structures differing only slightly from coenzyme $B_{12}$ may be useful in studies on the relationship between activity and chemical structure of coenzyme $B_{12}$. Therefore it was decided to synthesize analogues of coenzyme $B_{12}$ containing in the place of adenosine, 1-methyladenosine and $N^6$-methyladenosine, and to test their coenzymic activity.

## MATERIAL AND METHODS

Vitamin $B_{12}$ and coenzyme $B_{12}$ were isolated from cultures of *Propionibacterium shermanii* [20]. 1-Methyladenosine was synthesized by the method of Jones & Robins [12] by methylating adenosine with methyl iodide in *N,N*-dimethylformamide solution. Traces of iodide were removed by passing the aqueous solution of the preparation through a short column with Dowex-1 in the $HCO_3^-$ form. $N^6$-Methyladenosine was obtained by heating 1-methyladenosine dissolved in 0.25 N-sodium hydroxide [12]. It was observed that heating in concentrated ammonia also converts 1-methyladenosine quantitatively into isomeric $N^6$-methyl-

adenosine. 2′,3′-*O*-Isopropylidene derivatives of methyladenosines were prepared as follows: 0.5 m-mole of methyl adenosine, thoroughly pulverized and dried at 105° over $P_2O_5$ at 0.1 mm. Hg for 4 hr., was suspended in 30 ml. of anhydrous acetone, and 5 m-moles of anhydrous *p*-toluenesulphonic acid were added, the acid being previously dried for 4 hr. over $P_2O_5$ at 64° and 0.1 mm. Hg. The mixture was shaken for about 15 min. to dissolve the nucleoside and left for 5 hr. at room temperature, during which time the solution took a light yellow colour. Then 20 mg. of Celite was added, and the clear filtrate was added with 2 g. of thoroughly pulverized natrium bicarbonate; the suspension was mixed for at least 1 hr. to make the solution neutral. Then the suspension was placed in the extraction capsule of a Soxhlet apparatus and extracted with anhydrous acetone for 12 hr. The extract was evaporated to dryness and the chromatographic homogeneity of the reaction product was checked with solvents *A* and *B* (see below). Small amounts of methyladenosine found in some of the preparations were removed by passing the solution through a short column with Dowex-1 (borate form). From the dry residue, 2′,3′-*O*-isopropylidene-1-methyladenosine was crystallized from warm methanol or methanol and ethyl acetate. The yield was 80%; m.p. 255°. 2′,3′-*O*-Isopropylidene-$N^6$-methyladenosine was obtained only as a glassy residue with 75% yield. 5′-*O*-Tosyl-2′,3′-*O*-isopropylidene nucleosides were obtained by the action of *p*-toluenesulphonyl chloride on appropriate isopropylidene derivatives dissolved in anhydrous pyridine distilled over KOH. The following procedure was applied: 50 mg. of well pulverized derivative, previously dried for 10 hr. at 105° over $P_2O_5$ at 0.1 mm. Hg, were suspended in 1.5 ml. of anhydrous pyridine and warmed. After cooling on an ice bath, 29 mg. of *p*-toluenesulphonyl chloride were added, with mixing. After 10 min. the mixture was removed from the ice and left for 15 hr. at room temperature. The sediment formed was removed by filtration and pyridine was evaporated from the filtrate and washings at room temperature under reduced pressure. The viscous, resinous residue obtained, although non-homogeneous on chromatography, was used directly for the synthesis of coenzyme $B_{12}$ analogues.

Synthesis of analogues of coenzyme $B_{12}$ and purification of crude preparations by electrophoresis and paper chromatography were carried out as previously described [22] except that double amounts of vitamin $B_{12}$ and of the tosyl derivative were used, and the time of reaction was prolonged to 10 min.

Enzymic studies were performed according to the methods already described [22], $\beta$-hydroxypropionaldehyde being assayed according to Smiley & Sobolov [16] and acetaldehyde and propionaldehyde after Böhme & Winkler [3]. The enzymic system from *Aerobacter aerogenes* cells (strain no. 572, PZH, Warszawa) was prepared according to the

method previously described [21]. For colorimetric measurements a Bausch & Lomb Spectronic 20 photocolorimeter was used.

Spectral analyses were performed with a Hilger H 700 spectrophotometer with 1 cm. long quartz cuvettes. Concentrations of coenzyme $B_{12}$ and its analogues were determined in samples of the solution after conversion into dicyano derivatives, by measuring the extinction at 580 mμ and taking the molar extinction coefficient value as $10.1 \times 10^3$ [1].

For identification of purines and their derivatives, the following solvents were used for paper chromatography: (*A*), 5% aqueous solution of ammonium bicarbonate [12]; (*B*), propan-2-ol - water - conc. ammonia (70:25:5, by vol.); and (*C*), *n*-butanol - water - conc. ammonia (86:13:1, by vol.) [18]. The spots were detected by a low-pressure quartz lamp (Philips 57413 P/40 TUV 30W) equipped with a filter made from a quartz tube containing solutions of nickel sulphate and cobaltous sulphate [5].

For chromatographic purification and identification of corrins, two solvents were used: (*D*), *n*-butanol - propan-2-ol - water - acetic acid (100:70:99:1, by vol.) and (*E*), *n*-butan-2-ol - water - acetic acid (70:99:1, by vol.). Paper electrophoresis was carried out in 1 M-acetic acid at 6 - 8 V/cm. Both chromatography and electrophoresis were carried out in complete darkness.

1-Methyladenine and $N^6$-methyladenine used as standards were obtained from the appropriate nucleosides by hydrolysis in 2 N-hydrochloric acid. Adenosine was a commercial product of Nutritional Biochemicals Co., U.S.A., and P-cellulose was prepared according to Peterson & Sober [15]. Other reagents were obtained from Fabryka Odczynników Chemicznych, Gliwice, Poland.

## RESULTS AND DISCUSSION

### *Synthesis and physico-chemical properties of the analogues*

The method described recently by Jones & Robins [12] for the synthesis of methyladenosine nucleosides, permitted to carry out the synthesis of coenzyme $B_{12}$ analogues. 1-Methyladenosine and $N^6$-methyladenosine were found to react readily with acetone in the presence of anhydrous *p*-toluenesulphonic acid and to be transformed almost quantitatively into 2′,3′-*O*-isopropylidene derivatives. Tosylation of these derivatives followed the same course as for the nucleoside compounds described thus far. The chromatographic properties of the obtained purine compounds are summarized in Table 1.

Incorporation of nucleosides in the chemical synthesis of coenzyme $B_{12}$ analogues was confirmed by isolating the nucleosides after photolysis, or free purine base after treatment with potassium cyanide. The reactions with potassium cyanide were performed as follows: about 1 mg. of the analogue was dissolved in 10 ml. water, added with a few mg.

Table 1

$R_F$ *values of 1-methyladenine,* $N^6$*-methyladenine and their derivatives*

Chromatograms were developed on Whatman no. 1 filter paper by the descending technique. In solvent *A*, chromatograms were developed at 18°.

| Compound | Solvent | | |
|---|---|---|---|
| | *A* | *B* | *C* |
| Adenosine | 0.54 | 0.52 | 0.23 |
| 1-Methyladenosine | 0.77 | 0.43 | 0.20 |
| $N^6$-Methyladenosine | 0.64 | 0.64 | 0.47 |
| 2′,3′-*O*-Isopropylidene-1-methyladenosine | 0.77 | 0.68 | |
| 2′,3′-*O*-Isopropylidene-$N^6$-methyladenosine | 0.64 | 0.79 | |
| 1-Methyladenine | | | 0.25 |
| $N^6$-Methyladenine | | | 0.63 |

KCN and left in complete darkness for several hours. Then the solution was adjusted to pH 6 with dilute acetic acid, added with water to 15 ml. and applied to a P-cellulose column (2 cm. × 2 cm.). The pigmented corrinoids were eluted with water and then free purine with 0.1 N-HCl. The spectral analysis and paper chromatography of the isolated compounds showed that potassium cyanide decomposed both coenzyme $B_{12}$ analogues, liberating free purine similarly as in the reaction with coenzyme $B_{12}$ in which adenine was liberated [10]. The base liberated from an analogue synthesized from a 1-methyladenosine derivative had an absorption maximum at 258 - 259 mμ in acid, and at 270 - 271 mμ in alkaline solution, and its spot on the chromatograms corresponded to 1-methyladenine. The base liberated from the analogue synthesized from the $N^6$-methyladenosine derivative had $E_{max}$ at 265 - 267 mμ in 0.1 N-HCl, and at 274 mμ in alkaline solution; on chromatography, the compound behaved like $N^6$-methyladenine standard. The absorption maxima of the bases were in agreement with data found in the literature [13, 18].

The photolysis was performed by irradiating solutions of 1 mg. of the analogue showed $E_{max}$ at 258 mμ in 0.1 N-HCl, and 259 - 260 mμ in alkaline on a P-cellulose column. The column was washed with dilute solution of hydrogen cyanide to convert aquo(hydroxy)-cobalamin into the cyano form. The purine derivatives were eluted with 0.1 N-HCl, collecting ten 3-ml. fractions. Each of the analysed samples contained at least three products, with marked predominance of one compound. The main fraction eluted from the photolysis products of the 1-methyladenine analogue in 10 ml. water in direct sunlight, and the product adsorbed solution. The main fraction of the $N^6$-methyladenine derivative showed $E_{max}$ at 262 - 263 mμ in acid, and at 267 mμ in alkaline solution. These data are in agreement with those for 1-methyladenosine and $N^6$-methyladenosine [13, 18].

In the course of photolysis of coenzyme $B_{12}$ under aerobic conditions, the nucleoside moiety is split off in the form of a radical which stabilizes as the 8,5′-cyclic adenosine [7], and moreover adenosine-5′-aldehyde [8] and adenosine-5′-carboxylic acid are formed [11]. As in spectral analysis all these compounds behaved like adenosine [1], it may be assumed that the products of photolytic degradation of the synthesized analogues containing 1-methyladenine or $N^6$-methyladenine also contain cyclic nucleosides, aldehyde and possibly carboxyl derivatives.

The method by which the analogues were synthesized, their behaviour on photolysis and in the reaction with potassium cyanide, corroborate their structure as Co-5′-deoxy-(1-methyladenosyl)-α-(5,6-dimethylbenzimidazolyl)-cobamide (Co-5′-d(1-MAdo)-DMBIA-cobamide), and Co-5′-deoxy-($N^6$-methyladenosyl)-α-(5,6-dimethylbenzimidazolyl)-cobamide (Co-5′-d($N^6$-MAdo)-DMBIA-cobamide). The schematic structural formulae of the two synthesized compounds are shown in Fig. 1. On electrophoresis both analogues exhibited greater mobility than coenzyme $B_{12}$

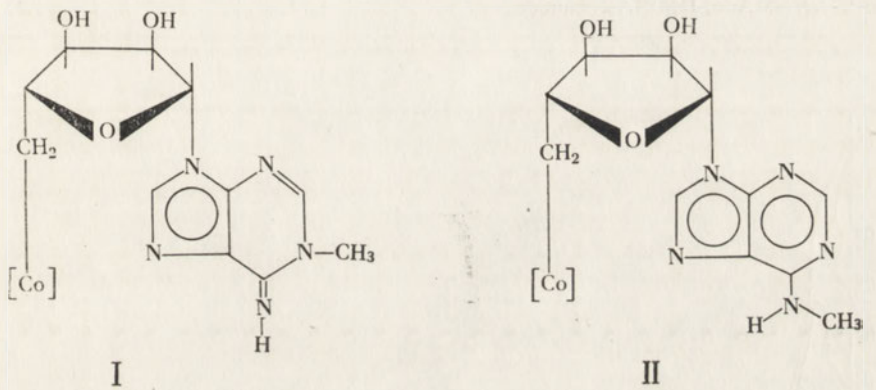


Fig. 1. Structural formulae of coenzyme $B_{12}$ analogues. (I), Co-5′-deoxy-(1-methyladenosyl)-α-(5,6-dimethylbenzimidazolyl)-cobamide; abbreviation used: Co-5′-d(MAdo)-DMBIA-cobamide. (II), Co-5′-deoxy-($N^6$-methyladenosyl)-α-(5,6-dimethylbenzimidazolyl)-cobamide; abbreviation used: Co-5′-d($N^6$-MAdo)-DMBIA-cobamide.

(Table 2) owing to the more basic character of the methyl derivatives of adenosine. Of the two methyladenosines, 1-methyladenosine was more basic [6] and the analogue containing this nucleoside had greater electrophoretic mobility. On paper chromatography, the mobilities of the two analogues differed very markedly and it may be assumed that Co-5′-d(1-MAdo)-DMBIA-cobamide is more polar than its $N^6$-MAdo isomer.

The two analogues synthesized, similarly as coenzyme $B_{12}$, are photosensitive and, although the rate of degradation has not been measured, the observations indicate that they undergo photolysis at least as rapidly as coenzyme $B_{12}$.

Table 2

*Chromatographic and electrophoretic mobility of analogues of coenzyme $B_{12}$*

Chromatograms were developed in a dark room at 20° by the descending technique on Whatman no. 3 MM paper. Electrophoresis was carried out on Whatman no. 3 MM paper in 1 M-acetic acid. The relative rate of migration is given in terms of $R_B$ denoting the distance moved by the compound divided by the distance moved by cobinamide. The position of cyanocobalamin defines the starting point.

| Compound | Paper chromatography | | Paper electrophoresis $R_B$ |
|---|---|---|---|
| | Solvent *D* | Solvent *E* | |
| | $R_{coenzyme\ B_{12}}$ | | |
| Coenzyme $B_{12}$ | 1.00 | 1.00 | 1.18 |
| Cyanocobalamin | | | 0.00 |
| Aquocobinamide cyanide | | | 1.00 |
| Co-5′-d(1-MAdo)-DMBIA-cobamide | 0.54 | 0.29 | 1.30 |
| Co-5′-d($N^6$-MAdo)-DMBIA-cobamide | 1.30 | 1.37 | 1.22 |

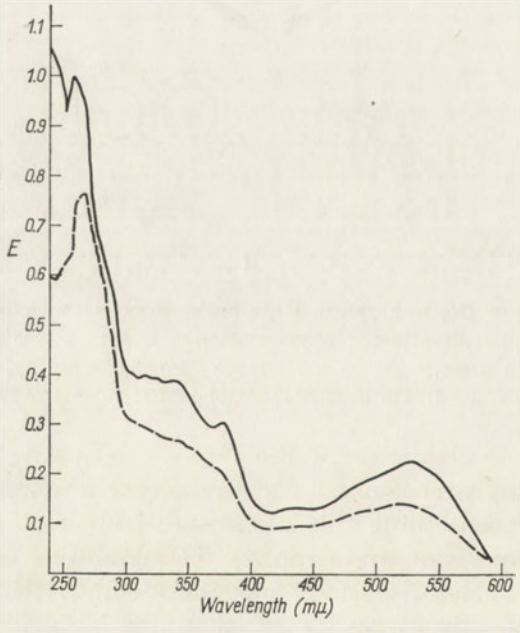


Fig. 2. Absorption spectra of coenzyme $B_{12}$ analogues in aqueous solution: (——), Co-5′-d(1-MAdo)--DMBIA-cobamide, concn. $2.92 \times 10^{-5}$ M; (— — —), Co-5′-d($N^6$--MAdo)-DMBIA-cobamide, concn. $1.78 \times 10^{-5}$ M.

The absorption spectra of the analogues (Fig. 2) resembled the spectrum of coenzyme $B_{12}$, except that the maximum in the 260 mμ region was shifted toward 257 - 258 mμ for 1-methyladenosyl analogue, and toward 265 - 267 mμ for the $N^6$-methyl derivative.

*Coenzymic activity of the coenzyme $B_{12}$ analogues*

The main enzymic test employed in this laboratory for studying the analogues of coenzyme $B_{12}$ is the transformation of glycerol to β-hydroxypropionic aldehyde. This reaction is catalysed by an enzymic system present in a preparation obtained from *A. aerogenes* [21]. This preparation catalyses as well the coenzyme $B_{12}$ dependent transformations of ethylene glycol to acetic aldehyde, and of 1,2-propandiol to propionic aldehyde; these two reactions were usually chosen by other authors for studying the analogues in a system isolated from a different strain of *A. aerogenes*.

As observed previously [22], and confirmed in the present experiments, the specificity of these reactions in relation to naturally occurring corrin coenzymes varies, although they are catalysed by the same enzymic system and belong to the same type of intramolecular oxyredox transformations. For instance, the coenzymic form of cobinamide was active with diols but was inactive when glycerol was used as substrate. With some enzymic preparations, the cobinamide coenzyme was inactive also in the reaction of transformation of ethylene glycol, while showing activity in the transformation of propandiol [22]. The reasons for this behaviour are not clear.

The 1-methyladenosine analogue was inactive in all three reactions even when the incubation was prolonged to 30 min. The analogue was considered to be practically inactive if after 10 min. incubation the amount of β-hydroxypropionic aldehyde formed was practically the same in the tested sample and in the blank test not containing the analogue. In the transformation of glycerol, the 1-methyladenosine analogue showed a competitive inhibition. The index of inhibition defining the ratio of the concentration of the analogue to the concentration of coenzyme $B_{12}$ at which the velocity of the reaction was reduced to one-half, was 2.3. For determinations of the inhibition index, coenzyme $B_{12}$ and the analogue were added to the reaction mixture together. The order in which coenzyme $B_{12}$ and the analogue were added influenced markedly the results because of the incomplete reversibility of the process of binding corrin compounds by apoenzyme [16, 17]. The concentrations of coenzyme $B_{12}$ used in these experiments were chosen so as to ensure complete saturation of the apoenzyme (this value was determined from kinetic data and the details will be described in a separate paper). The reaction mixture used previously [22] was found to be suitable; however, depending on the activity of the preparation studied, it was sometimes necessary to reduce the concentration of the enzyme from 0.5 to 0.2 mg. of protein per sample.

The Co-5′-d($N^6$-MAdo)-DMBIA-cobamide was found to act as a coenzyme of ethylene glycol and propandiol transformations. With glycerol

it was practically inactive during 10 min. incubation, but gave traces of $\beta$-hydroxypropionic aldehyde when the sample was incubated for 30 min. Under these conditions the 1-methyladenosyl analogue was inactive. Nevertheless, the $N^6$-methyladenosyl analogue appeared to be a competitive inhibitor in the reaction with coenzyme $B_{12}$. The inhibition index for this compound was 1.4, indicating an almost identical with coenzyme $B_{12}$ affinity to the apoenzyme.

The relation of the amount of acetaldehyde formed from ethylene glycol, to the concentration of the $N^6$-methyladenosyl analogue in the Lineweaver-Burk plot is shown in Fig. 3a. The graphically determined Michaelis constant defining the dissociation constant of the apoenzyme--coenzyme complex ($K'_m$) was $4 \times 10^{-7}$ M. The $K'_m$ for coenzyme $B_{12}$ for the same reaction was found to be identical: $4 \times 10^{-7}$ M (Fig. 3b). In the reaction with propandiol the $K'_m$ value for $N^6$-methyladenosyl analogue was $2.5 \times 10^{-7}$ M, and for coenzyme $B_{12}$, $1.4 \times 10^{-7}$ M (Fig. 4 a, b),

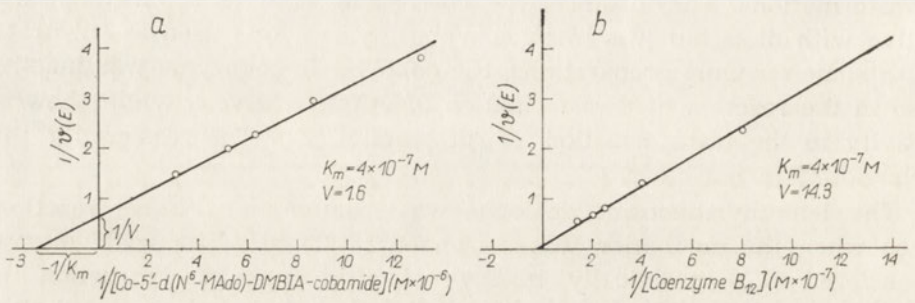


Fig. 3. Lineweaver-Burk plot of acetaldehyde formation versus: (a), Co-5′-d($N^6$--MAdo)-DMBIA-cobamide and (b), coenzyme $B_{12}$ concentration. Conditions: 50 µmoles of ethylene glycol; 0.2 ml. of 0.2 M-potassium phosphate buffer, pH 8.0; 1.6 mg. of protein and Co-5′-d($N^6$-MAdo)-DMBIA-cobamide or coenzyme $B_{12}$ at concentrations indicated, in a final volume of 1 ml., were incubated for 10 min. at 37°. Acetaldehyde was determined according to Böhme & Winkler [3]. The concentrations of acetaldehyde are given in extinction units.

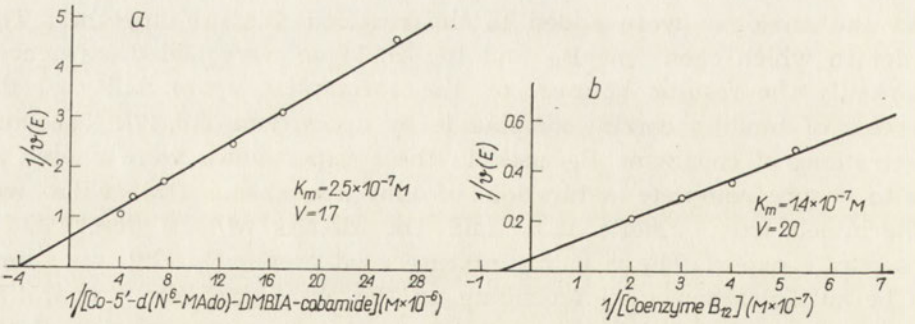


Fig. 4. Lineweaver-Burk plot of propionaldehyde formation versus: (a), Co-5′--d($N^6$-MAdo)-DMBIA-cobamide and (b), coenzyme $B_{12}$ concentration. Conditions as in the experiment shown in Fig. 3 except that 1,2-propandiol instead of ethylene glycol was used as substrate.

indicating similar affinity of coenzyme $B_{12}$ and the analogue to the enzyme. In spite of very similar values of the dissociation constants of the complex of apoenzyme with coenzyme $B_{12}$ and with the analogues, the maximum velocity ($V$) of the reaction with ethylene glycol was 14.3 extinction units for coenzyme $B_{12}$ but only 1.6 for $N^6$-methyladenosyl analogue (Fig. 3). It seems, therefore, that the apoenzyme-coenzyme $B_{12}$ complex is much more active than the apoenzyme-analogue complex. The results of kinetic studies of the reaction of propandiol (Fig. 4) showed similar relations.

The suggestion that the two enzyme complexes differ in their catalytic activity was confirmed by the Michaelis constant values for propandiol determined in the presence of an excess of the methylated analogue and coenzyme $B_{12}$ (Fig. 5 a, b). These $K''_m$ values were, respectively, $3.1 \times 10^{-4}$ M and $1.1 \times 10^{-3}$ M, differing by one order of

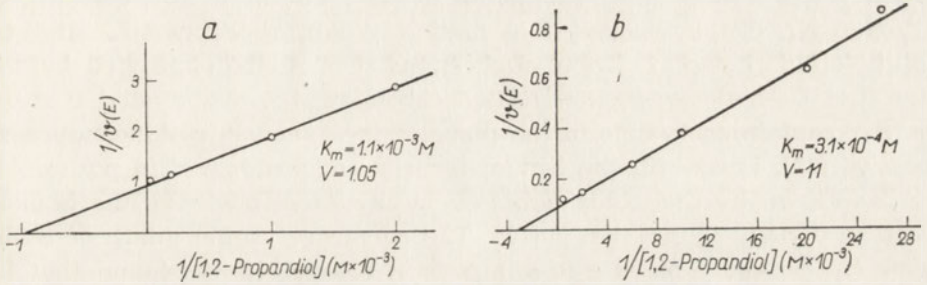


Fig. 5. Lineweaver-Burk plot of propionaldehyde formation versus 1,2-propandiol concentration in the presence of: (*a*), Co-5′-d($N^6$-MAdo)-DMBIA-cobamide and (b), coenzyme $B_{12}$. Conditions as in the experiment shown in Fig. 3 except that in (*a*) 3.8 μm-moles of the analogue and in (b) 0.1 μm-mole of coenzyme $B_{12}$ were used.

magnitude. Hence it appears that Co-5′-d($N^6$-MAdo)-DMBIA-cobamide gives with apoenzyme a complex that is equally stable as the one formed with coenzyme $B_{12}$, but the holoenzyme formed is catalytically many times less active than the natural complex. These kinetic data are summarized in the scheme:

$$\text{Apoenzyme} + \text{coenzyme } B_{12} \rightleftharpoons \text{Holoenzyme} \quad (K'_m \approx 10^{-7})$$

$$\text{Apoenzyme} + \begin{matrix}\text{analogue of}\\ \text{coenzyme } B_{12}\end{matrix} \rightleftharpoons \begin{matrix}\text{Analogue of}\\ \text{holoenzyme}\end{matrix} \quad (K'_m \approx 10^{-7})$$

$$\text{Substrate} + \text{holoenzyme} \rightleftharpoons \left[\begin{matrix}\text{Substrate-}\\ \text{-holoenzyme}\end{matrix}\right] \longrightarrow \text{Product} + \text{holoenzyme} \quad (K''_m \approx 10^{-4})$$

$$\text{Substrate} + \begin{matrix}\text{analogue of}\\ \text{holoenzyme}\end{matrix} \rightleftharpoons \left[\begin{matrix}\text{Substrate-}\\ \text{-analogue of}\\ \text{holoenzyme}\end{matrix}\right] \longrightarrow \text{Product} + \begin{matrix}\text{analogue of}\\ \text{holoenzyme}\end{matrix} \quad (K''_m \approx 10^{-3})$$

Co-5′-d(1-MAdo)-DMBIA-cobamide and Co-5′-d($N^6$-MAdo)-DMBIA--cobamide differed from coenzyme $B_{12}$ (Co-5′-dAdo-DMBIA-cobamide)

only in having an additional methyl group in position 1 or $N^6$ of adenosine. The first one was completely inactive, the second was only weakly active as coenzyme. Each, however, possessed the ability to combine with apoenzyme like coenzyme $B_{12}$.

It seems that the following tentative proposition may be advanced concerning the relation between the activity of coenzyme $B_{12}$ and its structure. The nitrogen $N_1$ of the adenosyl group seems to play an essential role in the coenzymic function. Adenosine $N_1$ nitrogen is known to possess the highest density of electrons and to undergo protonation most readily [6, 12], and it seems that in coenzyme $B_{12}$ the $N_1$ may be the site at which the coenzyme is attached to the substrate or to the apoenzyme, this binding being essential for its activity. For instance, combining with apoenzyme it may give rise to appropriate conformation of the protein, which is not formed when the coenzyme combines with apoenzyme through other functional groups. Therefore the blocking of nitrogen $N_1$, e.g. by means of a methyl group, abolishes the catalytic ability of the complex. These suggestions seem to be supported by the fact that Co-5′-deoxyinosine-DMBIA-cobamide, i.e. deaminated coenzyme $B_{12}$, containing inosine in the place of the adenosyl residue, was also inactive [22]. Inosine in the lactam form is not protonated in position 1, but, as shown by the experiments on methylation, possesses the highest electron density in position 7 [12]. The adenosine amino group of coenzyme $B_{12}$ probably plays a less important role, as it was found that its substitution by a methylamino group does not abolish the catalytic properties of the compound, although it results in their marked decrease. This reduced activity may be due either to a spatial obstacle or to increased basicity of the amino nitrogen. The data presented are too scarce to justify more than a tentative suggestion on the coenzyme $B_{12}$ function, and further experiments on other analogues of coenzyme $B_{12}$ are required.

## SUMMARY

The synthesis and properties of two analogues of coenzyme $B_{12}$ containing 1-methyladenosine or $N^6$-methyladenosine in the place of adenosine, are described. The influence of the analogues on enzymic transformation of glycerol into $\beta$-hydroxypropionic aldehyde and of ethylene glycol and 1,2-propandiol into acetic and propionic aldehydes, respectively, was studied. Both analogues proved to be strong competitive inhibitors of the reaction of glycerol. The 1-methyladenosine analogue was also inactive in the transformation of diols, which was catalysed by the $N^6$-methyladenosyl analogue. The dissociation constants of complexes of apoenzyme with both analogues and with coenzyme $B_{12}$ were of the same order ($K'_m = 10^{-7}$ M). The apoenzyme-coenzyme $B_{12}$ complex was much more active than the complex of apoenzyme and the $N^6$-methyl-

adenosyl analogue. $K''_m$ for 1,2-propandiol was $3.1 \times 10^{-4}$ M, and in the presence of the $N^6$-methyladenosyl analogue, $1.1 \times 10^{-3}$ M. The relation between coenzymic activity and chemical structure of coenzyme $B_{12}$ is discussed.

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## SYNTEZA I WŁASNOŚCI ANALOGÓW KOENZYMU $B_{12}$ METYLOWANYCH W CZĘŚCI ADENOZYLOWEJ CZĄSTECZKI

### Streszczenie


Opisano syntezę i własności dwóch analogów koenzymu $B_{12}$ zawierających w miejsce adenozyny odpowiednio 1-metyloadenozynę i $N^6$-metyloadenozynę. Zbadano wpływ tych analogów na enzymatyczne przekształcenie glicerolu w aldehyd $\beta$-hydroksypropionowy oraz glikolu etylenowego w aldehyd octowy i 1,2-propandiolu w aldehyd propionowy. Oba związki okazały się inhibitorami kompetytywnymi w reakcji glicerolu. Analog zawierający 1-metyloadenozynę był także nieaktywny w przemianie dioli, natomiast analog z $N^6$-metyloadenozyną katalizował te reakcje. Stałe dysocjacji kompleksów apoenzymu z analogami jak i z koenzymem $B_{12}$ są tego samego rzędu ($K'_m$ $10^{-7}$ M). Kompleks apoenzymu z koenzymem $B_{12}$

jest aktywniejszy od kompleksu apoenzymu z analogiem $N^6$-metyloadenozylowym. Stała Michaelisa dla reakcji przemiany 1,2-propandiolu w aldehyd propionowy w obecności koenzymu $B_{12}$ wynosi $3.1 \times 10^{-4}$ M, zaś w obecności analogu z $N^6$-metyloadenozyną $1.1 \times 10^{-3}$ M. Przedyskutowano zależność aktywności koenzymatycznej koenzymu $B_{12}$ od jego budowy chemicznej.

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# SEPARATION OF PROTEINS FROM *PROPIONIBACTERIA* ON CELLULOSE ION EXCHANGERS

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The purpose of this study was to develop a method suitable for the separation of proteins from *Propionibacteria* on cellulose ion exchangers. As control of reproducibility of results and for the identification of fractions, the activity of two hydrolytic enzymes, namely alkaline phosphatase and β-galactosidase, was assayed. The bacteria were grown on two culture media, one containing glucose and the other lactose as energy sources. Lactose was applied to induce the formation of β-galactosidase by *Propionibacteria*.

## EXPERIMENTAL

*Organisms.* The following strains were used: *Propionibacterium shermanii* (National Collection of Dairy Organisms, no. 839), *P. freudenreichi* (American Type Culture Collection, no. 6207), *P. petersoni* (A.T.C.C., no. 4870) and *P. arabinosum* (A.T.C.C., no. 4965).

Cultures of the bacteria were carried out according to Zodrow & Pawełkiewicz [14] in media containing casein hydrolysate and glucose or lactose, and the acid produced during growth of bacteria was neutralized with aqueous solution of ammonia. The cultures were centrifuged and each bacterial pellet divided into two parts. One was used to prepare the acetone powder by the method described by Bartosiński [1], the other was dried in a thin layer on a glass plate at room temperature and then ground manually for 4 hr. in a porcelain mortar with powdered glass.

*Substrates.* Tetraacetyl-α-bromogalactose was obtained by the method of Pasternak *et al.* [11], and p-nitrophenyl-β-D-galactopyranoside by a method based on the procedure of Glaser & Wulwek [4] for the synthesis of o-nitrophenyl derivative of glucose. Phenolphosphoric acid was obtained by the method described for p-nitrophenylphosphoric acid [6].

*Ionites.* Carboxymethyl(CM)-cellulose and diethylaminoethyl(DEAE)--cellulose were prepared according to Ellis & Simpson [3], the synthesis

of 2-chloro-*N*,*N*-diethylaminoethyl hydrochloride being carried out after Hall & Stephenson [5]. The obtained CM-cellulose contained 0.65 mEq. per gram and DEAE-cellulose 0.70 mEq. per gram. Phosphato(P)-cellulose prepared by the method of Peterson & Sober [12] contained 0.55 mEq. per gram.

*Analytical methods.* The extinctions were measured in a Pulfrich photocolorimeter equipped with an Elpho attachment.

Protein was determined by the phenol Folin reagent after Lowry *et al.* [7], the extinction of the sample being read at 660 mμ. Ovalbumin (Nutritional Biochem. Corp., Cleveland, Ohio, U.S.A.) was used as standard.

β-Galactosidase activity was measured by incubating 5 ml. of a solution containing 2.5 mg. of *p*-nitrophenyl-β-D-galactopyranoside dissolved in 0.01 M-phosphate buffer of pH 6.8, with 0.2 to 3.0 ml. of protein solution, and the whole adjusted to 8 ml. with the same buffer [13]. The incubation was carried out for 1 hr. at 40°, and the reaction was stopped by adding two drops of concentrated NaOH solution; the amount of *p*-nitrophenolate released was determined at 420 mμ, against a blank sample containing no protein. A solution of *p*-nitrophenol in 0.01 M-phosphate buffer of pH 6.8, adjusted to pH about 11 by adding several drops of NaOH, was used as standard. The unit of galactosidase activity was defined as that amount of enzyme which caused the liberation of 1 μg. of *p*-nitrophenol during 1 hr. incubation.

Alkaline phosphatase activity was measured in an incubation mixture consisting of 1 ml. of a solution containing 2 mg. of phenolphosphoric acid in 0.01 M-phosphate buffer, pH 9.4, and 0.2 to 3.0 ml. of protein solution, the whole being brought to the volume of 5.5 ml. with the same buffer [9]. Another sample containing no phenolphosphoric acid was prepared simultaneously. After incubation for 1 hr. at 38°, the Folin reagent was added and both samples were left for 1 hr. in the refrigerator; then the extinctions at 660 mμ were determined. In this way in the first sample the phenol liberated during incubation was determined together with the protein present in the sample, and in the second one only protein was determined. The difference between the two values gave the amount of liberated phenol. Analytic grade phenol (Fabryka Odczynników Chemicznych, Gliwice, Poland) dissolved in 0.01 M-phosphate buffer, pH 9.4, served as standard. The unit of phosphatase activity was defined as that amount of enzyme which liberated 1 μg. of phenol from the substrate during 1 hr.

## RESULTS

*Protein extraction.* One to three grams of acetone-dried or air-dried bacteria were ground in a mortar and 40 to 80 ml. of appropriate buffer or water was added. The thoroughly homogenized suspension was left

for 16 hr. at 0° and then centrifuged for 10 min. at 5° at 26 000 *g*. The clear or only slightly opalescent yellowish supernatant was dialysed in a cellophane tubing for 24 hr. at 2 - 3° against the same buffer. If the extraction was carried out with water, dialysis was performed against the solution which was to be used for initiating elution from the column. A small amount of precipitate which sometimes formed was removed by centrifugation and the protein solution was submitted to chromatographic separation.

*Column chromatography.* One to three grams of cellulose exchanger were suspended in 100 ml. of a buffer of the same pH and ionic strength as that used for extraction, or in water. The suspension was poured into a chromatographic tube closed at the lower end with a plate of Jena sintered glass G-2. The height of the column varied between 20 and 90 mm. depending on the amount of exchanger used, the diameter being 15 to 20 mm. The excess of fluid was allowed to flow out so that only a top layer of 1 - 2 mm. was left.

The bacterial protein solution was transferred to the tube very carefully to avoid stirring up the evenly distributed exchanger. The adsorbed proteins were eluted using stepwise and continuous pH gradient or ionic strength gradient procedures. The continuous gradient elution was performed according to the method of Parr [10] using two or more connected vessels. The separation which lasted from 4 to 10 hr. was performed at room temperature. The flow-rate was 2 - 8 ml. per minute, regulated by changing the pressure from 50 to 150 mm. water. The volume of the fractions ranged from 5 to 15 ml., and 100 to 200 fractions were collected by means of a fraction collector. The elution of the protein adsorbed on the cation exchangers, CM- and P-cellulose, with the two procedures gave unsatisfactory and non-reproducible separation, and the enzymes were inactivated. With a buffer of increasing pH, nearly 90% of protein was eluted in one peak and the remainder in 4 - 6 very small, indistinct and enzymically inactive fractions. The pH values of the effluent were either higher or lower that the pH of the eluent. Also when neutral salt solution (NaCl) was used as eluent, the pH of the effluent changed from 7 to less than 2; about 85% of the protein was eluted in the first peak, and the remainder in several indistinct, enzymically inactive fractions. With buffers consisting of weakly dissociated acids and salts the changes in pH values were less marked but still apparent. Very strong buffering of the cationite, which theoretically should assure the stability of the pH, diminished as a rule the adsorption capacity of the exchanger or lead to its complete loss at pH near 7.

The separation on DEAE-cellulose in the $OH^-$ form gave results similar to those obtained with CM-cellulose and P-cellulose. Anions were exchanged by the $OH^-$ ions causing a sharp increase in pH, outflow of almost all the protein, and usually inactivation of the enzymes. Chroma-

tography on DEAE-cellulose in the $Cl^-$ form, from which the adsorbed proteins were eluted with a pH gradient, gave similar results. When the eluents were buffered too strongly, the adsorption ability was practically abolished.

Distinct and reproducible separation of proteins could be obtained only on DEAE-cellulose in the Cl form using stepwise ionic strength gradient elution at constant pH 7. The ionic strength was changed by increasing the concentration of the phosphate buffer and by adding sodium chloride. Fig. 1 represents the separation of proteins extracted from acetone-dried *P. shermanii* grown on the lactose medium. Nine

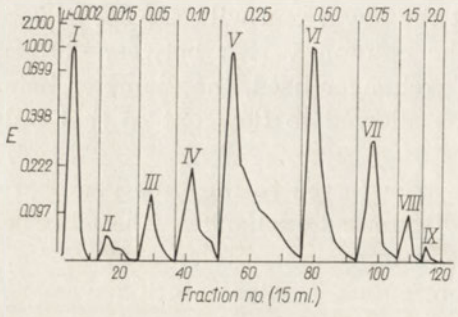


Fig. 1

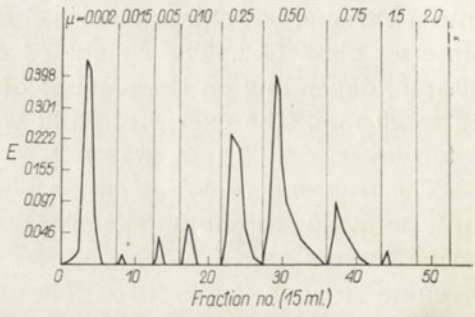


Fig. 2

Fig. 1. Stepwise elution on a DEAE-cellulose column of proteins from acetone-dried *P. shermanii*. Acetone powder, 3 g., was extracted with 60 ml. of phosphate buffer, ionic strength 0.002 μ, pH 7, the cells were removed by centrifugation and the extract containing 0.37 g. of protein was dialysed against the same buffer. A tube 50 mm. high was filled with 2 g. of DEAE-cellulose (Cl form) suspended in the same buffer. Stepwise elution was carried out with phosphate buffers, pH 7, at ionic strengths from 0.002 μ to 0.1 μ, and at ionic strengths above 0.2 μ with the addition of appropriate amounts of NaCl. Flow-rate about 7 ml./min. Ionic strength of the eluent in this and subsequent Figures is indicated in the upper part of the diagram.

Fig. 2. Rechromatography of peak *I* in Fig. 1 (eluted with 0.002 μ solution). Protein solution from peak *I*, 75 ml. (fractions 4 - 8), was dialysed against phosphate buffer, μ 0.002, then concentrated to 30 ml. and applied on the column of 1 g. DEAE--cellulose ($Cl^-$ form). Stepwise elution was performed in the same way as for the primary chromatography.

protein fractions were obtained from each of the four *Propionibacteria* strains studied. Attempts at further separation by increasing the number and changing the composition of the eluents, were unsuccessful. The results obtained for bacteria grown on the lactose medium were practically the same as when the glucose or fructose medium was used.

The rechromatography of the obtained protein fractions was performed under the same conditions and with the same eluents as the primary separation. Protein solutions from a peak (3 - 15 fractions of 15 ml.) were

dialysed, concentrated to about 20 ml. by ultrafiltration and then applied on a DEAE-cellulose column. Dialysis and concentration were performed at 1° to avoid losses in enzymic activity. Peak *I*, similarly as the whole protein, separated on rechromatography into nine fractions (Fig. 2) which upon second rechromatography did not further separate. Other protein peaks from the first column did not further separate upon rechromatography and were recovered in the same conditions (Fig. 3).

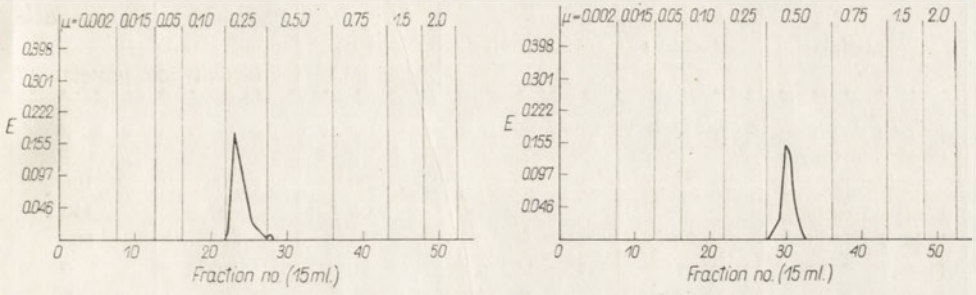


Fig. 3. Rechromatography of (*a*), peak *V*; and (b), peak *VI*, in Fig. 1 (eluted with 0.25 μ and 0.50 μ solution, resp.). Other conditions as for Fig. 2.

Peak *I*, which seemed to contain only the protein not adsorbed by the exchanger, proved to be a mixture of all proteins present in the bacterial extract submitted to separation. Presumably its non-homogeneity was due to the fact that the protein solution applied on the column passed through without being adsorbed until the ionite became saturated. By increasing the amount of ionite in relation to protein and by uniform distribution of the adsorbent in the column as well as by applying a slower flow-rate, the protein content of peak *I* could be diminished; however, even so it was not possible to obtain a homogeneous protein.

All the separations were carried out on short columns of exchanger; this facilitated the flow through the column and permitted a rapid separation of proteins. Clean-cut elution of protein fractions could be obtained with relatively small amounts of the exchanger and eluent solutions. With longer columns, the separation was less distinct and lasted longer.

*Enzymic activity.* The data pertaining to the distribution and activity of alkaline phosphatase and β-galactosidase are summarized in Table 1. Galactosidase was found in all strains grown in the presence of lactose and was localized in peak *VI* of the chromatogram eluted at ionic strength of 0.5 μ. Moreover, small amounts were present in the peak *I* which, as already mentioned, contained all the proteins of the studied mixture. Peak *V* also contained trace amounts of the enzyme, this, however, may be regarded as contamination by the neighbouring fraction; after rechromatography, peak *V* no longer contained any traces of the enzyme. The

## Table 1

*Distribution and activity of alkaline phosphatase and β-galactosidase in protein fractions from* Propionibacteria

Proteins extracted from acetone-dried bacteria which had been grown on media containing glucose or lactose, were separated on DEAE-cellulose column as described under Methods. The enzymic activity in protein peaks was assayed on three combined 15 ml. fractions indicated in the Table.

| Material | Ionic strength of eluent (μ) | Fractions no. | Protein concn. (μg./ml.) | Phosphatase (activity/mg. protein) | Galactosidase (activity/mg. protein) |
|---|---|---|---|---|---|
| *P. shermanii* (grown in lactose medium) | | | | | |
| Crude centrifuged extract | | | 6200 | 15 | 106 |
| Dialysed extract | | | 5000 | 20 | 140 |
| Peak *I* | 0.002 | 3, 4, 5 | 750 | 110 | 800 |
| Peak *II* | 0.015 | 13, 14, 15 | 30 | 0 | 0 |
| Peak *III* | 0.050 | 27, 28, 29 | 85 | 125 | 0 |
| Peak *IV* | 0.100 | 40, 41, 42 | 115 | 600 | 0 |
| Peak *V* | 0.250 | 54, 55, 56 | 700 | 154 | 10 |
| Peak *VI* | 0.500 | 79, 80, 81 | 730 | 0 | 3760 |
| Peak *VII* | 0.750 | 98, 99, 100 | 220 | 0 | 0 |
| Peak *VIII* | 1.500 | 109, 110, 111 | 60 | 0 | 0 |
| Peak *IX* | 2.000 | 114, 115, 116 | 20 | 0 | 0 |
| *P. shermanii* (grown in glucose medium) | | | | | |
| Crude centrifuged extract | | | 6300 | 13 | 2 |
| Dialysed extract | | | 5300 | 16 | 3 |
| Peak *I* | 0.002 | 2, 3, 4 | 820 | 80 | 6 |
| Peak *II* | 0.015 | 14, 15, 16 | 35 | 0 | 0 |
| Peak *III* | 0.050 | 29, 30, 31 | 100 | 200 | 0 |
| Peak *IV* | 0.100 | 40, 41, 42 | 120 | 690 | 0 |
| Peak *V* | 0.250 | 53, 54, 55 | 710 | 100 | 0 |
| Peak *VI* | 0.500 | 80, 81, 82 | 750 | 0 | 0 |
| Peak *VII* | 0.750 | 96, 97, 98 | 250 | 0 | 0 |
| Peak *VIII* | 1.500 | 108, 109, 110 | 60 | 0 | 0 |
| Peak *IX* | 2.000 | 114, 115, 116 | 25 | 0 | 0 |
| *P. arabinosum* (grown in lactose medium) | | | | | |
| Crude centrifuged extract | | | 6100 | 20 | 120 |
| Dialysed extract | | | 5000 | 40 | 150 |
| Peak *I* | 0.002 | 3, 4, 5 | 800 | 120 | 750 |
| Peak *II* | 0.015 | 15 | 15 | 0 | 0 |
| Peak *III* | 0.050 | 26, 27, 28 | 50 | 0 | 0 |
| Peak *IV* | 0.100 | 37, 38, 39 | 65 | 0 | 0 |
| Peak *V* | 0.250 | 51, 52, 53 | 690 | 840 | 0 |
| Peak *VI* | 0.500 | 77, 78, 79 | 610 | 100 | 3900 |
| Peak *VII* | 0.750 | 98, 99, 100 | 200 | 0 | 30 |
| Peak *VIII* | 1.500 | 107, 108, 109 | 85 | 0 | 0 |
| Peak *IX* | 2.000 | 115 | 20 | 0 | 0 |

activity of galactosidase per mg. protein in peak *VI* was 30 to 40 times as high as in the crude protein extract. Cohen and Monod [2, 8] demonstrated the induction of β-galactosidase in normal strains of *E. coli*. Similarly, the fact that *Propionibacteria* grown in the lactose-containing medium form β-galactosidase seems to indicate induction of this enzyme in *Propionibacteria*. In the glucose-containing medium the strains studied did not form galactosidase. The presence of traces of this enzyme was probably due to very small amounts of galactose present in the commercial casein hydrolysates used for the preparation of the culture media, which may have induced formation of some β-galactosidase.

The activity of alkaline phosphatase was found in three or two protein fractions; in the main peaks the activity per mg. protein was 40 to 50 times as high as in the crude protein extracts. Phosphatases from *P. shermanii*, *P. petersoni* and *P. freudenreichi* were located in peaks *III*, *IV* and *V*, the greatest amount being present in peak *IV*. Phosphatase from *P. arabinosum* was eluted by a solution of a different ionic strength in peaks *V* and *VI*, the majority being found in peak *V*.

The presence of the same enzyme in several protein fractions none of which could be further separated on rechromatography may be regarded as confirmation of heterogeneity of alkaline phosphatases. As regards *P. arabinosum*, the localization of phosphatase is another characteristic in respect to which this strain, known from microbiological studies to possess some distinctive properties, differs from other *Propionibacteria*.

## SUMMARY

A method was developed for separating the proteins of *Propionibacteria* on DEAE-cellulose column employing stepwise ionic strength gradient elution at constant pH. The proteins of four strains of *Propionibacteria* (*P. shermanii*, *P. petersoni*, *P. freudenreichi* and *P. arabinosum*) were separated each into nine fractions in which β-galactosidase and alkaline phosphatase activity were determined. β-Galactosidase activity which was not present in *Propionibacteria* grown on glucose media could be induced when the bacteria were grown on a lactose medium. The distribution of phosphatase in *P. arabinosum* was different from that in the remaining strains.

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## ROZDZIAŁ BIAŁEK BAKTERII KWASU PROPIONOWEGO NA CELULOZOWYCH WYMIENNIKACH JONOWYCH

Streszczenie

Opracowano metodę rozdziału białek bakterii kwasu propionowego na kolumnach z DEAE-celulozą, stosując elucję buforami o skokowo wzrastającej sile jonowej przy stałym pH. Białka szczepów *Propionibacterium shermanii*, *P. petersoni*, *P. freudenreichi* i *P. arabinosum* rozdzielono na 9 frakcji, w których oznaczono aktywność $\beta$-galaktozydazy i fosfatazy alkalicznej. $\beta$-Galaktozydaza nie występowała w szczepach hodowanych na pożywkach z glukozą, natomiast była indukowana podczas wzrostu bakterii na pożywkach zawierających laktozę. Rozmieszczenie na chromatogramie fosfatazy było u *P. arabinosum* inne niż u pozostałych szczepów.

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# Vi-PHAGE RECEPTOR PROPERTIES OF Vi-POLYSACCHARIDES ISOLATED BY DIFFERENT METHODS

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It often occurs that biologically active macromolecular substances isolated by different methods show considerable differences in their properties. As far as the Vi-antigen is concerned, which is a bacterial polysaccharide composed mainly of acetylated aminogalacturonic acid (2-amino-2-deoxy-D-galacturonic acid) [2], several isolation methods are known; moreover, several bacterial species of Enterobacteriaceae may serve as starting material. The most thoroughly hitherto examined are two Vi-polysaccharide preparations from *Escherichia coli*, one isolated by chemical fractionation by Webster *et al.* [11], and the other by continuous flow electrophoresis by Jarvis *et al.* [3]. The antigenic, immunogenic and some physical and chemical properties of these preparations were compared [5], but their activity as Vi-phage receptors was not studied. Our previous paper on the Vi-receptor from *Salmonella typhi* indicated that not every Vi-antigenic material possesses the receptor properties [8]. It seemed therefore interesting to examine the receptor properties of the preparations of Jarvis and Webster, and to compare them with the properties of a Vi-polysaccharide obtained from the same bacterial strain by a procedure worked out in this Laboratory.

## MATERIALS

Vi-polysaccharide preparation from *E. coli* 5396/38, purified by ethanol fractionation in the presence of high concentrations of sodium chloride and heating in 0.1 M-acetic acid [10], was kindly supplied by Dr. Marion E. Webster (National Heart Institute, Bethesda, U.S.A.) and Dr. M. Landy (National Cancer Institute, Bethesda, U.S.A.). This preparation is denoted as Vi-W, according to the established nomenclature [5].

Vi-polysaccharide preparation from *E. coli* 5396/38, purified by continuous flow electrophoresis [3], was kindly given by Dr. T. G. Jarvis (Department of Microbiology, Idaho University, Pocatello, U.S.A.). This

preparation is denoted as Vi-J, according to the established nomenclature [5].

Vi-polysaccharide preparation from *Salmonella typhi* 21802 was purified by chromatography on erythrocyte stroma in this Laboratory [8], and is denoted here as Vi-T typhi.

*Escherichia coli* 5396/38, kindly sent by Doc. Dr. T. Lachowicz from the Hygienic Laboratory of the Army, Kraków, Poland, was used for preparation of Vi-T by a procedure described in this paper.

Vi-phage II type A [1] was obtained from the International Reference Laboratory for Enteric Phage Typing, London, England.

Rabbit antiserum against *Paracolon ballerup* (serum anti-Vi) was prepared by the National Reference Laboratory for Enteric Phage Typing, Gdańsk, Poland, and preserved with 0.2% phenol.

*Reagents*: Kieselgel G according to Stahl (Merck, Darmstadt, West Germany); ninhydrin, crystalline bovine serum albumin, and Celite 535 (B.D.H., Poole, England); pancreatine (K and K Laboratories, New York, U.S.A., lot 18896 F); tubings for dialysis (Kalle, Wiesbaden, West Germany). Other reagents were products of Fabryka Odczynników Chemicznych (Gliwice, Poland).

## METHODS

Vi-receptor activity was estimated by the method previously described [10].

Precipitin test was carried out after Landy & Webster [6]. The protein in the precipitate was determined according to Lowry *et al.* [7], crystalline bovine serum albumin being used as standard.

Haemagglutinin test was carried out as described in the previous paper [8].

Immunoelectrophoresis was carried out as described previously [8] except that agar was prepared in 0.1 M-diethylbarbiturate buffer containing 5 mM-$MgCl_2$.

Specific viscosity was determined as described previously [8]. Ultraviolet absorption spectrum was examined as previously except that Spektromom 201 (Hungary) spectrophotometer was used.

Acetyl groups, acid polysaccharides and sugars were estimated by the methods given previously [8].

Chromatography of hydrolysates was performed in the following way. The Vi-preparation, 3 mg., was heated with conc. HCl in boiling water for 2 hr., diluted with 2 ml. of distilled water and decolorized with a small quantity of charcoal. Hydrochloric acid was eliminated by threefold distillation *in vacuo* with small quantities of water; the residue was left overnight over soda-lime. Two-dimensional thin-layer chromatography was carried out on plates (75 × 120 mm.) covered with Kieselgel G, dried for 2 hr. at 130°, 200 μg. of the hydrolysate being applied at a time.

The chromatograms were developed twice in the same direction in the system: *n*-butanol - acetic acid - water (4 : 1 : 5, by vol.), then once in the other direction in the system: phenol - water (4 : 1, v/v) with 0.1% cupron. The spots were located with 0.3% solution of ninhydrin in *n*-butanol acidified with acetic acid. One-dimensional thin-layer chromatography was carried out on plates (26 × 75 mm.), 100 μg. of the hydrolysate being applied at a time. The chromatograms were developed twice in the system: *n*-butanol - acetic acid - water (4 : 1 : 5, by vol.) or in the system: phenol - water (4 : 1, v/v) with 0.1% cupron. The spots were located with aniline oxalate.

## RESULTS

### *Isolation of Vi-polysaccharide from* E. coli *by chromatography on erythrocyte stroma*

*Preparation of acetone-dried bacteria.* The bacteria (*E. coli* 5396/38) were cultured on 2.5% agar medium on meat broth containing 1% of peptone (Mikrokolor, Poland) in Petri dishes, 20 cm. in diameter. Ten turbid bacterial colonies giving strong agglutination with anti-Vi serum were suspended in meat broth. After incubation at 30° for 1 hr., the suspension was plated on Petri dishes. After incubation at 30° for 20 hr., the bacteria were quickly washed off from the agar surface with 15 ml. water and the suspension was immediately poured into 3 vol. of acetone. After 1 hr. the supernatant was decanted and the bacteria were centrifuged at 2200 *g* for 3 min. The sediment was suspended in acetone and left overnight at 37°. During the next 3 days acetone was changed daily, and the bacterial material was subsequently air-dried. The yield from 25 Petri dishes (i.e. from 5 litres of agar medium) amounted to about 10 g. of acetone-dried material.

*Preparation of crude Vi-polysaccharide.* One part (weight) of acetone-dried bacteria was shaken with 100 parts of 0.9% NaCl solution for 30 min. at room temperature and centrifuged for 15 min. at 12 000 *g*. To the supernatant, 3 vol. of acetone were added. The precipitate was centrifuged for 3 min. at 2200 *g*, washed with acetone and dried. The yield of this preparation amounted to about 20% of the weight of dry bacteria. Examined in the haemagglutinin test with anti-Vi serum, it possessed about 20 times higher activity per weight unit than the corresponding material from *S. typhi* [8].

*Acetone precipitation at* pH 5 *and pancreatine digestion.* The crude preparation, 3 g., was suspended in 30 ml. of 0.01 N-acetic acid and was shaken for 1 hr. The yellowish fluid was adjusted to pH 5 with 1 N-acetic acid, mixed with 300 ml. of acetone, left for 1 hr. in the refrigerator and centrifuged at 2200 *g* for 15 min. The inactive supernatant was discarded and the sediment suspended in 170 ml. of PBS (phosphate-

-buffered saline: 0.80 g. NaCl, 0.22 g. KCl, 0.29 g. $Na_2HPO_4 \cdot 12H_2O$, 0.02 g. $KH_2PO_4$, 0.01 g. $CaCl_2$ and 0.01 g. $MgCl_2 \cdot 6H_2O$ per 100 ml.). Pancreatine solution (100 mg./25 ml. PBS) was added and the mixture was dialysed against 2 litres of PBS at 39° in a rotating bag, under toluene, PBS being changed every hour. After 4 hr. the mixture was dialysed overnight against running tap water, filtered to remove the toluene, adjusted to pH 3 with acetic acid and mixed with 3 vol. of ethanol, and with sodium acetate to 0.1% concentration. The mixture was kept for 1 hr. in the refrigerator, then the sediment was collected by centrifuging at 2200 *g* for 5 min., washed three times with 90% ethanol, dissolved in about 300 ml. of 0.1 M-NaCl and adjusted to pH 6.5 with a solution of sodium bicarbonate. If opalescent, the nearly colourless solution was centrifuged for 30 min. at 18 000 *g* and the slight sediment was discarded. The clear solution was completed to 1 litre with 0.1 M-NaCl and left in the refrigerator, ready for chromatography.

*Chromatography on erythrocyte stroma.* The column, filled with human erythrocyte stroma set on Celite 535 was prepared as described in the previous paper [8], with the exception that a larger column (7 × 13.5 cm.) containing 100 ml. of stroma and 200 g. of Celite was used.

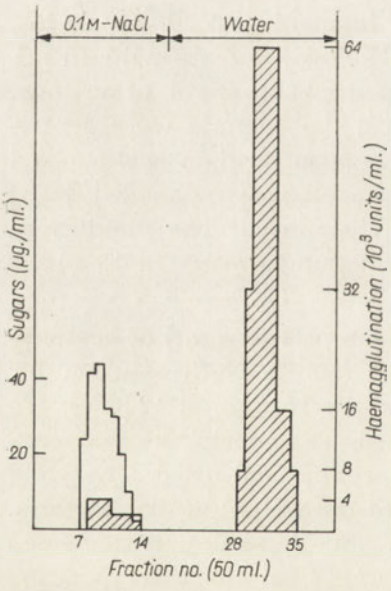


Fig. 1. Chromatography of the crude preparation of Vi-polysaccharide from *E. coli* on the erythrocyte stroma - Celite 535 column. (▩), Haemagglutination; (□), contents of sugars, estimated by the anthrone method.

The preparation, 200 ml., was introduced slowly into the column, followed by 20 ml. of 0.1 M-NaCl. The column was left overnight to permit the full adsorption of the Vi-polysaccharide and was then eluted with 0.1 M-NaCl; 50 ml. fractions of the effluent were collected, the flow-rate being adjusted to one fraction per 8 - 10 min. The effluent was tested for sugar by the anthrone method and for Vi-substance by haemagglutination with anti-Vi serum or by the turbidimetric method. It is

worth mentioning that Vi-polysaccharide is not detected by the anthrone test, hence sugars estimated by this test correspond to contaminations. The elution was continued until the effluent contained no sugar, then the column was eluted with bidistilled water (Fig. 1). The fractions containing the Vi-activity were pooled, dialysed overnight against distilled water and concentrated *in vacuo*. After addition of NaCl to obtain 0.1 M concentration, a slight quantity of the insoluble was removed by centrifugation for 30 min. at 18 000 *g*. This preparation did not contain sugars detected by the anthrone test, but still possessed a slight quantity of substances reacting with the Folin reagent in the method of Lowry *et al.* These substances were removed by subsequent rechromatography. The material obtained from two chromatographic runs, in the volume of 200 ml., was rechromatographed. The active fractions were pooled, dialysed overnight against distilled water, concentrated *in vacuo* to a volume of 20 ml. and mixed in the cold successively with 20 ml. of 6 M-formic acid, and 120 ml. of ethanol. As there was no precipitate at this stage, 200 ml. of ethyl ether were added in the cold, and the mixture left for 1 hr. in an ice bath. The precipitate formed after

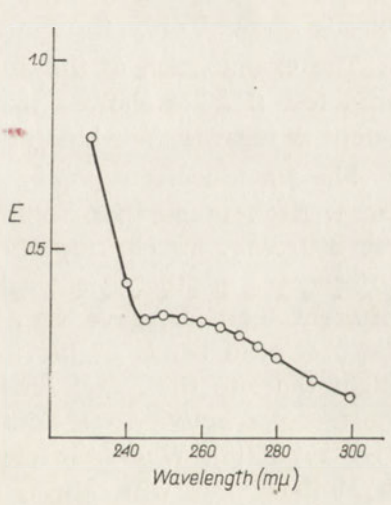


Fig. 2. UV-spectrum of the Vi-polysaccharide preparation from *E. coli* purified by chromatography on erythrocyte stroma (Vi-T). Conditions: 0.25% water solution, 1 cm. cell.

the addition of ethyl ether was spun down, dissolved in 20 ml. of water, dialysed against distilled water for 2 days, centrifuged for 30 min. at 12 000 *g* and lyophilized. This preparation was called Vi-T; its yield amounted to about 1% of the dry bacterial weight and was 20 times higher than that of Vi-T typhi [8].

### *Some properties of Vi-polysaccharide preparations*

Vi-T did not contain protein, sugars detectable by the anthrone test, nor contaminations derived from nucleic acids: it did not show any peak at 260 mμ (Fig. 2). The preparation possessed 19% of acetyl groups.

Its specific viscosity amounted to 0.48. The specific viscosities of Vi-W, Vi-J and Vi-T typhi amounted to 0.37, 1.04 and 0.24, respectively, in agreement with the data found in the literature [11, 3, 8].

All four preparations were subjected to hydrolysis with conc. HCl. Two-dimensional thin-layer chromatograms of the hydrolysates were sprayed with ninhydrin (Fig. 3). The most intensive spot on all chromatograms corresponded to aminogalacturonic acid, the main component of the preparations examined. In all chromatograms a distinct yellow spot corresponding to an unidentified substance was found. The chromatograms of Vi-J, Vi-T and Vi-T typhi hydrolysates were almost identical, while the Vi-W preparation contained a larger number of components. The chromatograms of hydrolysates obtained in milder conditions (4 N-HCl, 17 hr., 100°) contained all ninhydrin-positive spots shown in Fig. 3 except the unidentified yellow spot, which might represent an artifact due to the drastic hydrolysis conditions. The aminogalacturonic acid spot was much fainter than after hydrolysis with conc. HCl.

One-dimensional thin-layer chromatograms of the conc. HCl hydrolysates developed in the two solvent systems and sprayed with aniline oxalate showed only the aminogalacturonic acid spot.

The examination of the antigenic activity by the quantitative precipitin test (Fig. 5) showed that the four preparations are nearly equivalent as regards their binding of Vi-antibodies.

The immunoelectrophorograms of the preparations are shown in Fig. 4. Each preparation contained two fractions antigenically identical but differing in electrophoretic mobility, which may be related to differences in their molecular weight. Vi-J and Vi-T, isolated by different methods, gave very similar results. The strong inflection of the precipitin bands of the slower moving fraction of Vi-J and Vi-T might indicate their high molecular weight. It is worth to recall that the receptor activity was found only in the slower moving fraction of Vi-T typhi [8]. When the electrophoresis was performed as presented in Methods but with $MgCl_2$ omitted, the separation of the fractions was somewhat less distinct. The addition of sodium versenate to 5 mM concentration resulted in the trailing of the preparation during electrophoresis.

### *Vi-phage receptor properties of Vi-polysaccharides*

Till now, the ability to bind Vi-phage II was studied on the Vi-polysaccharide from *S. typhi*. In the present study, three Vi-preparations from *E. coli* (Vi-W, Vi-J and Vi-T) were examined and compared with a Vi-T typhi preparation. Although all four preparations had similar antigenic properties, they showed differences in receptor activity to Vi-

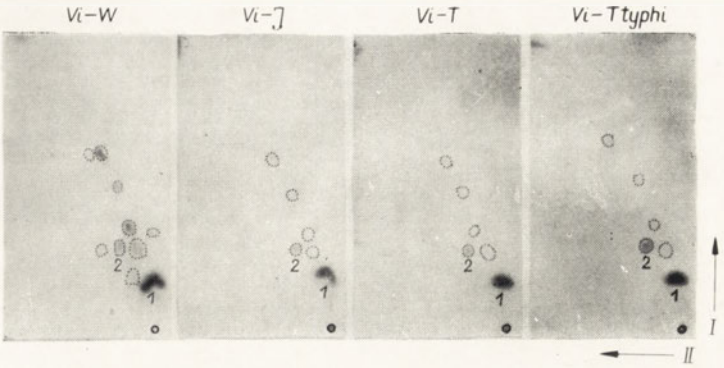


Fig. 3. Two-dimensional thin-layer chromatography of the hydrolysis products of Vi-polysaccharide preparations (conc. HCl, 2 hr., 100°). *I* direction: *n*-butanol - acetic acid - water (4 : 1 : 5, by vol.), twice; *II* direction: phenol - water (4 : 1, v/v) and 0.1% of cupron. The spots were located with ninhydrin. (*1*), Brown spot of aminogalacturonic acid; (*2*), yellow spot; other spots were violet.

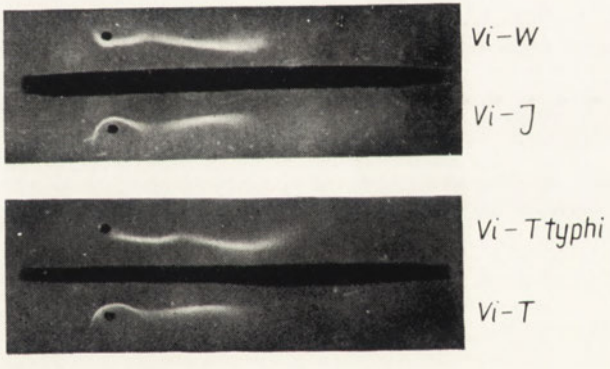


Fig. 4. Immunoelectrophorograms of Vi-polysaccharide preparations in agar gel, pH 8.6, developed with anti-Vi serum.

-phage II (Table 1). Vi-W exhibited no activity; Vi-T and Vi-J both possessed considerable receptor activity but it was markedly lower than in Vi-T typhi.

Table 1

*Receptor activity of Vi-polysaccharide preparations*

The figures represent mean values from two estimations. For conditions see text.

| Preparations | Receptor activity (units/mg.) |
|---|---|
| Vi-W | 0 |
| Vi-J | 185 |
| Vi-T | 270 |
| Vi-T typhi | 500 |

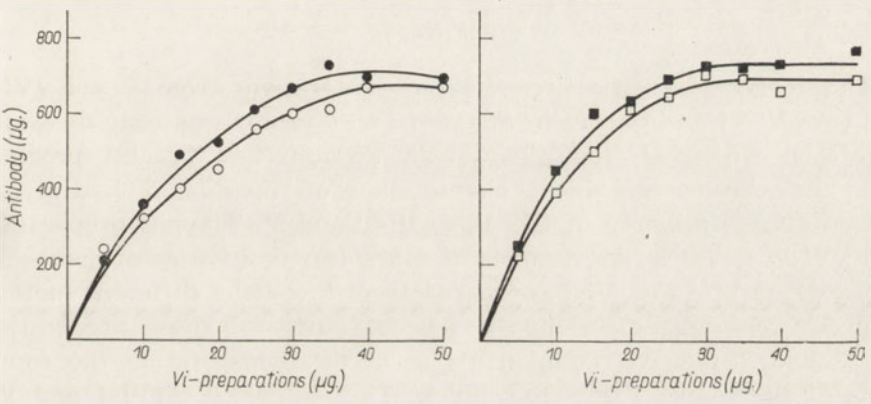


Fig. 5. Precipitation of Vi-polysaccharide preparations by the same anti-Vi serum. (■), Vi-W; (○), Vi-J; (●), Vi-T; (□), Vi-T typhi.

The Vi-polysaccharide isolated from *S. typhi* loses its receptor activity in the course of incubation with Vi-phage II, probably as the result of the phage enzyme action [4]. To find whether the Vi-receptor isolated from *E. coli* also undergoes this process, the preparations (100 receptor units/ml.) were incubated with Vi-phage II ($2 \times 10^{11}$ particles/ml.) in 0.1 M-ammonium acetate at 37°. Every 15 min. a sample was taken, heated for 2 min. at 100° to inactivate the phage, and the receptor activity was estimated. For the detailed description of the procedure see [9]. The results of these experiments (Fig. 6) indicate that the Vi--receptor from *E. coli* is also destroyed by Vi-phage II. The initial rates

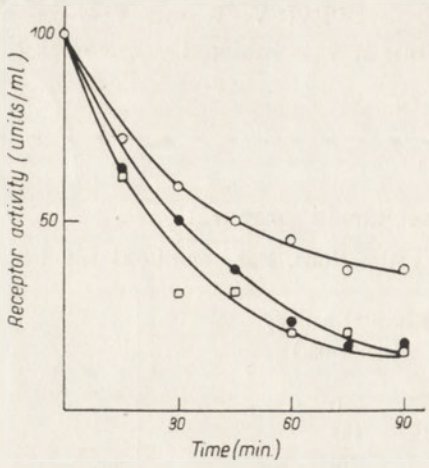


Fig. 6. The destruction of Vi-receptor activity of (○), Vi-J, (●), Vi-T; and (□), Vi-T typhi in the course of incubation with Vi-phage II ($2 \times 10^{11}$/ml.) at 37° in 0.1 M-ammonium acetate.

of the destruction of the Vi-receptor activity were different and amounted approximately to 40, 50 and 60 receptor units per ml. per 30 min. for Vi-J, Vi-T and Vi-T typhi, respectively.

## DISCUSSION

The degree of purification of three preparations from *E. coli* (Vi-W, Vi-J and Vi-T) and one from *S. typhi* (Vi-T typhi) was roughly similar as can be concluded from their antigenic activities in the precipitin test. The chromatographical examination of their acid hydrolysates showed great similarity of the Vi-J, Vi-T and Vi-T typhi preparations, while Vi-W had a larger number of ninhydrin-positive components. The similarity of Vi-J and Vi-T, prepared from *E. coli* by different methods, to Vi-T typhi suggests a high degree of purity of these preparations; the ninhydrin-positive components seem to correspond to the components of the native Vi-macrocomplex. The similarity of Vi-J and Vi-T is confirmed by the results of immunoelectrophoresis; on the other hand, the ultraviolet absorption spectrum shows that Vi-T in contrast to Vi-J [3], is not contaminated by nucleotides.

Webster *et al.* [12] examining the Vi-W preparation by free electrophoresis demonstrated its homogeneity; also the Vi-J preparation was found by Jarvis *et al.* [3], applying continuous flow electrophoresis, to be homogeneous. On the other hand, the same preparations when subjected to immunoelectrophoresis in agar-gel revealed the presence of two fractions each. This discrepancy requires further examination.

It is very difficult to ascertain the native state of Vi-T. This preparation cannot be compared with Vi-W because the latter was shown to undergo partial depolymerization and deacetylation during isolation [5], but it can be compared with Vi-J. However, Vi-J was obtained from bacteria growing in liquid medium while Vi-T was isolated from

bacteria growing on solid medium and this could have an effect on the polymerization degree. The acetyl group contents are difficult to compare owing to the different methods used for their estimation. The mild procedure of Vi-T isolation and the high receptor activity of this preparation may speak for its native state.

The results of receptor activity estimations deserve special attention. It has been shown that in spite of its high antigenic activity, Vi-W does not bind Vi-phage II at all. It seems that this is due to the isolation procedure. A drastic drop in receptor activity was observed in the course of heating with acetic acid [8] which constitutes an essential step of Vi-W isolation.

In further experiments it was found that the preparations from *E. coli* possessing the receptor activity (Vi-J and Vi-T) are losing this activity during incubation with Vi-phage II, similarly as Vi-T typhi. Somewhat different rates of destruction of the receptor activity of the preparations may be connected with their different viscosities, the lower rate corresponding to more viscous solutions. Should the destruction of receptor activity due to phage action be an enzymic reaction, which however is not yet proved, all the three active preparations could be regarded as substrates of the same phage enzyme.

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## SUMMARY

Highly purified Vi-polysaccharide was isolated from *Escherichia coli* 5396/38 by column chromatography on human erythrocyte stroma set on Celite. This preparation (Vi-T) was compared with two Vi-polysaccharide preparations from the same bacterial strain, one isolated by Webster *et al.* by chemical fractionation (Vi-W), the other isolated by Jarvis *et al.* by continuous flow electrophoresis (Vi-J). Vi-J and Vi-T possessed receptor activity in relation to Vi-phage II but Vi-W was inactive. Vi-phage II, when incubated with Vi-J or Vi-T, destroyed their receptor activity.

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## WŁASNOŚCI RECEPTOROWE WIELOCUKRÓW Vi IZOLOWANYCH RÓŻNYMI METODAMI

Streszczenie

Z bakterii *Escherichia coli* 5396/38 wyizolowano znacznie oczyszczony preparat wielocukru Vi metodą chromatografii na błonkach krwinek, osadzonych na celicie. Preparat ten (Vi-T) porównano z dwoma preparatami wielocukru Vi, otrzymanymi z tego samego szczepu bakterii, a mianowicie z preparatem otrzymanym metodą chemicznego frakcjonowania przez Webster i wsp. (Vi-W) i z preparatem otrzymanym metodą elektroforezy ciągłej przez Jarvisa i wsp. (Vi-J).

Preparaty Vi-J i Vi-T posiadały aktywność receptorową w stosunku do bakteriofaga Vi II, natomiast preparat Vi-W był tej aktywności pozbawiony. Bakteriofag Vi II inkubowany z preparatami Vi-J i Vi-T niszczył ich aktywność receptorową.

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# INTERACTION OF MACROCATIONS AND MACROANIONS WITH MITOCHONDRIA

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A number of investigators have reported an inhibitory effect of basic proteins, as protamine, histones, lysozyme, ribonuclease [17, 18], on the enzymes of mitochondrial respiratory chain as well as their uncoupling effect on oxidative phosphorylation [3, 19, 12]. In this laboratory a mitochondrial basic protein (MBP) has been isolated by Rzeczycki *et al.* [21] from hog kidney, and its inhibitory effect on electron transport was demonstrated [20]. Wolfe & McIlwain [23] have observed that in brain slices in the cold the migration of histones from nuclei to the subcellular particles occurs, and reported the inhibitory effect of histones [13, 23] and protamine [15] on electric excitability. As the excitability could be restored by polyacidic molecules or aggregates, e.g. gangliosides, McIlwain [15] assumed the bonding of protamine to the acidic components of the subcellular particles.

The inhibitory effect on electron transport and uncoupling of oxidative phosphorylation by basic proteins may be due to the binding of these macrocations to mitochondria. This could also influence mitochondrial swelling. Recently [4] we have demonstrated that mitochondria can bind basic proteins. This binding was overcome by ganglioside and high salt concentrations and seems therefore to be of an ionic character. In the present work, the effect of macrocations and macroanions on mitochondrial swelling was studied.

## MATERIALS AND METHODS

Wistar albino rats were starved for 24 hr., then killed by decapitation. Liver and kidney mitochondria were prepared in 0.25 M-sucrose - - 0.04 M-tris-HCl buffer, pH 7.4. Mitochondria were centrifuged between 800 and 7000 *g*, washed three times and resuspended in sucrose-tris solution.

Swelling of mitochondria was followed at room temperature by measuring the decrease in extinction at 520 mμ [6, 9], 1 cm. light-path

cuvettes and Unicam spectrophotometer being used. The following solutions were added to the cuvette: 10 - 100 µl. of substances inducing swelling; and 10 - 100 µl. of basic protein; the volume was made up to 2.9 ml. with 0.33 M-sucrose - 0.04 M-tris buffer, pH 7.4 [6] or 0.125 M-KCl - - 0.04 M-tris, pH 7.4 [9], then at zero time 0.1 ml. of suitably diluted mitochondria (about 500 µg. protein) was added. Initial extinction was about 0.6. The extinction was measured every 2 min. during 30 min. of incubation.

Binding of mitochondrial basic protein and mucin to mitochondria was determined as described previously [4].

Mitochondrial basic protein (MBP) was prepared from hog kidney as described previously [21], with a slight modification. To precipitate protein by tannin from the sulphosalicylic acid extract, tannin to protein ratio 20:1 was used instead of 2:1, because it appeared necessary to apply greater concentration of the tannin preparation (Friedrich August Thin, Germany) used in this work, to obtain satisfactory precipitation of protein.

Ganglioside was prepared from bovine brain as described by McIlwain [14]. Hydrolysis of the ganglioside was carried out in 0.1 N-$H_2SO_4$ at 80° for 1 hr., followed by dialysis [15]. The ganglioside preparation contained 26% of sialic acid before, and 7% after hydrolysis. Mucin was prepared from bovine submaxillary glands according to Nisizawa & Pigman [16]. The preparation contained 13% of sialic acid. Ribonuclease, lysozyme, and protamine sulphate were commercial products (B.D.H, England); ascorbic acid (Politechnika Śląska, Poland); L-malic acid (Light, England); succinic acid (Eisenach, Germany); phosphate-Na (Xenon, Poland); glutathione reduced (Biuro Obrotu Odczynnikami, Gliwice, Poland).

Protein in mitochondria was determined by the biuret method [8]. Sialic acid was determined with the orcinol reagent, using as standard *N*-acetylneuraminic acid prepared according to Martensson, Raal & Svennerholm [11] from serum proteins.

## RESULTS

To characterize the effect of basic proteins on mitochondrial swelling, we have introduced an index, $k_{20}$, calculated from the extinction values after 20 min. incubation, according to the equation:

$$k_{20} = \frac{E_{inh} - E_{ind}}{E_{sp} - E_{ind}}$$

where $E_{inh}$ is the extinction value for mitochondria incubated with both inducer and inhibitor of swelling; $E_{ind}$, the extinction value for mitochondria incubated with inducer only; and $E_{sp}$, the extinction value for

spontaneous swelling, the sample being incubated also for 20 min. The extinction values after 20 min. incubation were corrected for zero-time difference in extinction in relation to control. For instance, for calculation of $k_{20}$ for the mitochondrial basic protein presented in Fig. 1a, the respective extinction values were: $E_{inh} = 0.52 - 0.06$ (correction) $= 0.46$; $E_{ind} = 0.31 + 0.01 = 0.32$; $E_{sp} = 0.56$. Hence $k_{20} = \frac{0.46 - 0.32}{0.56 - 0.32} = 0.58$, approximately 0.6. When the value for $k_{20}$ is 0, this indicates lack of inhibitory or stimulating effect on induced mitochondrial swelling: $k_{20} < 0$ indicates enhanced swelling of mitochondria; $1 > k_{20} > 0$ indicates inhibitory effect on induced swelling; $k_{20} > 1$ indicates inhibition not only of induced but also of spontaneous swelling.

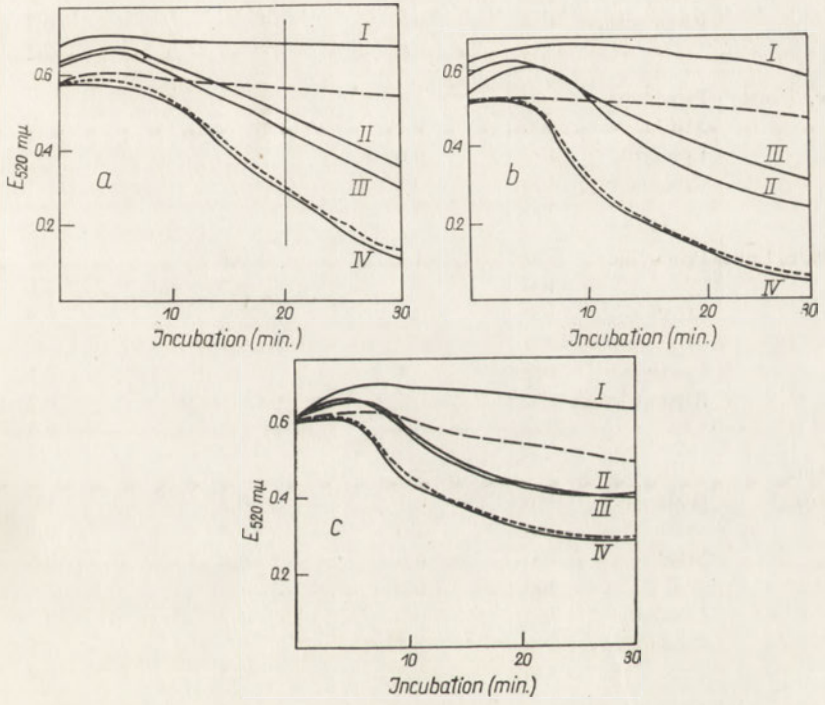


Fig. 1. Effect of basic proteins on the swelling of rat liver mitochondria induced by: (*a*), 1 mM-ascorbate; (*b*), 10 mM reduced glutathione; (*c*), 1 mM-succinate. Medium: 0.33 M-sucrose - 0.04 M-tris buffer, pH 7.4. (— — —), Spontaneous swelling; (- - -), swelling agent alone; (———), with the addition of: (*I*), 40 μg. protamine sulphate; (*II*), 100 μg. mitochondrial basic protein; (*III*), 100 μg. lysozyme; (*IV*), ribonuclease, 100 μg. in experiment *a*, and 300 μg. in experiments *b* and *c*.

The effects of basic proteins on swelling of rat liver mitochondria induced by ascorbate, reduced glutathione and succinate, are illustrated in Fig. 1. Table 1 gives the $k_{20}$ values both for the liver and kidney mitochondria, which ranged from $-1.2$ to $+3.1$. The values varied from

Table 1

*Effect of basic proteins on induced swelling of rat liver and kidney mitochondria*

Figures represent values for index $k_{20}$ characterizing the effect of basic proteins on induced mitochondrial swelling after 20 min. incubation (for details see text).

| Swelling agent | Basic protein (μg.) | | Liver mitochondria | | Kindey mitochondria |
|---|---|---|---|---|---|
| | | | in 0.33 M-sucrose | in 0.125 M-KCl | in 0.33 M-sucrose |
| Succinate, 1 mM | Protamine | 40 | 1.8 | 0.5 | |
| | | 100 | | 1.0 | 3.1 |
| | MBP | 100 | 0.4 | 0.0 | 0.9 |
| | Lysozyme | 100 | 0.4 | | |
| | Ribonuclease | 100 | 0.0 | | 0.7 |
| | | 300 | 0.0 | | —0.2 |
| Malate, 1 mM | Protamine | 40 | 1.4 | | |
| | MBP | 100 | 0.1 | | |
| | Lysozyme | 100 | 0.6 | | |
| | Ribonuclease | 100 | —0.1 | | |
| | | 300 | —0.2 | | |
| Ascorbate, 1 mM | Protamine | 40 | 1.1 | 0.5 | |
| | | 100 | | 0.7 | 2.0 |
| | MBP | 100 | 0.6 | | 0.9 |
| | | 200 | | 0.3 | |
| | Lysozyme | 100 | 0.4 | | 0.5 |
| | Ribonuclease | 100 | —0.3 | | 0.5 |
| | | 200 | | | 0.3 |
| Phosphate, 16 mM | Protamine | 40 | 1.1 | 0.5 | 1.0 |
| | | 100 | 1.0 | 0.7 | 0.9 |
| | MBP | 100 | 0.0 | —0.6 | 0.8 |
| | | 200 | 0.0 | | |
| | Lysozyme | 100 | 0.0 | —1.2 | 0.7 |
| | Ribonuclease | 100 | —0.4 | | |
| | | 300 | —0.5 | —0.2 | 0.2 |
| Glutathione reduced, 10 mM | Protamine | 100 | 1.2 | | |
| | MBP | 100 | 0.5 | | |
| | Lysozyme | 100 | 0.7 | | |
| | Ribonuclease | 100 | 0.0 | | |
| | | 300 | 0.0 | | |

one experiment to another but they gave a rough idea of the effect produced by basic proteins. The greatest inhibitory effect on induced swelling in all experiments was exerted by protamine, next by MBP and lysozyme; ribonuclease either had no effect at all or even stimulated

the swelling. Comparison of values obtained from parallel experiments carried out in 0.33 M-sucrose and 0.125 M-KCl media showed that in ionic medium the inhibition of swelling by basic proteins was much smaller. In sucrose medium there was an increase in extinction at zero time, especially when mitochondria were added with protamine or MBP (Fig. 1a). This was not observed or observed to a lesser degree in ionic medium.

The effect of ganglioside on the inhibition by MBP and protamine of spontaneous swelling is presented in Fig. 2; no inhibition of swelling was observed when the ganglioside was added together with the protein (curves *IV* and *V*) and a reversal of inhibition occurred when it was added after 4 min. of incubation of mitochondria with protamine (curve *III*). It was also observed that ganglioside as well as mucin stimulated swelling (Fig. 3). When the greatest part of sialic acid present

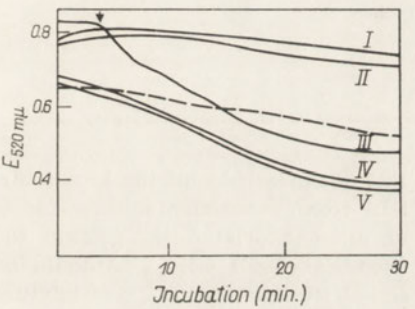


Fig. 2

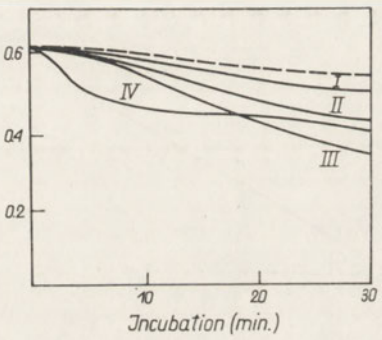


Fig. 3

Fig. 2. Effect of ganglioside on the basic protein-produced inhibition of spontaneous swelling of rat liver mitochondria. Medium: 0.33 M-sucrose - 0.04 M-tris buffer, pH 7.4. (— — —), Spontaneous swelling; (*I*), 40 μg. protamine sulphate; (*II*), 100 μg. mitochondrial basic protein; (*III*), 40 μg. protamine sulphate and 200 μg. ganglioside added at the fourth minute (indicated with arrow); (*IV*), 40 μg. protamine sulphate and 200 μg. ganglioside added at zero time; (*V*), 100 μg. mitochondrial basic protein and 300 μg. ganglioside added at zero time.

Fig. 3. Effect of ganglioside and mucin on swelling of rat liver mitochondria. Medium: 0.33 M-sucrose - 0.04 M-tris buffer, pH 7.4. (— — —), Spontaneous swelling; (*I*), 100 μg. ganglioside; (*II*), 200 μg. ganglioside; (*III*), 400 μg. ganglioside; (*IV*), 200 μg. mucin.

in the ganglioside was removed by hydrolysis in 0.1 N-$H_2SO_4$ at 80° for 1 hr. and dialysis, the ganglioside became unable to overcome the inhibition produced by basic proteins (Fig. 4). This indicated that the reversal was due to the presence of acidic compounds, i.e. sialic acid in a ganglioside.

The experiments reported above suggest that binding of basic proteins to mitochondria was responsible for their effects on swelling. The reversal of these effects by macroanions can be explained by their interaction with basic proteins bound to mitochondria. The results of experi-

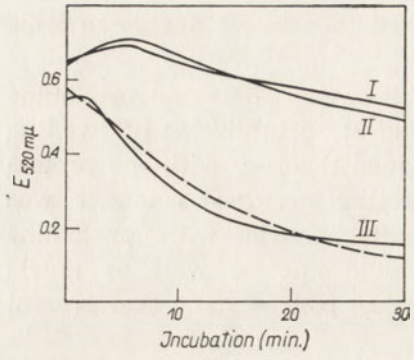


Fig. 4. Effect of native and hydrolysed ganglioside on the inhibition by protamine of spontaneous swelling of rat liver mitochondria. Medium: 0.33 M-sucrose - 0.04 M-tris buffer, pH 7.4. (— — —), Spontaneous swelling; (———), 50 μg. protamine sulphate: (*I*), alone: (*II*), with 200 μg. hydrolysed ganglioside (7% sialic acid); (*III*), with 200 μg. native ganglioside (26% sialic acid).

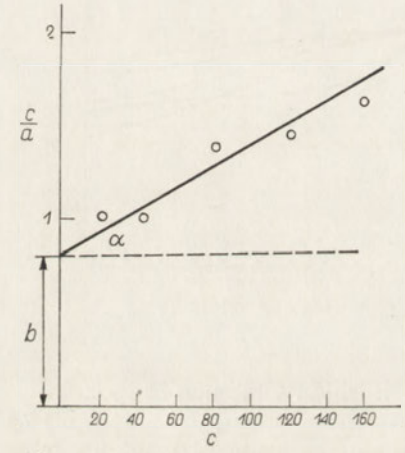


Fig. 5. Linear plot of the Langmuir equation from experimental data for binding of mitochondrial basic protein to mitochondria at pH 8.0. (*c*), Amount of MBP added to the incubation mixture; (*a*), amount of MBP bound to mitochondria; tg$\alpha$ equals $\frac{1}{a_0}$ and the segment b is $\frac{B}{a_0}$. For details see text.

ments on the binding of MBP to mitochondria were in agreement with the adsorption equation given by Langmuir [7]. The theoretical Langmuir plot was calculated for MBP adsorbed at pH 8 from the equation:

$$a = a_0 \frac{c}{B - c}$$

where $a$ is the amount of bound MBP; $c$, the amount of MBP added to the incubation mixture. Coefficients $a_0$ and $B$ were calculated graphically using linear plot for the transformed equation:

$$\frac{c}{a} = \frac{B}{a_0} + \frac{c}{a_0}$$

Fig. 5 presents this plot obtained from experimental data for binding of MBP to mitochondria. The values for coefficients $a_0$ and $B$ were 172 and 141, respectively. Varying hydrogen ion concentrations had but

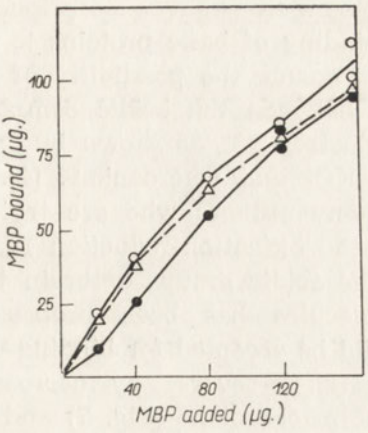


Fig. 6

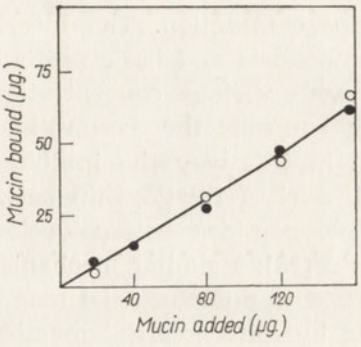


Fig. 7

Fig. 6. Binding of mitochondrial basic protein to rat liver mitochondria (●), at pH 6.0 and (○), at pH 8.0; (△), theoretical curve calculated from the Langmuir equation [7].

Fig. 7. Binding of mucin to rat liver mitochondria (●), at pH 6.0 and (○), at pH 8.0.

a slight effect on the shape of the curve (Fig. 6). As it has been observed that ganglioside and mucin stimulate mitochondrial swelling, binding of mucin to mitochondria was examined. The results of experiments shown in Fig. 7 indicate that mucin is also bound to mitochondria although to a smaller extent than MBP.

## DISCUSSION

The mitochondrial membrane which consists (according to Green & Fleischer [2]) of phospholipids surrounded by structural protein, possesses both negative and positive charged groups and can bind acidic as well as basic macroions. Changes in the structure of the mitochondrial membrane affect the metabolic processes of mitochondria, and the inhibitory effect on oxidoreduction processes and uncoupling of oxidative phosphorylation by basic proteins are generally accepted [17, 18, 19, 12]. According to some authors, there is a close connection between the oxidoreduction and mitochondrial swelling, as KCN, Antimycin A, and Amytal inhibit both electron transport and swelling. On the other hand, uncoupling agents such as thyroxine, calcium ions or dinitrophenol (100 μM) stimulate swelling [5]. Thus it seems very likely that the effect of basic proteins on oxidoreduction processes in mitochondria may find its reflection in the swelling too. This latter effect was observed in our experiments. The initial increase in absorbancy at 520 mμ observed when the mitochondria were incubated with basic proteins, may be explained either by rapid contraction or, what is more probable, by binding of basic

proteins to mitochondria which changes their light absorption. Our earlier experiments [4] indicated that binding of basic proteins to mitochondria does occur, but they did not exclude the possibility of mitochondrial contraction. The interaction between the acidic components of mitochondria and basic proteins is electrostatic, as shown by experiments with various concentrations of KCl and macroanions [4]. This binding supports the assumption of many authors who are trying to explain in this way the inhibition of the oxidation-reduction reaction [10, 22]. Such a relationship between the acidic groups of brain tissue, basic polypeptides and polyacidic molecules has been proposed by McIlwain [15]; a similar mechanism could be accepted for binding of basic protein to mitochondrial membrane and its reversal by macroanions. Binding by mitochondrial membrane of macroanions (Fig. 7) and their stimulating effect on swelling (Fig. 3) also suggest the possibility of acidic macroions influencing the mitochondrial metabolism. It seems very probable that basic protein naturally occurring in mitochondria [21] may be bound with the negative charged groups present in mitochondria.

## SUMMARY

It was shown that basic proteins inhibit both induced and spontaneous swelling of mitochondria. This effect can be overcome by the addition of macroanionic compounds, as ganglioside and mucin, which, when added alone, promote mitochondrial swelling. The effect of basic proteins is due to their binding to mitochondria.

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## WPŁYW MAKROKATIONÓW I MAKROANIONÓW NA MITOCHONDRIA

Streszczenie

Wykazano hamujący wpływ białek zasadowych na samoistne oraz indukowane pęcznienie mitochondriów. Efekt ten daje się usunąć przez dodatek makroanionów jak gangliozydu lub mucyny. Gangliozyd i mucyna same stymulują pęcznienie.

Wpływ białek zasadowych spowodowany jest wiązaniem ich do mitochondriów.

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# CHANGES IN CHEMICAL COMPOSITION OF ISOLATED RAT BRAIN AND LIVER NUCLEI

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Several metabolic reactions have been observed in nuclei isolated from calf thymus, e.g. incorporation of labelled amino acids into protein [1, 2, 5] and of orotic acid and adenine into nuclear RNA [3, 5, 9]. The same reactions were found to occur in isolated rat liver [17] and kidney [16] nuclei.

In our earlier experiments concerning the metabolism of RNA in the central nervous system, a rather rapid turnover of RNA in brain nuclei has been observed [6, 7, 13]. In the present work, chemical changes occurring in nuclei *in vitro* were studied.

## MATERIAL AND METHODS

For the experiments, adult albino rats of the Wistar strain were used. For each experiment 10 animals were decapitated and the brains and livers removed. The brains were homogenized in a Potter homogenizer in 1.5 M-sucrose - 5 mM-$CaCl_2$ solution and the nuclei sedimented by centrifugation at 20 000 *g* for 60 min. The details of the procedure have been described previously [15, 6]. Liver nuclei were prepared in 2.2 M-sucrose solution according to the method of Chauveau *et al.* [10]. The obtained nuclei preparations were examined with a phase-contrast microscope. Consumption of oxygen was measured in the Warburg apparatus.

The isolated nuclei were incubated at 37° in isotonic sucrose solution buffered with tris [12] or phosphate [4, 5]. In experiments in which the nuclei were labelled *in vivo*, media with phosphate or tris were used; in experiments on the incorporation of $^{32}P$ *in vitro*, only the tris-containing medium was used. The incubation mixture *A* containing phosphate buffer, pH 7.4, was composed of: 1.0 ml. 0.1 M-phosphate - 0.25 M-sucrose; 0.8 ml. 0.1 M-glucose containing 85.7 μmoles NaCl and 21 μmoles $MgCl_2$; 0.2 ml. 2.5 mM-ATP-Na or 0.2 ml. $H_2O$; 2.0 ml. nuclei suspension in 0.25 M-sucrose containing 10 μmoles $CaCl_2$. The

incubation mixture *B* containing tris buffer, pH 7.4, was composed of: 0.7 ml. 0.2 M-tris - 0.25 M-sucrose; 0.6 ml. 0.1 M-glucose containing 64.2 μmoles NaCl and 15.8 μmoles $MgCl_2$, or 0.6 ml. glucose containing 134 μmoles NaCl; 0.1 ml. [$^{32}$P]$Na_2HPO_4$ (30 μc) or 0.1 ml. 1.5% SDS; 1.6 ml. nuclei suspension in 0.25 M-sucrose containing 10 μmoles $CaCl_2$ or 160 μmoles EDTA. RNA, DNA, free nucleotides and phospholipids were assayed before and after incubation by the methods described previously [14, 8, 6]. The dry weight of the residue remaining after extraction of lipids and acid-soluble compounds, was taken as protein. When the medium was also to be analysed, the suspension of nuclei was divided into two equal parts; one was added to the incubation mixture and incubated for 1 hr., the other was immediately centrifuged at 0° and the obtained sediment and supernatant were analysed, giving the zero--time values. Radioactivity was assayed in a layer of wet preparation by a Geiger-Müller counter with a mica window (1.5 mg./cm.$^2$) from a distance of 5 mm.

For *in vivo* $^{32}$P-labelling, rats were injected with [$^{32}$P]$NaH_2PO_4$, the dose of 10 μc per 100 g. body wt. being administered directly into the fourth ventricle of the brain; in some experiments an additional dose of 150 μc $^{32}$P was administered intraperitoneally. After 24 hr. the animals were killed and the nuclei isolated.

## RESULTS

In isolated brain and liver nuclei after incubation in isotonic sucrose solution at 37° for 4 hr., the content of RNA decreased by 30 - 50%, that of protein by 25 - 30%, while the content of free nucleotides increased. Microscopic examinations showed that the nuclei were coagulated to a marked degree but their internal structure was intact.

Changes in brain and liver nuclear RNA labelled *in vivo* with radioactive phosphorus are shown in Table 1. After 1 hr., as well as after 4 hr. of incubation, a marked decrease of RNA content was observed while changes in phospholipids and free nucleotides were but slight. The presence of ATP and the composition of the medium had almost no effect. Similarly, the removal of divalent ions from the medium by using EDTA was without effect. The addition of EDTA caused, however, a decrease of the oxygen consumption quotient from 1.95 to 0.4 μl.$O_2$/hr./ /mg. protein for brain nuclei and from 2.7 to 0.8 μl.$O_2$/hr./mg. protein for liver nuclei.

In all experiments in which the nuclei were incubated *in vitro* with radioactive phosphate, $^{32}$P was incorporated into RNA in spite of the decrease of RNA content in the nuclei. When $^{32}$P was administered *in vivo*, the specific activity of nuclear RNA decreased during incubation, while that of free nucleotides was unchanged. The specific activity

of brain nuclear RNA was higher than the activity of liver nuclear RNA probably as the result of the intraventricular administration of $^{32}P$.

In the second series of experiments, both the nuclei and the incubation media were analysed (Table 2). For the *in vivo* RNA labelling, $^{32}P$ was administered both intraperitoneally and into the fourth ventricle of the brain. In this experiment the effect of sodium dodecyl sulphate (SDS), known as a nuclease inhibitor [11], was also studied. After 1 hr. of incubation, the content of RNA in brain and liver nuclei markedly decreased while the decrease of free nucleotides was but slight. The specific activity of the RNA released from the nuclei into the medium was lower than that of RNA remaining in the nuclei, while the specific activity of free nucleotides in the medium increased in experiments with the brain but not with the liver. Microscopic examination of the nuclei after incubation with SDS showed that some of them underwent morphological changes. Incubation in the presence of SDS caused the destruction of a part of the nuclei, as shown by the presence of DNA in the incubation medium.

## DISCUSSION

The experiments on the chemical composition of nuclei isolated from brain and liver indicated that the nuclei, while retaining their morphological structure, lost during incubation 30 - 50% of the initial RNA content. This loss was not due to destruction of the nuclei since incubation did not cause a decrease in nuclear DNA. On the contrary, the amount of DNA found in the nuclei after incubation was consistently somewhat higher; this seems to be due to the conditions of incubation enhancing the extractability of DNA.

The experiments of Scholtissek *et al.* [20, 19] and of Samarina & Zbarskij [18] indicate that under certain conditions the metabolically active fractions may be released from the nuclei. This suggestion seems to be supported by the decrease in specific activity of nuclear RNA labelled *in vivo*, observed in the presented experiments. Still, the analysis of the nuclei and of the incubation media offered no confirmation to this view; the specific activity of the RNA released from the nuclei was found to be not higher, but even lower than that of the RNA remaining in the nuclei.

The decrease during incubation of the sum of RNA found in the nuclei and in the medium seems to indicate partial depolymerization of RNA; this was also suggested by the increased concentration in the medium of free nucleotides the specific activity of which was in the liver nearly the same as, and in the brain higher than, that of the nucleotides remaining in the nuclei.

Although during incubation marked decomposition of nuclear RNA was observed which was not overcome by ATP addition, $^{32}P$ was incor-

Table 1

*Effect of the incubation medium on the chemical composition of isolated brain and liver nuclei*

*A*, sucrose - phosphate medium; *B*, sucrose - tris medium; for details see Methods.

| Labelling | Medium | Time (hr.) | DNA (μg. P) | Dry wt. (mg.) | RNA | | Free nucleotides | | Phospholipids | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | (μg. P) | (counts/min./ μg. P) | (μg. P) | (counts/min./ μg. P) | (μg. P) | (counts/min./ μg. P) |
| | | | | | **Brain nuclei** | | | | | |
| *In vivo* | *A*, with ATP | 0 | 127.5 | 11.5 | 26.4 | 42 | 12.7 | 53 | 143.5 | 32 |
| | | 4 | 149.5 | 10.0 | 19.7 | 27 | 16.6 | 53 | 135.0 | 29 |
| | *A*, without ATP | 0 | 133.0 | 13.0 | 34.5 | 96 | 9.6 | 120 | 180.0 | 75 |
| | | 4 | 146.0 | 10.7 | 26.3 | 67 | 11.3 | 117 | 166.0 | 91 |
| *In vitro* | *B*, with $CaCl_2$ | 0 | 277.0 | 24.6 | 58.5 | — | 16.0 | — | 305.0 | — |
| | | 1 | 290.0 | 21.2 | 42.0 | 200 | 11.3 | — | 290.0 | 11.5 |
| | *B*, with EDTA | 0 | 111.5 | 12.0 | 25.7 | — | 12.7 | — | 109.5 | — |
| | | 1 | 124.0 | 11.8 | 14.3 | 130 | 11.8 | — | 90.9 | 10 |
| | | | | | **Liver nuclei** | | | | | |
| *In vivo* | *A*, with ATP | 0 | 188.0 | 20.5 | 80.0 | 6 | 46.2 | 6 | 84.0 | 4 |
| | | 4 | 188.5 | 17.0 | 53.0 | 4 | 43.5 | 6 | 85.3 | 4 |
| | *A*, without ATP | 0 | 245.0 | 21.0 | 85.5 | 8 | 15.0 | 8 | 99.0 | 5 |
| | | 4 | 299.0 | 17.4 | 59.3 | 8 | 31.4 | 8 | 90.3 | 5 |
| *In vitro* | *B*, with $CaCl_2$ | 0 | 540.0 | 30.0 | 99.0 | 0 | 15.3 | — | 99.3 | 0 |
| | | 1 | 580.0 | 25.3 | 73.0 | 70 | 12.4 | — | 100.0 | 18 |
| | *B*, with EDTA | 0 | 593.0 | 34.0 | 128.5 | 0 | 28.0 | — | 126.0 | 0 |
| | | 1 | 583.0 | 24.6 | 59.6 | 81 | 38.0 | — | 90.4 | 13 |

## Table 2

*Effect of sodium dodecyl sulphate on the chemical composition of brain and liver nuclei and of incubation media*

Sucrose - tris medium (*B*) was used; for details see Methods.

| Labelling | Medium | Time (hr.) | Analysed material | DNA (μg. P) | Dry wt. (mg.) | RNA (μg. P) | RNA (counts/min./μg. P) | Free nucleotides (μg. P) | Free nucleotides (counts/min./μg. P) | Phospholipids (μg. P) | Phospholipids (counts/min./μg. P) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **Brain nuclei** | | | | | |
| *In vivo* | *B*, with SDS | 0 | nuclei | 268.0 | 24.0 | 47.0 | 185 | 9.4 | 329 | 234.0 | 151 |
| | | | medium | — | — | 6.6 | 70 | 17.4 | 353 | 5.8 | 200 |
| | | 1 | nuclei | 272.0 | 20.0 | 19.3 | 129 | 8.9 | 285 | 245.0 | 134 |
| | | | medium | 4.7 | — | 18.7 | 92 | 15.0 | 636 | 9.2 | 191 |
| *In vitro* | *B*, without SDS | 0 | nuclei | 465.0 | 34.5 | 85.0 | — | 16.0 | — | 480.0 | — |
| | | | medium | — | — | 7.0 | — | 2.9 | — | 3.1 | — |
| | | 1 | nuclei | 480.0 | 29.0 | 49.2 | 353 | 11.3 | — | 470.0 | 20 |
| | | | medium | — | — | 5.0 | 4 | 27.7 | — | 5.8 | — |
| | | | | | | **Liver nuclei** | | | | | |
| *In vivo* | *B*, with SDS | 0 | nuclei | 540.0 | 22.7 | 104.0 | 144 | 13.2 | 120 | 123.5 | 186 |
| | | | medium | 0 | — | 8.6 | 40 | 10.3 | 213 | 1.3 | 170 |
| | | 1 | nuclei | 460.0 | 17.3 | 43.2 | 131 | 12.2 | 283 | 138.0 | 136 |
| | | | medium | 43.8 | — | 34.9 | 80 | 51.1 | 210 | 5.8 | 207 |
| *In vitro* | *B*, without SDS | 0 | nuclei | 622.0 | 30.0 | 99.0 | — | 15.3 | — | 99.0 | — |
| | | | medium | 0 | — | 3.3 | — | 4.7 | — | 4.5 | — |
| | | 1 | nuclei | 691.0 | 25.3 | 71.0 | 240 | 12.4 | — | 100.0 | 8.4 |
| | | | medium | 0 | — | 6.2 | 0 | 44.2 | — | 4.4 | — |

porated simultaneously from the incubation medium into nuclear RNA. At the same time, incorporation of $^{32}P$ into phospholipids was but slight.

The removal from the incubation medium of dilavent ions inhibited neither RNA decomposition nor the incorporation of $^{32}P$ into the RNA remaining in the nuclei; it resulted only in decreased oxygen consumption.

The addition of SDS, an inhibitor of pancreatic ribonuclease [12], did not inhibit nuclease activity in liver nuclei as indicated by the increase in the content of free nucleotides in the medium. In brain nuclei no increase of free nucleotides was observed which might suggest a different mode of action of SDS on brain nuclear nuclease.

Both brain and liver nuclear membranes were damaged by SDS, as shown by the release into the medium of nearly 50% of nuclear RNA and of small quantities of DNA.

The presented experiments demonstrate the difficulties encountered in studying *in vitro* the synthesis of nuclear RNA. In the nuclei isolated both from brain and from liver, during incubation in isotonic sucrose solution the catabolic processes were the prevailing ones, leading to the destruction of nuclear RNA.

SUMMARY

Isolated rat brain and liver nuclei were incubated in a glucose-containing sucrose medium. During incubation, added $^{32}P$ was incorporated into nuclear RNA although the content of RNA decreased by about 50% and an increase of nucleotides occurred.

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## ZMIANY W SKŁADZIE CHEMICZNYM IZOLOWANYCH JĄDER KOMÓRKOWYCH Z MÓZGU I WĄTROBY SZCZURA

Streszczenie

Izolowane jądra komórek mózgowych i wątrobowych inkubowano w izotonicznym roztworze sacharozy z dodatkiem glukozy. Podczas inkubacji dodany $^{32}P$ był włączany do RNA jądrowego nawet wówczas, gdy jednocześnie stwierdzano ubytek RNA jądrowego o około 50% oraz wzrost zawartości wolnych nukleotydów.

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# UTILIZATION OF CARBON-BOUND PHOSPHORUS BY MICROORGANISMS

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The natural occurrence [3, 5] of 2-aminoethylphosphonic acid ($H_2N$—$CH_2$—$CH_2$—$PO_3H_2$), the phosphorus analogue of taurine and $\beta$-alanine, indicates that enzymes synthesizing phosphorus-carbon (P—C) bonds are present in some at least living organisms. In fact, Kanoatsu & Horiguchi [4] and Rosenberg [7] demonstrated recently that 2-aminoethylphosphonic acid is synthesized from inorganic phosphate by some protozoa, but the nature of this process is still obscure. The possible pathways of P—C bond decomposition are also unknown.

Zeleznick, Myers & Titchener [9] showed that *Escherichia coli* Crookes strain can grow in a medium containing methyl- or ethylphosphonate as sole sources of phosphorus. It seems probable that enzymes splitting P—C bonds chemically resistant to cleavage, may be present in other microorganisms. In the present work this possibility was studied and an attempt was made to find organisms giving, when grown on substrates with carbon-bound phosphorus, a large yield of cells and thus better suited for more detailed biochemical studies.

## MATERIALS AND METHODS

*Potassium ethylphosphonate* ($CH_3CH_2$—$PO_3HK$). Crude ethylphosphonic acid prepared after Kosolapoff [6] was neutralized with one equivalent of potassium hydroxide and the resulting potassium salt was crystallized from ethyl alcohol. Several crystallizations were necessary until the product was free from inorganic phosphate according to the Fiske & Subbarow test.

*Dilithium-2,3-dihydroxypropylphosphonate* ($CH_2OH$—$CHOH$—$CH_2$—$PO_3Li_2$). This was prepared after Rosenthal & Geyer [8] and purified until

free from inorganic phosphate by repeated precipitation with alcohol from aqueous solution.

*Organisms. Escherichia freundi* was isolated from sewage. Other organisms were obtained from the following Departments of the Institute of Immunology and Experimental Therapy: *Escherichia coli* 306, 307 and 18, and *Proteus* from the Department of Genetics; *Bacillus subtilis, Nocardia brasiliensis, Streptomyces globisporus* and *Streptomyces globisporus* var. *flavofuscus*, from the Department of Antibiotics, and *Mycobacterium phlei* from the Department of Mycology.

*Media.* The growth of microorganisms was studied in synthetic media as simple as possible in order to keep contamination by inorganic phosphate below the level of interference. Four kinds of media were used: (1), Simple medium of Zeleznick *et al.* [9] with inorganic phosphate: $NH_4Cl$, 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; NaCl, 0.5 g.; $CH_3COOH$, 2.2 ml.; $K_2HPO_4$, 6.0 g.; $KH_2PO_4$, 3.0 g.; water 1000 ml.; pH 7.4. (2), Simple medium with phosphonates; the composition of this medium was the same as above, except that 9.1 g. of potassium ethylphosphonate or 1.0 g. of dilithium-2,3-dihydroxypropylphosphonate were added instead of inorganic phosphates. (3), Synthetic medium of Sauton: citric acid, 2.0 g.; $K_2HPO_4$, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; ammonio-ferric citrate, 0.05 g.; asparagine, 4.0 g.; glycerol, 10 ml.; water, 1000 ml.; pH 7.0. (4), Sauton medium with phosphonates: instead of inorganic phosphate, 0.42 g. of potassium ethylphosphonate or 0.48 g. dilithium-2,3-dihydroxypropylphosphonate were added per 1000 ml. of medium; pH 7.0. The pH value of the media was adjusted with 0.5 M-KOH or 0.5 M-HCl.

*Transfers.* Organisms were transferred to fresh media every three days with the exception of the slower growing *M. phlei* which was transferred to fresh medium after every seven days of growth. The size of inoculum was 0.1 ml. of suspension (density of $10^9$ bacterial cells per 1 ml. according to McFarland scale).

*Evaluation of growth of organisms.* The growth of bacteria was followed by nephelometric measurements at 650 mμ, with the exception of *M. phlei* which does not grow in suspension. The growth of this organism was evaluated on the basis of the area of surface growth.

*Determination of phosphorus.* The method of Fiske & Subbarow [2] or the micromethod of Chen *et al.* [1] were used.

## RESULTS

As shown in Table 1, six out of ten organisms studied could be adapted to grow in consecutive transfers in simple medium of Zeleznick *et al.* [9] with inorganic phosphate but only two survived when phosphate was replaced with ethylphosphonate. The utilization of 2,3-dihydroxypropylphosphonate was studied on three organisms only and it was

found that it supported both strains of *Escherichia* growing on ethylphosphonate and, in addition, was utilized by *M. phlei* which was unable to grow on ethylphosphonate.

Table 1

*Adaptation of microorganisms to the simple medium with orthophosphate and phosphonates*

Composition of media as described in text. (+), Growth of bacteria; (—), absence of growth of bacteria.

| Organism | Simple medium with | | |
|---|---|---|---|
| | orthophosphate | ethylphosphonate | dihydroxypropyl-phosphonate |
| *Escherichia freundi* | ++ | + | + |
| *Escherichia coli* 306 | ++ | — | |
| *Escherichia coli* 307 | ++ | + | + |
| *Escherichia coli* 18 | — | — | |
| *Proteus* | — | — | |
| *Bacillus subtilis* | ++ | — | |
| *Mycobacterium phlei* | ++ | — | +— |
| *Nocardia brasiliensis* | — | — | |
| *Streptomyces globisporus* 5 | ++ | — | |
| *Streptomyces globisporus* 55 | — | — | |

Table 2

*The effect of glycerol on the utilization of phosphonates by* M. phlei *growing on Sauton and simple media*

Composition of the media as described in text; 1% glycerol was added where indicated.

| Additions | Simple medium | | | | Sauton medium | | | |
|---|---|---|---|---|---|---|---|---|
| | Transfers | | | | Transfers | | | |
| | 1 | 2 | 3 | 4-9 | 1 | 2 | 3 | 4-20 |
| Orthophosphate | ++ | ++ | ++ | ++ | — | — | — | — |
| Orthophosphate with glycerol | +++ | +++ | +++ | +++ | ++++ | ++++ | ++++ | ++++ |
| Ethylphosphonate | ++ | + | — | — | | | | |
| Ethylphosphonate with glycerol | ++ | + | — | — | +++ | ++ | — | — |
| Dihydroxypropyl-phosphonate | ++ | + | +— | +— | | | | |
| Dihydroxypropyl-phosphonate with glycerol | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Glycerol | ++ | + | — | — | +++ | ++ | — | — |
| None | ++ | + | — | — | | | | |

In view of the well known fact that glycerol stimulates the growth of acid-resistant bacilli, its effect on the utilization of carbon-bound phosphorus by *M. phlei* in simple and Sauton media was studied. The results summarized in Table 2 showed that glycerol stimulated the growth of *M. phlei* with 2,3-dihydroxypropylphosphonate as source of phosphorus but had no effect in case of ethylphosphonate.

The growth of *E. coli* 307 and *E. freundi* in simple medium with phosphonates and with inorganic phosphate was followed through many consecutive transfers (Fig. 1); both these strains of *Escherichia* were able to utilize carbon-bound phosphorus but their growth was slower.

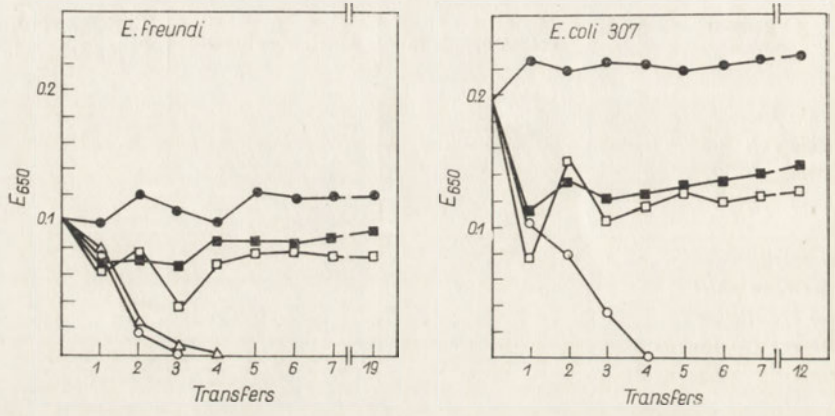


Fig. 1. Growth of *E. freundi* and *E. coli* 307 on simple medium. The growth of bacteria was determined by changes in extinction at 650 mμ after three days of incubation: (○), on simple medium (no orthophosphate); (△), with traces of orthophosphate (1 μg. P in 1 ml.); (●), with orthophosphate; (□), with ethylphosphonate; and (■), with dihydroxypropylphosphonate.

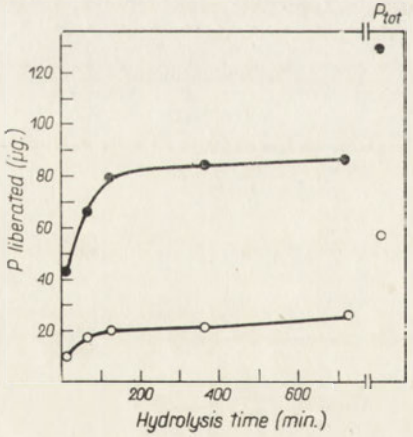


Fig. 2. Liberation of phosphorus during acid hydrolysis of *M. phlei*. The eight-day-old cultures were washed three times with water and dried to constant weight at 105°. Dried bacteria, 10 mg., were hydrolysed in 2 N-$H_2SO_4$ at 100°. Phosphorus was determined according to the micromethod of Chen *et al.* [1]. Bacteria grown on Sauton medium supplemented with 1% glycerol: (○), with dihydroxypropylphosphonate; (●), with orthophosphate.

The dry weight and total phosphorus content of *M. phlei* cells grown in Sauton medium with inorganic phosphate or dihydroxypropylphosphonate were compared. After 8 days of incubation in 5 ml. of medium

with inorganic phosphate, 18.8 mg. of dry cells containing 1.1% of total phosphorus were harvested, while in medium with dihydroxypropylphosphonate 13.7 mg. of cells were obtained with only 0.7% of total P.

Dry *M. phlei* cells were hydrolysed at 100° in 2 N-sulphuric acid and the rate of inorganic phosphate liberation was determined. The results shown in Fig. 2 indicate that *M. phlei* incorporated carbon-bound phosphorus into acid-labile compounds. In control experiments it was established that 2,3-dihydroxypropylphosphonate hydrolysed for many hours at 100° in 2 N-sulphuric acid did not release inorganic phosphate. Attempts to detect the decomposition of P—C bonds in 2,3-dihydroxypropylphosphonates by disrupted cells of *M. phlei* were unsuccessful.

## DISCUSSION

To study bacterial growth by substituting inorganic phosphate with other sources of phosphorus, it is necessary to use simple synthetic media and this considerably limits the number of strains suitable for such studies (Table 1). Our results indicate also that the ability to utilize carbon-bound phosphorus is not frequently encountered. Zeleznick *et al.* [9] demonstrated this ability in *E. coli* Crookes and we found two more strains of *Escherichia* possessing the same property, viz. *E. coli* 307 and *E. freundi*. Two other strains tested, *E. coli* 306 and 18, could not be adapted to ethylphosphonate. In view of the growing interest in the biochemistry of compounds with P—C bonds, further screening of microorganisms for ability to utilize carbon-bound P appears to be desirable.

Zeleznick *et al.* studied the growth of *E. coli* Crookes on methyl-, ethyl- and hydroxymethylphosphonates and found that the hydroxymethyl derivative is not utilized, while in the presence of methyl- and ethylphosphonates growth rate and phosphorus accumulation were the same as with inorganic phosphate. In our experiments, the growth of *Escherichia* was considerably slower when inorganic phosphate was substituted with phosphonates.

2,3-Dihydroxypropylphosphonate used in the present work is a compound which bears some structural resemblance to phosphoglycerol. It supported the growth of *E. coli* 307 and *E. freundi* equally well as ethylphosphonate and was also utilized by *M. phlei*. As *M. phlei* gives a higher yield of cells than *Escherichia*, it could be used in studies on P—C bond decomposition.

The slow growth and low phosphorus content of *M. phlei* grown on 2,3-dihydroxypropylphosphonate implies that carbon-bound-phosphorus is not readily available for this organism and not more than the necessary minimum of inorganic phosphate is produced. This was confirmed by the fact that in no case was inorganic phosphate released into the medium. It follows that *M. phlei* has or produces only a limited amount of enzyme (or enzymes) decomposing the P—C bond. The hydrolysis

curve of phosphate compounds accumulated in *M. phlei* grown on 2,3-dihydroxypropylphosphonate indicates that a large part of phosphorus is present in the form of acid-labile compounds, presumably esters whose formation would not be possible without breaking P—C bonds. Unfortunately we have not been able to demonstrate the splitting of P—C bonds in cell-free homogenates of *M. phlei*.

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We would like to express our gratitude to Prof. Dr. Tadeusz Baranowski for helpful discussion and advice.

## SUMMARY

1. It was found that *Escherichia coli* 307 and *E. freundi* grow on synthetic medium when inorganic phosphate is replaced with ethylphosphonate or 2,3-dihydroxypropylphosphonate.

2. *Mycobacterium phlei* is not able to utilize phosphorus from ethylphosphonate but grows on 2,3-dihydroxypropylphosphonate. The growth is slower, however, and the cells have a lower phosphorus content.

3. *M. phlei* produces acid-labile phosphorus compounds from the acid-resistant 2,3-dihydroxypropylphosphonate.

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## ZUŻYTKOWANIE FOSFORU ZWIĄZANEGO Z WĘGLEM PRZEZ MIKROORGANIZMY

### Streszczenie

1. Stwierdzono, że *Escherichia coli 307* i *Escherichia freundi* rosną na pożywce mineralnej zawierającej kwas etylofosfonowy albo kwas 2,3-dwuhydroksypropylofosfonowy w miejsce ortofosforanu.

2. Wykazano, że *Mycobacterium phlei* nie rośnie na pożywkach zawierających w miejsce fosforanu kwas etylofosfonowy, natomiast rośnie na pożywkach z kwasem 2,3-dwuhydroksypropylofosfonowym.

3. *M. phlei* w trakcie wzrostu na 2,3-dwuhydroksypropylofosfonianie tworzy związki fosforowe kwasolabilne.

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# A STUDY ON THE LOSS OF THE RECEPTOR ACTIVITY OF Vi-POLYSACCHARIDE DURING INCUBATION WITH Vi-PHAGE II*

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In 1954 Koziński & Opara [7] have shown that Vi-antigen preparation lost its receptor activity in relation to Vi-phage II as a result of incubation with a bacterial lysate containing the Vi-phage II. As the phage infectivity was maintained after incubation, the authors concluded that the action of Vi-phage on Vi-substance is of an enzymic character. Later [15, 5] it was demonstrated that the Vi-substance is a polysaccharide composed mainly of acetylated aminogalacturonic acid, and recently [12] an improved method for obtaining a highly purified preparation has been described.

In the present work an attempt was made to elucidate the nature of the reaction between Vi-phage II and Vi-polysaccharide leading to the destruction of the receptor activity.

## MATERIALS AND METHODS

Vi-polysaccharide preparations from *Salmonella typhi* 21802 were purified by chromatography on erythrocyte stroma [12], and contained 450 - 500 receptor units per mg.

Crude Vi-phage II suspensions were obtained by lysis of phage-infected bacteria *S. typhi* in modified Stokes & Bayne medium [14] and contained $2 - 8 \times 10^{11}$ phage particles per ml.; they were kept in the cold with chloroform in order to prevent the growth of contaminating bacteria.

Purified Vi-phage II preparation was obtained using the modified Creaser & Taussig method [3] in the following way: 500 ml. of crude Vi-phage II suspension was dialysed overnight in the cold against 10 liters of distilled water saturated with chloroform. The next day

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the suspension was filtered through a Hyflo-Supercel layer 3 - 5 mm. thick, with very gentle suction. A column (3 × 4 cm.) was prepared from 5 g. of ECTEOLA-cellulose, washed successively with 1 N-NaOH, water, 1 M-$KH_2PO_4$, and 0.01 M-phosphate buffer, pH 7.0, and then the Hyflo-Supercel filtrate was allowed to flow through the column at a rate of 5 ml. per minute. After washing with 0.01 M-phosphate buffer, pH 7.0, the adsorbed phage was eluted with 0.3 M-NaCl - 0.01 M-phosphate buffer, pH 7.0, the flow-rate being 0.5 ml. per minute. The first 10 ml. of the turbid effluent were collected and subjected to two cycles of differential (12 000 *g*, 60 min.; 3000 *g*, 15 min.) centrifugation at 0° in Janetzki K 14/A centrifuge. The phage pellet was always suspended in 0.15 M-NaCl - 0.01 M-phosphate buffer, pH 7.0, by gentle pipetting, after overnight standing with this salt solution in the cold. The course of purification is presented in Table 1. In the final product only slight quantities of bacterial impurities were found by electron microscopy (Fig. 1).

Table 1

*Purification of Vi-phage II*

| Preparation | Volume (ml.) | $10^{-13}$ × Phages | Protein (mg.) | Purity ($10^{-13}$ × Phages/mg. protein) | Purification | Yield (%) |
|---|---|---|---|---|---|---|
| Crude phage suspension | 500 | 21.2 | 175.0 | 0.12 | | 100 |
| After ECTEOLA treatment | 10 | 20.2 | 22.5 | 0.88 | 7.3 | 95 |
| After I centrifugation | 10 | 13.8 | 9.5 | 1.45 | 12.1 | 65 |
| After II centrifugation | 10 | 11.2 | 6.9 | 1.62 | 13.5 | 53 |

Modified Stokes & Bayne (mSB) medium [11], and formolized sheep erythrocytes were prepared as described previously [14].

*Reagents used:* sodium ethylenediaminetetraacetate (EDTA), tris and Hyflo-Supercel (Light, Colnbrook, England); *p*-chloromercuribenzoic acid (B.D.H., Poole, England); *N*-ethylmaleimide (Schuchardt, München, West Germany); tubings for dialysis (Kalle, Wiesbaden, West Germany). ECTEOLA-cellulose containing 0.26 mEq. nitrogen per gram, was prepared according to Peterson & Sober [9] from Whatman cellulose powder, triethanolamine (Schuchardt, München, West Germany) and 1-chloro-2:3-epoxypropane (epichlorohydrin, Light, Colnbrook, England). Other reagents were products of Fabryka Odczynników Chemicznych (Gliwice, Poland).

Protein was determined according to Lowry *et al.* [8], after wolframate precipitation, crystalline bovine serum albumin (B. D. H., Poole,


Fig. 1. Electron micrograph of the purified Vi-phage II preparation. Agar-diffusion technique [6] with a slight modification [13] was applied. Chromium shadowing. Electron microscope: Zeiss D2. The arrow points to the tip of the phage's tail, by means of which phage reacts with Vi-polysaccharide [13].

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England) being used as standard. For protein determination in the crude phage, the suspension was previously dialysed against distilled water.

Phage counts were made in triplicate by the agar layer method [1], as previously described [14].

Vi-receptor estimation was performed as described previously [14]. The quantity of Vi-polysaccharide which, under the conditions used, reduced the Vi-phage content by $1 \times 10^{11}$/ml. was adopted as Vi-receptor unit (RU).

Determination of the receptor-destroying activity was based on the receptor estimation [14]. The Vi-polysaccharide solution containing 200 RU per ml. was mixed with an equal volume of the Vi-phage II suspension and incubated in Hoeppler ultrathermostat at 37°. At definite intervals, samples equivalent to 1 - 2 RU (0.02 - 0.25 ml.) were taken to thick-walled test-tubes (100 mm. × 12 mm.) and immersed into boiling water for 2 min. to inactivate the receptor-destroying agent. After adjusting the volume to 1 ml. with 0.15 M-NaCl - 0.01 M-phosphate buffer, pH 7.0, the sample was added with 0.5 ml. of 10% (v/v) suspension of formolized sheep erythrocytes in the same salt solution. The mixture was incubated in a water bath at 37° for 1 hr. with occasional stirring and left overnight in the cold to permit the adsorption of the Vi-polysaccharide on erythrocytes. Throughout the experiment stirring was performed by means of a rubber cork fixed excentrically on the motor spindle. The next day the supernatant was discarded and the sediment of erythrocytes washed once with NaCl-phosphate buffer; the final sediment was then drained off thoroughly and the test-tube was put into ice-water. The Vi-phage II crude suspension was adjusted to the concentration of $3 - 4 \times 10^{11}$ particles/ml. by suitable dilution with mSB medium and after cooling in ice-water, 2 ml. were added to the test-tube and the contents were stirred vigorously. The mixture was incubated for 5 min. in ice-water and centrifuged for 2 min. at 1000 *g* in cooled centrifuge vessel. The supernatant was immediately separated and taken for phage estimation. The quantity of unadsorbed phage was the measure of the receptor activity of the examined sample, its value being found graphically (Fig. 2). As throughout this work different crude phage suspensions were applied, the receptor activity values were referred to a standard Vi-polysaccharide preparation.

In preliminary experiments it was found that heating for 1 min. in boiling water inactivated the receptor-destroying agent and that incubation even for 10 min. at 100° of Vi-polysaccharide with the crude Vi-phage II suspension inactivated by heating, did not interfere with the estimation of the receptor activity.

## RESULTS

### *Properties of the receptor-destroying agent*

In order to identify the receptor-destroying agent, the crude phage suspension ($4 \times 10^{11}$/ml.) was centrifuged in MSE-Major centrifuge at 24 000 *g* for 90 min. at 0°. The sediment, containing practically the whole original quantity of the phage, was suspended in the initial volume of mSB medium. Both this suspension and the supernatant were incubated with equal volumes of Vi-polysaccharide solutions (200 RU/ml.) and the receptor activity was estimated at 15 min. intervals. The results shown in Fig. 3 indicate that the whole receptor-destroying agent was present

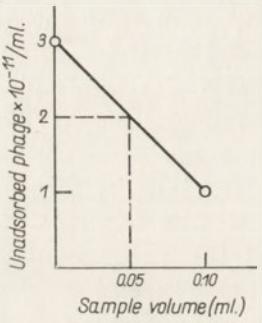


Fig. 2

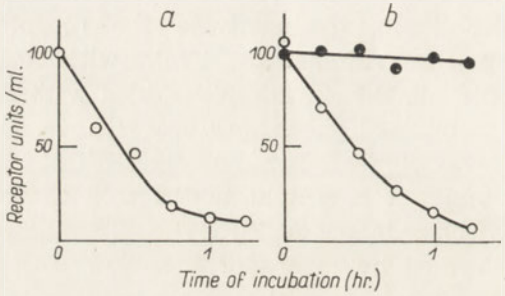


Fig. 3

Fig. 2. Estimation of Vi-polysaccharide receptor activity. The volume of the examined sample amounted to 0.1 ml., the concentration of unadsorbed Vi-phage II to $1 \times 10^{11}$/ml. The amount of Vi-phage in the control sample was $3 \times 10^{11}$/ml. One receptor unit corresponds here to 0.05 ml., hence 1 ml. of the reaction mixture contained 20 receptor units at the sampling time.

Fig. 3. Destruction of the receptor activity of Vi-polysaccharide by (*a*), crude Vi-phage II suspension ($2 \times 10^{11}$/ml. of the incubation mixture) and (*b*), the suspension after centrifugation for 90 min. at 24 000 *g*; (○), sediment, (●), supernatant. Conditions: mSB medium, pH 7.2, 37°.

in the sediment. As it was also found that the receptor-destroying activity per phage particle was the same for crude preparations as for the purified ones, it seems that the phage particle can be considered as the receptor-destroying agent. The findings that the receptor-destroying agent is non-diffusible and is inactivated by short heating at 100° are in accordance with this conclusion. The rate of destruction of the receptor activity at 37° plotted against phage concentration (Fig. 4) showed a linear relationship.

Purified Vi-phage II suspension was incubated with Vi-polysaccharide solution and the loss of the receptor activity and the amount of

infective phage particles were estimated (Fig. 5). The results indicate that the phage, while it destroyed the receptor activity of the polysaccharide, did not lose its infectivity.

Vi-polysaccharide solution was incubated with purified Vi-phage II suspension and samples were taken every 15 min. for receptor activity estimation. After 2 hr. incubation, a second portion of Vi-polysaccharide

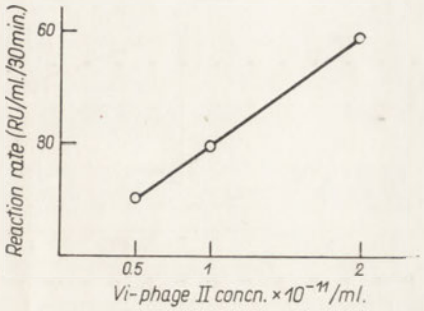


Fig. 4. Effect of Vi-phage II concentration on the destruction rate of the receptor activity of Vi-polysaccharide. Conditions: mSB medium, pH 7.2, 37°.

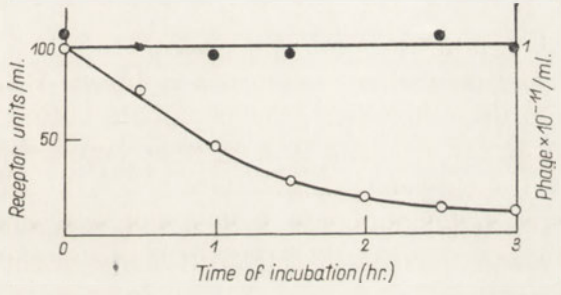


Fig. 5. Effect of incubation of Vi-polysaccharide with purified Vi-phage II preparation on (○), receptor activity of the polysaccharide and (●), infectivity of phage. Conditions: mSB medium, pH 7.2, 37°.

was added and the receptor activity was determined again. The results presented in Fig. 6 indicate that although the receptor activity was almost completely destroyed after 2 hr. incubation, the phage maintained its ability to act on a new portion of polysaccharide. In the first part of the experiment the initial reaction rate amounted to 30 RU/ml./30 min. and in the second one to 15 RU/ml./30 min.; however, two-fold phage dilution should be taken into consideration.

### *Simple reaction medium*

In the experiments presented above, the destruction of the receptor activity due to the phage was studied in mSB medium, composed of enzymic lactalbumin hydrolysate, glucose, citrate, and inorganic salts. It seemed advisable to simplify as far as possible the medium in which

purified phage preparations and Vi-polysaccharide were to be suspended and the reaction studied; for this purpose three salt solutions were chosen: Na,K-phosphate buffer, pH 7.0, $\mu$ 0.1 [4]; tris-HCl buffer, pH 7.0, $\mu$ 0.1 [4], and 0.1 M-ammonium acetate adjusted to pH 7.0 with aqueous ammonia solution. In ammonium acetate solution the initial rate of destruction of the receptor activity was the same as in mSB medium,

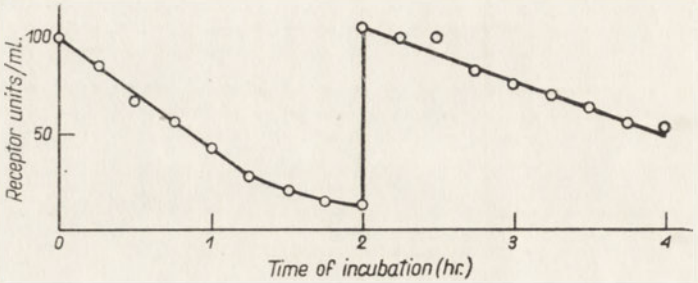


Fig. 6. Destruction of the receptor activity of Vi-polysaccharide by the purified Vi-phage II preparation ($1 \times 10^{11}$/ml. of the incubation mixture). After 2 hr. incubation, an equal volume of Vi-polysaccharide (200 receptor units/ml.) was added. Conditions: mSB medium, pH 7.2, 37°.

about 60 RU/ml./30 min. for the phage concentration amounting to $2 \times 10^{11}$/ml. of the incubation mixture and in tris-HCl buffer it was a little lower. On the other hand, the phosphate buffer caused a significant inhibition of the reaction rate. Further experiments were therefore carried out in 0.1 M-ammonium acetate.

Koziński & Opara [7] found it difficult to reproduce the loss of receptor activity and thought that this difficulty might be due to the presence of unidentified inhibitors. Taking into account their results and the observed inhibition of the reaction by phosphate, an examination was undertaken of the effect of EDTA and two of the thiol group inhibitors, namely *p*-chloromercuribenzoate and *N*-ethylmaleimide. The reaction was carried out in 0.1 M-ammonium acetate, pH 7.0, at 37°, the concentration of the polysaccharide in the incubation mixture being 100 RU/ml. and that of the phage $2 \times 10^{11}$/ml. Neither $10^{-4}$ M-*p*-chloromercuribenzoate nor $10^{-3}$ M-*N*-ethylmaleimide diminished the initial reaction rate. On the other hand, $10^{-2}$ M-EDTA significantly inhibited the reaction. As it has been found that Vi-polysaccharide adsorbed on the formolized erythrocytes has a much lower ability to adsorb Vi-phage II in the presence of EDTA, in these experiments the estimation of receptor activity was slightly modified: to the solution used for completing the sample to the volume of 1 ml. before the addition of erythrocytes, 0.01 M-$MgCl_2$ was added and 0.01 M-phosphate buffer was replaced by 0.01 M-tris-HCl buffer, pH 7.0. By this modified method it

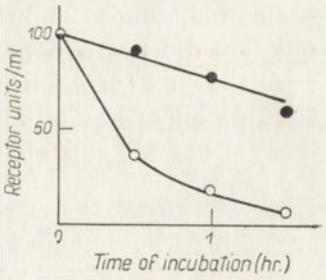


Fig. 7. Effect of EDTA on the destruction of the receptor activity of Vi-polysaccharide by the purified Vi-phage II preparation ($2 \times 10^{11}$/ml. of the incubation mixture); incubation: (○), without, and (●), with 0.01 M-EDTA. Conditions: 0.1 M-ammonium acetate, pH 7.0, 37°.

was shown that EDTA in $10^{-2}$ M concentration caused a decrease in the initial reaction rate by about 80% (Fig. 7). The addition of $10^{-2}$ M-$MgCl_2$ to the incubation mixture had no marked effect on the course of the reaction.

### *Effect of temperature and* pH

The reaction was carried out in 0.1 M-ammonium acetate, pH 7.0, the concentration of the polysaccharide being 100 RU/ml. and that of the phage $2 \times 10^{11}$/ml. The mixture was incubated in Hoeppler ultrathermostat at 17°, 27° or 37° (Fig. 8). Within the range of 17 - 37° an increase of temperature by 10° caused an 1.6 to 1.7-fold increase of the reaction rate.

The effect of pH presented in Fig. 9 indicates a broad pH optimum on the alkaline side. The experiments were performed in 0.1 M-ammonium acetate adjusted to appropriate pH with 0.1 M-acetic acid, or 0.1 M-

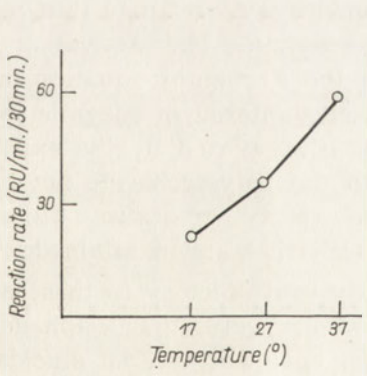


Fig. 8

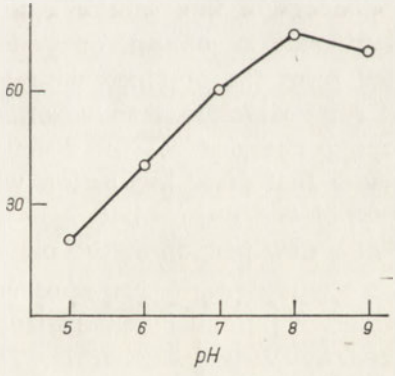


Fig. 9

Fig. 8. Effect of temperature on the destruction of the receptor activity of Vi-polysaccharide due to Vi-phage II. Vi-polysaccharide concentration: 100 receptor units/ml., Vi-phage II concentration: $2 \times 10^{11}$/ml. Conditions: 0.1 M-ammonium acetate, pH 7.0.

Fig. 9. Effect of pH on the destruction of the receptor activity of Vi-polysaccharide by Vi-phage II. Vi-polysaccharide concentration: 100 receptor units/ml., Vi-phage II concentration: $2 \times 10^{11}$/ml. Conditions: 0.1 M-ammonium acetate, 37°.

-ammonia, under toluene. In these conditions the pH value of the medium did not change in the course of the incubation (only at pH 9.0, a fall to 8.9 was observed); toluene had no effect on the reaction. Control experiments have also shown that the receptor activity of Vi--polysaccharide is stable in the chosen pH range.

## DISCUSSION

In the present study a simple, defined medium and approximate pH optimum for the reaction between Vi-phage II and Vi-polysaccharide were determined, and the effect of temperature on the reaction rate was examined. It has been observed that neither toluene nor thiol group reagents inhibit this reaction.

EDTA was found to influence the reaction of destruction of the receptor activity of Vi-polysaccharide by Vi-phage II, by lowering the ability of the polysaccharide to adsorb the phage. This seems to indicate that divalent cations are involved in the binding of the phage by the polysaccharide. Maybe that the divalent cations act by lowering the negative charge of the acid Vi-polysaccharide by forming complexes with carboxyl groups.

The presented results seem to support the suggestion concerning the enzymic character of the action of Vi-phage II on Vi-polysaccharide. It was shown that the phage particle itself may be considered as the agent causing the destruction of the receptor activity of the polysaccharide. The phage loses this ability after short heating at 100°. The dependence of the reaction rate on temperature may (but need not) be a manifestation of an enzymic reaction. The activation energy, calculated from the obtained data by the Arrhenius equation, amounts to about 9000 cal./mole, a value often encountered in enzymic reactions. The enzymic character of the reaction is supported by the experiments which show that after incubation with the polysaccharide not only the infectivity of the phage but also its ability to destroy the receptor activity of a new portion of the polysaccharide, are maintained.

The presented results are apparently contradictory to those obtained by Baron *et al.* [2] and by Staub [10] who reported that Vi-phage II was inactivated by Vi-polysaccharide. This, however, can be elucidated by a quantitative comparison. In the experiments of Baron *et al.* after 18 hr. of incubation at 37°, 1 μg. of the polysaccharide inactivated about $10^3$ phages, and in the experiments of Staub after 1 hr. of incubation at 44°, up to $10^8$ phages (personal communication). In our experiments [14] during 18 hr. of incubation at 37° the maximum quantity of phages inactivated by 1 μg. of the polysaccharide amounted to $3 \times 10^6$. So small quantities of inactivated phages could not be detected in the experiments presented in this paper, performed at 37° at phage concentration

of the order of $10^{11}$/ml. This trace inactivation does not seem to be connected with the destruction of the receptor activity of Vi-polysaccharide.

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## SUMMARY

It has been found that the destruction of the receptor activity of Vi-polysaccharide due to Vi-phage II action, may occur in 0.1 M-ammonium acetate medium. The effect of temperature and pH on the reaction rate was studied. The reaction was inhibited by EDTA but *p*-chloromercuribenzoate and *N*-ethylmaleimide had no effect.

After incubation with the polysaccharide, the infectivity of the phage as well as its ability to destroy the receptor activity of a new portion of the polysaccharide, were maintained.

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## BADANIE UTRATY AKTYWNOŚCI RECEPTOROWEJ WIELOCUKRU Vi, ZACHODZĄCEJ PODCZAS INKUBACJI Z FAGIEM Vi II

Streszczenie

Stwierdzono, że utrata aktywności receptorowej wielocukru Vi wskutek działania faga Vi II może zachodzić w środowisku 0.1 M-octanu amonu. Zbadano wpływ temperatury i pH na szybkość tej reakcji. Reakcja jest hamowana przez EDTA, natomiast *p*-chlororteciobenzoesan i *N*-etylomaleimid nie wywierają na nią wpływu.

Po inkubacji z wielocukrem zostaje zachowana zarówno infekcyjność faga, jak i jego zdolność rozkładania aktywności receptorowej nowej porcji wielocukru.

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# COMPARATIVE INVESTIGATION ON TYROSINE METABOLISM IN ANIMALS

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In mammals, the main oxidative metabolism of tyrosine proceeds through *p*-hydroxyphenylpyruvate, homogentisate and fumarylacetoacetate to fumarate and acetoacetate.

Canellakis & Cohen [2] demonstrated the presence in pigeon liver of tyrosine-*α*-ketoglutarate aminotransferase (EC 2.6.1.5), and Crandall & Halikis [3] of homogentisate oxygenase (EC 1.13.1.5); in the previous work [16] the activity of *p*-hydroxyphenylpyruvate oxidase (EC 1.14.2.2) has been demonstrated. These observations suggest that in the pigeon the breakdown of tyrosine follows the same pathway as in mammals. In other animals the main catabolic pathway of tyrosine is still unknown, and in the present work the oxidation of tyrosine has been studied on some poikilothermic vertebrates and lower animals. Some preliminary results have been reported [16, 17].

## EXPERIMENTAL

*Material.* For experiments, hepatopancreas of snail, *Helix pomatia*, and livers of the following animals were used: perch, *Lucioperca sandra*; pike, *Esox lucius*; frog, *Rana esculenta*; sand lizard, *Lacerta agilis*; pigeon, *Columba domestica*; and albino rat. The frogs were tested in winter (during hibernation), in spring (during the mating season) and in summer.

Isolated livers were immediately homogenized at 4° with half their volume of water, and acetone-dried preparations were made according to La Du & Greenberg [8]. For experiments, freshly prepared extracts were used; acetone powder was homogenized with 10 or 20-fold volume of an appropriate buffer in a Potter homogenizer, and after 20 min. centrifuged, the supernatant being used for the assays. Protein in the extracts was determined by the tannin micromethod of Mejbaum-Katzenellenbogen [15].

*Determination of enzymic activity.* Two to four preparations of acetone-dried liver were prepared, several separate extracts being made from each preparation. The enzymic activity of the preparations kept in an evacuated desiccator remained unaltered for some weeks. The amount of extract used for incubation was chosen so as to obtain a linear relationship between the enzymic activity and the amount of protein, two different concentrations of protein being used for each assay. For determination of activity, the time of reaction was 2 hr. for aminotransferase and 10 min. for the oxidases to keep within the range of the linear rate of the reaction, and the results were calculated per 100 mg. of protein.

*Chemicals used*: α-Ketoglutaric acid (B.D.H., England), pyridoxal phosphate and natrium diethyldithiocarbamate (California Corp. for Biochemical Research, U.S.A.), *p*-hydroxyphenylpyruvic acid (Mann Research Lab. Inc., New York, U.S.A.), reduced glutathione (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.), homogentisic acid and tris (Sigma Chemical Company, St. Louis, U.S.A.), hydrocortisone (Hydroadreson, N.V. Organon OSS, Holland). L-Tyrosine and other reagents were of Polish origin.

### *Tyrosine—α-ketoglutarate aminotransferase*

The activity of this enzyme was studied in a system containing in 2.5 ml.: 6 μmoles of tyrosine, 12 μmoles of α-ketoglutarate, 30 μgrams of pyridoxal phosphate, 50 μmoles of tris buffer, pH 8.1, 5 μmoles of natrium diethyldithiocarbamate, and extract from acetone-dried liver in 0.05 M-tris buffer, pH 8.1. The reaction mixture was incubated in a Höppler ultrathermostat at 30° for 2 hr. To stop the reaction, 0.5 ml. of 30% trichloroacetic acid was added and the precipitated protein was centrifuged off. The formed *p*-hydroxyphenylpyruvate was assayed spectrophotometrically by the enol borate-tautomerase method of Lin *et al.* [13] for arylketoacids, the extinction at 310 mμ of the complex being measured in an Unicam SP 500 spectrophotometer. In the control samples, in which either one of the components, tyrosine, α-ketoglutarate or enzyme, was omitted or inactivated enzyme was used, the complex did not form.

The second reaction product, glutamic acid, was identified by paper chromatography; 0.1 ml. of the deproteinized supernatant was applied on Whatman no. 1 paper and the chromatogram developed in water-saturated phenol for 15 hr. by the ascending technique. After drying for 24 hr., the spots were located by spraying with a 0.1% solution of ninhydrin in water-saturated *n*-butanol. For experiments aiming at identification of glutamic acid, dialysed extracts were used; this was found necessary due to the presence of small amounts of this amino acid in the extracts.

After incubation, on the chromatograms of the proper samples, in addition to the spot of tyrosine ($R_F$, 0.62), appeared a spot whose $R_F$ value, 0.28 - 0.32, corresponded to that of an authentic sample of glutamic acid. Glutamic acid was not found in the absence of active enzyme, of tyrosine, or of $\alpha$-ketoglutarate.

The addition of pyridoxal phosphate appeared to be necessary for the reaction (Fig. 1). In its absence the activity of aminotransferase did not exceed 1/5 of the maximum activity, so it seems that most of the endogenous coenzyme was removed during preparation of the acetone powder. The optimum pH for the aminotransferase activity in perch liver extract was from pH 7.8 to 8.5 (Fig. 2), the pH curve being similar

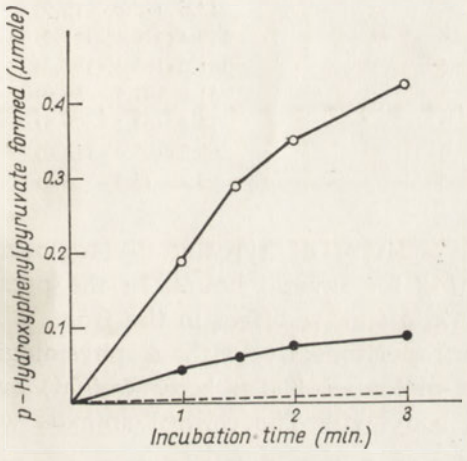


Fig. 1

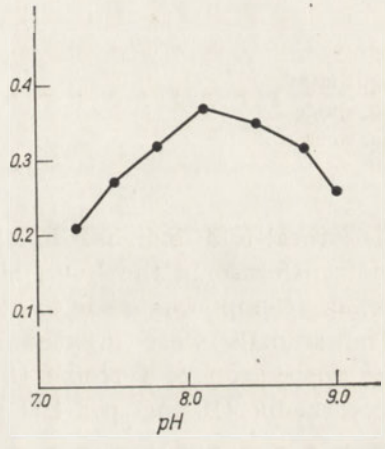


Fig. 2

Fig. 1. The effect of pyridoxal phosphate on the activity of tyrosine-$\alpha$-ketoglutarate aminotransferase in acetone-dried-liver extract from the perch. Conditions as described in text; amount of protein per sample, 0.2 mg.; (○), whole sample; (●), no pyridoxal phosphate; (- - -), no tyrosine or no $\alpha$-ketoglutarate.

Fig. 2. The effect of pH on the activity of tyrosine-$\alpha$-ketoglutarate aminotransferase in the acetone-dried-liver extract from the perch. Conditions as described in text; amount of protein per sample, 0.2 mg. For the pH values 7.0 - 8.0, phosphate buffer; for pH 8.0 - 9.0, tris buffer.

to that for rat liver aminotransferase, only slightly shifted towards the alkaline side. No difference was found using tris or phosphate buffer.

The activity of tyrosine-$\alpha$-ketoglutarate aminotransferase (Table 1) was found to be high in the perch, pike and frog, several times higher than in the rat. In the frog, the activity of the enzyme was higher in the spring and summer than in winter; in the mating season no differences between the two sexes were found.

Lin & Knox [11] observed that intraperitoneal administration to rats with intact adrenal glands of tyrosine or of hydrocortisone resulted

Table 1

*Activity of tyrosine aminotransferase in extracts from acetone-dried livers of various animals*

The amount of protein in the samples ranged from 0.05 mg. (in fishes) to 4 mg. (in the pigeon) depending on the activity. The results are mean values, with limit values in parentheses; they are expressed as μmoles of p-hydroxyphenylpyruvate formed/100 mg. protein/2 hr.

| Animal | No. of determinations | Activity |
|---|---|---|
| Rat | 11 | 25.7 (21.2 - 30.5) |
| Pigeon | 9 | 1.8 (1.6 - 2.0) |
| Lizard | 7 | 13.8 (12.6 - 16.9) |
| Pike | 6 | 115.4 (114.1 - 117.2) |
| Perch | 5 | 181.0 (168.5 - 188.3) |
| Frog, winter | 6 | 51.8 (47.5 - 56.9) |
| Frog, spring | 5 | 74.9 (63.5 - 79.5) |
| Frog, summer | 3 | 109.2 (107.4 - 112.0) |
| Snail | 5 | 3.4 (3.2 - 3.7) |

in a several-fold increase in the activity of tyrosine-α-ketoglutarate aminotransferase in the liver, lasting for several hours. In the present work, an attempt was made to obtain a similar effect in the frog.

The animals were injected intraperitoneally with a physiological saline suspension of tyrosine (1 m-mole per 300 g. body weight) or of hydrocortisone (10 mg. per 100 g. body wt.). The control animals were

Table 2

*Effect of tyrosine and hydrocortisone on the activity of tyrosine-α-ketoglutarate aminotransferase*

Tyrosine (1 m-mole/300 g. body wt.) or hydrocortisone (10 mg./100 g. body wt.) were administered intraperitoneally as a suspension in 0.9% NaCl. The control animals received 0.9% NaCl. At the time indicated in the Table, the animals were killed, the livers were acetone-dried, and in the extract the enzymic activity was assayed. The results are mean values from experiments on 3 animals; in parentheses the limit values are given. The activity is expressed as μmoles of p-hydroxyphenylpyruvate formed/100 mg. protein/2 hr.

| Animal | Time after injection (hr.) | 0.9% NaCl, control | Tyrosine | Hydrocortisone |
|---|---|---|---|---|
| Rat | 5 | 24 (23.7 - 24.6) | 46.6 (45.8 - 47.4) | 177.5 (174 - 182) |
| Frog, winter | 5 | 50 (47.5 - 52.5) | 53 (49.6 - 56.7) | 49 (48.4 - 51) |
| Frog, summer | 5 | 109 (107.8 - 110.6) | | 108 (104 - 115) |
| Frog, summer | 10 | 110.5 (107.3 - 113.7) | | 106.6 (91.4 - 115) |
| Frog, summer | 15 | 112.6 (108.6 - 116.6) | | 106 (91.2 - 118.3) |

injected with 0.9% NaCl solution only. In agreement with the experiments of Lin & Knox [11, 12] 5 hr. after the administration of tyrosine or hydrocortisone to the rats the activity of aminotransferase increased, respectively, 2- and 7-fold. In the frog neither in winter, when the protein metabolism is very low, nor in summer did this effect appear (Table 2). As the absorption of hydrocortisone could be expected to be slower in the frog than in the rat, the enzymic activity was tested not only 5 hr., but also 10 and 15 hr. after the injection. Even so, no increase of aminotransferase activity due to hydrocortisone, was observed.

### *p-Hydroxyphenylpyruvate oxidase*

*p*-Hydroxyphenylpyruvate, a tyrosine transamination product, is oxidized in mammals to homogentisate through a complex reaction involving oxidation, decarboxylation, and shifting of the side chain. The activity of this oxidase was assayed manometrically in a Warburg apparatus according to La Du & Zannoni [9]. The main compartment of the flask contained 0.5 ml. of 0.1 M-phosphate buffer, pH 6.5, 25 μg. of 2,6-dichlorophenolindophenol, 20 μmoles of reduced glutathione, 2 μmoles of $\alpha,\alpha'$-dipyridyl, and an appropriate amount of the acetone-dried-liver extract in 0.1 M-phosphate buffer, pH 6.5. Although the pH optimum for this reaction is above 7, the value of 6.5 was applied since according to Pitt [18] at pH values above 7 in the presence of oxygen, *p*-hydroxyphenylpyruvate is non-enzymically oxidized to *p*-hydroxybenzaldehyde and oxalate. The side arm of the manometric flask was filled with 5 μmoles of *p*-hydroxyphenylpyruvate, and the central well with 0.2 ml. of 10% KOH. The final volume of the fluid was 2.8 ml., with air as the gas phase and temp. 30°. After equilibration of temperature, the substrate was tipped in, and oxygen consumption was read every 10 min. Homogentisic acid was identified by paper chromatography after deproteinization with 0.42 ml. of 20% metaphosphoric acid. The separation was performed according to Knox & LeMay-Knox [7] in an atmosphere of formic acid vapour on Whatman no. 1 paper, using water-saturated *n*-butanol. The spots were located by spraying with ammoniacal $AgNO_3$ which gives with homogentisic acid a black-blue spot with $R_F$ 0.70.

In the livers of all animals tested except the frog, oxidation of *p*-hydroxyphenylpyruvate was observed. The oxygen consumption was at its maximum after about one hour of incubation, and when the endogenous oxidation was subtracted, the values obtained did not exceed 110 μl. $O_2$, that is the amount necessary to oxidize 5 μmoles of hydroxyphenylpyruvate. In the control samples containing no enzymic extract, no oxygen uptake was observed.

The activity was the highest in fishes (Table 3), being about 8 times higher than that found in the rat, and in the pigeon and lizard 2-3 times

Table 3

*Activity of p-hydroxyphenylpyruvate oxidase in acetone-dried-liver extracts*

The assays were made in a Warburg apparatus, as described in text. The amount of protein in samples was from 3.5 mg. (in fishes) to 50 mg. (in the snail). The results are mean values, with limit values in parentheses; they are expressed as µl. of utilized oxygen/100 mg. protein/10 min. of incubation. After incubation, the presence of homogentisic acid was assayed chromatographically.

| Animal | No. of determinations | Control (no substrate) | With substrate | $\Delta$ | Homogentisic acid |
|---|---|---|---|---|---|
| Rat | 8 | 17 (10 - 29) | 55 (51 - 68) | 38 | + |
| Pigeon | 4 | 49 (41 - 60) | 150 (138 - 164) | 101 | + |
| Lizard | 2 | 26 (22 - 40) | 109 (99 - 118) | 83 | + |
| Pike | 4 | 12 (9 - 15) | 255 (230 - 278) | 243 | + |
| Perch | 4 | 155 (115 - 202) | 491 (417 - 562) | 336 | + |
| Frog, winter | 4 | 37 (31 - 44) | 37 (28 - 41) | 0 | — |
| Snail | 4 | 26 (24 - 27) | 61 (56 - 64) | 35 | — |

higher than in the rat. It was found by chromatography that hydroxyphenylpyruvate gradually diminished during incubation and eventually disappeared, and homogentisate was simultaneously formed.

The oxygen uptake in snail hepatopancreas was similar to that found in the rat, but no formation of homogentisate could be demonstrated. In the frog, neither during hibernation nor in spring or summer could the activity of *p*-hydroxyphenylpyruvate oxidase be observed. Since some inhibitor could be expected to be present in the liver extracts, the extracts were dialysed for 18 hr. against water; this, however, had no effect on the enzymic activity. Also no inhibition was found to occur when the frog extract was added to the perch extract. Since in mammals the hydroxyphenylpyruvate oxidase is known to contain tightly bound copper [5] it was thought that in the frog this bonding may be less strong and that during acetone-drying the copper may become dissociated. When, however, the $Cu^{2+}$ ion was added to the incubation mixture, this resulted only in an increase of endogenous respiration but had no effect on the oxidation of hydroxyphenylpyruvate.

Since it appeared impossible to demonstrate the presence of hydroxyphenylpyruvate oxidase in frog liver, attempts were made to find it in the kidney, but the results obtained were also negative.

*Oxidation of homogentisate*

The conversion of homogentisate to fumarate and acetoacetate is catalysed by three-component enzymic system (Knox & Edwards [6]): oxygenase containing the $Fe^{2+}$ ion, oxidatively cleaves the aromatic ring

of homogentisate to form maleylacetoacetate which is converted by *cis-trans* isomerase activated by glutathione to fumarylacetoacetate which in turn is hydrolysed to yield fumarate and acetoacetate.

Homogentisate oxygenase was determined manometrically in a Warburg apparatus. The main compartment contained 100 μmoles of tris buffer, pH 7.2, 10 μmoles of reduced glutathione, 2 μmoles $FeSO_4$, and acetone-dried-liver extract in tris buffer, pH 7.2. To the side arm was added 10 μl. of homogentisic acid which had not been neutralized to prevent non-enzymic oxidation. The central well was filled with 0.2 ml. of 10% KOH. The final volume of fluid was 3.0 ml., with air as the gas phase and temp. 30°. After temperature equilibration, the substrate was tipped in, and oxygen consumption was read every 10 min., the values over the endogenous respiration of the extract being taken as the measure of enzymic activity. Maximum oxygen uptake never exceeded 220 μl. $O_2$, that is the value corresponding to the oxidation of 10 μmoles of homogentisate. In samples with no $Fe^{2+}$ added, there was no oxidation

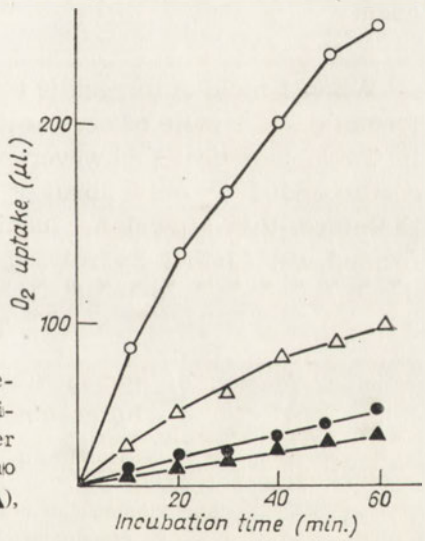


Fig. 3. Oxidation of homogentisate by the acetone-dried-liver extract from the perch. Conditions as described in text; amount of protein per sample, 15 mg.; (○), whole sample; (●), no homogentisic acid; (△), no glutathione; (▲), no $FeSO_4$.

of homogentisate indicating the loss of this ion during the preparation of the acetone-dried liver. When no glutathione was added, the reaction was much slower (Fig. 3).

In the livers of all animals tested except the snail, oxidation of homogentisate was observed (Table 4). However, the activities per 100 mg. protein per 10 min. of incubation were always lower than in the rat.

After 1 hr. incubation, acetoacetate was estimated by the method of Edson [4] which consists in estimating the amount of $CO_2$ resulting from decarboxylation of acetoacetate catalysed by aniline citrate. Within 12 min. at pH 4, all acetoacetic acid undergoes decarboxylation but no

fumarylacetic acid is decarboxylated [3]. In all samples in which the oxidation of homogentisate took place, formation of acetoacetate was observed; in the control samples no acetoacetate was detected.

Table 4

*Oxidation of homogentisate by acetone-dried-liver extracts*

Conditions of experiment as described in text. The amount of protein in samples from 7.5 mg. to 55 mg. The results are mean values, with limit values in parentheses; they are expressed as µl. of utilized oxygen/100 mg. protein/10 min. of incubation.

| Animal | No. of determinations | Control (no substrate) | With substrate | $\Delta$ |
|---|---|---|---|---|
| Rat | 4 | 91 (81 - 107) | 512 (450 - 568) | 421 |
| Pigeon | 4 | 8 (4 - 14) | 166 (155 - 180) | 158 |
| Lizard | 2 | 20 (12 - 28) | 120 (90 - 150) | 100 |
| Perch | 4 | 107 (84 - 140) | 383 (364 - 406) | 266 |
| Frog, winter | 4 | 39 (34 - 43) | 278 (236 - 310) | 239 |
| Snail | 4 | 17 (12 - 21) | 16 (11 - 24) | 0 |

When 1 mole of oxygen is utilized for the oxidation of homogentisate, formation of 1 mole of acetoacetate could be expected; the results shown in Table 5 indicate, however, that the amount of acetoacetate formed corresponded to only about a half of the oxygen utilized. It seems, therefore, that after 1 hr. incubation only a half of maleylacetoacetate formed was further hydrolysed to fumarate and acetoacetate.

Table 5

*Oxidation of homogentisate and formation of acetoacetate by acetone-dried-liver extracts*

Conditions as described in text.

| Animal | Amount of protein (mg./sample) | $O_2$ utilized (µmoles/1 hr./sample) | Acetoacetate formed (µmoles/1 hr./sample) |
|---|---|---|---|
| Rat | 24 | 5.1 | 3.4 |
| | 24 | 5.5 | 3.1 |
| Pigeon | 55 | 5.5 | 2.1 |
| | 55 | 6.1 | 2.5 |
| Lizard | 40 | 3.2 | 1.5 |
| | 40 | 3.1 | 1.6 |
| Perch | 15 | 8.6 | 5.0 |
| | 15 | 8.0 | 4.5 |
| Frog, winter | 22 | 4.2 | 2.2 |
| | 22 | 4.3 | 2.0 |
| Snail | 34 | 0.3 | 0 |
| | 34 | 0.28 | 0 |

## DISCUSSION

The presented experiments indicate that the oxidative breakdown of tyrosine in the pigeon, lizard, pike and perch follows the same pathway as that found in mammals. Since all determinations were carried out under the same conditions and at the same temperature, and the extracts from acetone-dried livers may be regarded as a fairly uniform material, it seemed that a comparison of the activities obtained may be allowed. Rat liver was taken as reference and activities found in other animals were expressed in relation to this activity, taken as 100 (Table 6). The values obtained were found to differ widely, and moreover there was no parallelism between the successive enzymes of the oxidative breakdown of tyrosine.

Table 6

*Comparison of activity of the enzymes of oxidative breakdown of tyrosine in liver*

The activities were referred to the activity found in the rat; the values given were calculated from Tables 1, 3 and 4.

| Animal | Tyrosine-α-keto-glutarate amino-transferase | *p*-Hydroxyphe-nylpyruvate oxidase | Homogentisate oxygenase |
|---|---|---|---|
| Rat | 100 | 100 | 100 |
| Pigeon | 7 | 266 | 37 |
| Lizard | 54 | 220 | 24 |
| Pike | 440 | 640 | — |
| Perch | 700 | 880 | 63 |
| Frog, winter | 200 | 0 | 57 |
| Frog, spring | 290 | 0 | — |
| Frog, summer | 420 | 0 | — |
| Snail | 13 | 93* | 0 |

* Only utilization of oxygen was found; homogentisate, the reaction product, was not demonstrated.

In the pigeon, the activity of tyrosine aminotransferase was rather low, the activity of *p*-hydroxyphenylpyruvate oxidase was 2.5 times that found in the rat, and the activity of homogentisate oxygenase again lower. Similar relations were found in the lizard. On the other hand, in fishes both aminotransferase and *p*-hydroxyphenylpyruvate oxidase were 4 - 9 times more active than the corresponding enzymes in the rat, while homogentisate oxygenase in the perch had but half of the activity found in the rat. It seems that in fish livers very high activity of aminotransferases is present as the activity of aspartate-α-ketoglutarate aminotransferase found in carp liver exceeded 30 times that in the rabbit and pigeon (Raczyńska-Bojanowska & Gąsiorowska, unpublished results).

Quite unexpected results were obtained with the frog. In all mature frogs, irrespective of the seasonal physiological conditions, the activity of aminotransferase was high, 2 - 4 times that found in the rat, but neither in spring and summer nor in winter was it possible to demonstrate the presence of *p*-hydroxyphenylpyruvate oxidase. Although the enzyme catalysing the formation of homogentisate was not present, the enzyme oxidizing homogentisate was found to be fairly active.

The lack of only one enzymic reaction in the tyrosine breakdown chain in the frog would be in some respect analogous to alkaptonuria, an inborn error of metabolism in man. In 1958 La Du, Zannoni, Laster & Seegmiller [10] studying a fragment of liver tissue taken *intra operationem* from an alkaptonuric patient, found that homogentisate oxygenase activity was the only intermediate step missing, while the activities of tyrosine aminotransferase, *p*-hydroxyphenylpyruvate oxidase, maleylacetoacetate isomerase and fumarylacetoacetate hydrolase did not differ from those of normal subjects. La Du *et al.* were also unable to demonstrate the presence of an inhibitor of the missing enzyme.

The lack of *p*-hydroxyphenylpyruvate oxidase in the frog seems to be analogous to another defect of tyrosine metabolism in man which has been described by Medes and termed tyrosinosis [14]. The patient excreted in the urine *p*-hydroxyphenylpyruvate and *p*-hydroxyphenyllactate, presumably due to the inability to oxidize *p*-hydroxyphenylpyruvate.

In the snail, the aminotransferase activity was but slight, and the subsequent enzymes of tyrosine metabolism could not be demonstrated. Although the extracts from the hepatopancreas in the presence of *p*-hydroxyphenylpyruvate utilized oxygen, the oxidation did not lead to the formation of homogentisate, and also homogentisate was not oxidized.

These results are similar to observations made on insects. Sekeris & Karlson [19] demonstrated the presence of tyrosine-*α*-ketoglutarate aminotransferase in *Calliphora* larvae, but the hydroxyphenylpyruvate formed was reduced to *p*-hydroxyphenylpropionate. A low activity of aminotransferase in pupae and adult moth of *Celerio euphorbiae* was observed by Bełżecka, Laskowska & Mochnacka [1], while the attempts at demonstration of the oxidation of *p*-hydroxyphenylpyruvate in homogenates from caterpillars, pupae in summer, and adult moth, were unsuccessful (Michałek-Moricca, unpublished results). Only in the diapauzing pupae, similarly as in the snail, oxygen consumption in the presence of *p*-hydroxyphenylpyruvate was observed, but the formation of homogentisate could not be demonstrated. It seems, therefore, that in the invertebrates tested so far, i.e. in the snail *Helix pomatia*, in *Calliphora* and in *Celerio euphorbiae*, the main pathway of tyrosine metabolism differs from that found in mammals.

SUMMARY

1. It was found that in the livers of pigeon, lizard, perch and pike, tyrosine is oxidatively decomposed to fumarate and acetoacetate, similarly as in mammals.

2. In adult frog, one of the intermediate steps, p-hydroxyphenylpyruvate oxidase, could not be demonstrated. It seems that a metabolic block is involved.

3. In snail hepatopancreas, only tyrosine aminotransferase was demonstrated, and the breakdown of tyrosine seems to proceed through a different pathway.

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BADANIA PORÓWNAWCZE NAD PRZEMIANĄ TYROZYNY U RÓŻNYCH ZWIERZĄT

Streszczenie

1. Stwierdzono, że w wątrobie gołębia, jaszczurki, sandacza i szczupaka tlenowy rozkład tyrozyny biegnie taką samą drogą jak u ssaków do fumaranu i acetooctanu.

2. U dorosłej żaby nie udało się wykazać jednego enzymu z ciągu reakcji, oksydazy p-hydroksyfenylopyrogronianu. Wydaje się, że jest to blok metaboliczny.

3. W hepatopancreas ślimaka wykazano jedynie aminotransferazę tyrozyny; prawdopodobnie rozkład tyrozyny idzie inną drogą.

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# ENERGY-LINKED REDUCTION OF THE MITOCHONDRIAL NICOTINAMIDE-ADENINE DINUCLEOTIDES BY CHOLINE AND SARCOSINE

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In 1957 Chance & Hollunger [2] found that nicotinamide-adenine dinucleotides of tightly coupled rat heart mitochondria could be largely reduced upon the addition of succinate. In mitochondria of locusta flight muscle and in rat skeletal muscle mitochondria the same result could be obtained when glycero-1-phosphate was used as substrate [8]. Further studies carried out in various laboratories showed that this effect was dependent on the supply of energy and it has been interpreted as an energy-linked reversal of the electron flow through the respiratory chain [6].

The purpose of this work was to study the influence of choline and sarcosine, two substrates oxidized directly by flavoprotein enzymes [3, 10] on the degree of reduction of the mitochondrial nicotinamide-adenine dinucleotides.

## MATERIALS AND METHODS

Wistar albino rats weighing from 220 to 250 g. were used without prior starving. The animals were killed by decapitation, the livers removed immediately, immersed in ice-cold 0.25 M-sucrose solution and washed several times with this medium. Mitochondria were prepared in 0.25 M-sucrose - 2 mM-EDTA - 0.03 M-nicotinamide by differential centrifugation (first centrifugation at 500 *g*, second at 4500 *g*), washed once and finally suspended in the same medium.

The oxygen uptake and oxidative phosphorylation were measured polarographically with Clark oxygen electrode in 0.25 M-sucrose - 0.01 M--triethanolamine hydrochloride (TRA) - 1 mM-EDTA medium, pH 7.2. The experiments on the reduction of the mitochondrial nicotinamide--adenine dinucleotides were carried out in the same medium (for details see Tables). To facilitate the penetration of choline into the mitochondria, the experiments were carried out at 37° and the time of incubation was at least 10 min. Reduced and oxidized nicotinamide-

-adenine dinucleotides were determined enzymically according to the method of Klingenberg [5], reduced forms in alkaline and oxidized in acid mitochondrial extracts; alcohol dehydrogenase was used to determine NAD, lactic dehydrogenase to determine reduced NAD, and glutamate dehydrogenase for determination of reduced NADP. Protein was determined by the biuret method as described by Szarkowska & Klingenberg [15].

Substrates and cofactors were commercial products: ATP (Pabst Lab., Milwaukee, Wisc., USA), ADP (Sigma, St. Louis, Mo. USA), choline chloride and sarcosine (Light Co. Ltd. Colnbrook, Bucks, England); betaine aldehyde (formylmethylammonium ion) was synthesized according to the method of Bergel *et al.* [1]. Alcohol dehydrogenase was prepared from baker yeast by the method of Racker [12], lactic dehydrogenase from beef heart muscle according to Neilands [11] and glutamate dehydrogenase from beef liver according to Strecker [14]. Other reagents were of Polish origin.

## RESULTS

### *Oxygen uptake and respiratory control with choline and sarcosine as substrates*

It is known that choline does not enter freely intact mitochondria and therefore only after repeating freezing and thawing or another treatment resulting in disintegration of the mitochondrial membrane, choline is

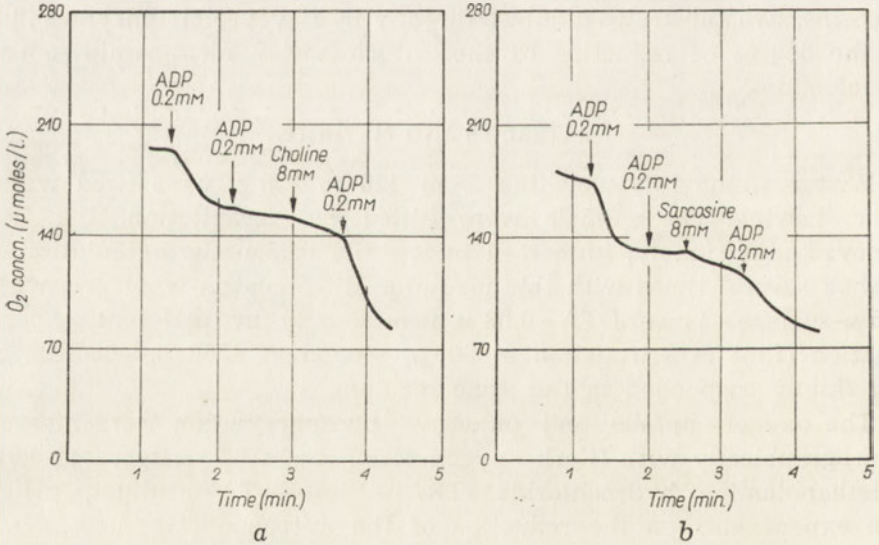


Fig. 1. Oxygen uptake and respiratory control (a), with choline and (b), with sarcosine. Rat liver mitochondria (12 mg. protein) were preincubated in 1 ml. sucrose - triethanolamine hydrochloride - EDTA medium, pH 7.2, with 2.5 mM-$P_i$ and 1.6 mM-ADP. The incubation mixture was aerated to prevent anaerobiosis.

oxidized to an appreciable extent [16]. Since in tightly coupled, freshly prepared mitochondria choline oxidation is not too efficient, in our experiments greater amount of mitochondria had to be used to obtain measurable results. The dense mitochondrial suspension used contained a great amount of endogenous substrates exhibiting a respiratory control and therefore, to obtain a clear picture, choline could be added only after all endogenous substrates had been exhausted by adding repeatedly small amounts of ADP. Under such conditions, mitochondria were able to oxidize choline and exhibit respiratory control (Fig. 1a). When sarcosine was used instead of choline, oxidation and respiratory control were also observed (Fig. 1b).

*Reduction of nicotinamide-adenine dinucleotides by choline*

Table 1, experiment 1, shows that when choline was used as substrate there occurred an appreciable reduction of the mitochondrial nicotinamide-adenine dinucleotide similar to that obtained with succinate. However, the addition of ATP in the presence of choline caused a decrease in the reduction. The direct product of choline oxidation, betaine aldehyde, is oxidized in mitochondria by NAD, and in our experiments, the reduction of NAD by betaine aldehyde was very pronounced (Table 1, expt. 2). On the other hand, betaine, the product of betaine aldehyde oxidation, upon the addition of ATP could be

Table 1

*The reduction of mitochondrial NAD by succinate, choline and betaine aldehyde*

Medium: 0.25 M-sucrose - 10 mM-TRA - 1 mM-EDTA, pH 7.2. Substrate concn., 10 mM; ATP, 3.3 mM; rat liver mitochondria, 14 mg. protein/ml. Incubation mixture was flushed with $O_2$ for 5 min., then incubated for 10 min. at 37°. Reduced and oxidized NAD was estimated. Σ NAD represents the sum of NAD and reduced NAD.

| Expt. no. | Addition | Reduced NAD (μmoles/g. protein) | Reduced NAD/Σ NAD |
|---|---|---|---|
| I | None | 0.16 | 0.05 |
| | Choline | 1.32 | 0.40 |
| | Choline and ATP | 1.07 | 0.32 |
| | Succinate | 1.36 | 0.40 |
| | Succinate and ATP | 1.81 | 0.56 |
| II | None | 0.40 | 0.12 |
| | Choline | 1.44 | 0.39 |
| | Choline and ATP | 1.07 | 0.28 |
| | Betaine aldehyde | 1.68 | 0.45 |
| | Betaine aldehyde and ATP | 1.68 | 0.45 |

expected to accept hydrogens from reduced NAD and thus push the reaction in the reverse direction, decreasing the level of reduced NAD. However, in our experiments ATP did not affect the reduction level of NAD.

To eliminate the possible effect of betaine aldehyde, arsenite was

Table 2

*The effect of arsenite on the reduction of NAD*

Medium: sucrose - TRA - EDTA, pH 7.2. Substrate concn., 8 mM; mitochondria, 16.4 mg. protein/ml. Incubation mixture was flushed with $O_2$ for 5 min., then incubated for 10 min. at 37°.

| Expt. no. | Addition | NAD (μmoles/g. protein) | |
|---|---|---|---|
| | | no arsenite | 2 mM-arsenite |
| I | None | 2.13 | 2.64 |
| | Succinate | 1.09 | 1.78 |
| | Choline | 1.21 | 1.84 |
| | Betaine aldehyde | 1.50 | 2.58 |
| II | None | 2.04 | 2.80 |
| | Sarcosine | 1.34 | 1.77 |
| | Formaldehyde | 0.73 | 2.91 |

Table 3

*The reduction of nicotinamide-adenine dinucleotides in the system inhibited by cyanide*

Medium: sucrose - TRA - EDTA, pH 7.2. Substrate concn. 8 mM; arsenite, 2 mM; mitochondria, 16 mg. protein/ml. Incubation mixture was flushed with $O_2$ for 5 min., then KCN was added to 1 mM concn., followed by the substrate, and 3.3 mM-ATP where indicated; then $N_2$ was passed for 3 min. The tubes were closed with glass stoppers under the stream of $N_2$. In one experiment after 10 min. of incubation with choline, ATP was added and the incubation was continued for further 5 min.

| Addition | Reduced NAD (μmoles/g. protein) | Reduced NAD/ Σ NAD | Reduced NADP (μmoles/g. protein) |
|---|---|---|---|
| None | 0.18 | 0.06 | 0.47 |
| ATP | 0.20 | 0.06 | 0.47 |
| Succinate | 0.74 | 0.25 | 1.50 |
| Succinate and ATP | 1.48 | 0.50 | 2.62 |
| Choline | 1.07 | 0.37 | 2.18 |
| Choline and ATP | 1.07 | 0.37 | 2.18 |
| Choline, and ATP added after 10 min. incubation | 1.62 | 0.53 | 3.03 |
| Betaine aldehyde | 0.35 | 0.11 | 0.62 |
| Betaine aldehyde and ATP | 0.35 | 0.11 | 0.62 |

added to the incubation mixture. It was found that arsenite, an inhibitor of oxidation of endogenous substrates [13], suppressed also the NAD--linked oxidation of betaine aldehyde (Table 2), therefore further experiments on choline oxidation were carried out in the presence of 2 mM--arsenite to eliminate the effect of betaine aldehyde. KCN was also added to the incubation mixture. In spite of the presence of arsenite, ATP added simultaneously with choline did not increase the reduction of NAD (Table 3). Since ATP is a mitochondria contracting agent, it might diminish the permeability of the mitochondrial membrane for choline; therefore ATP was added after preliminary 10 min. incubation of mitochondria with choline. Under these conditions ATP caused an increase in the level of reduced dinucleotides similar to that obtained with succinate. The same effect of ATP was obtained in the system to which Antimycin A was added instead of KCN (Table 4). Both these

Table 4

*The reduction of nicotinamide-adenine dinucleotides in the system inhibited by Antimycin A*

Conditions as described in Table 3, except that Antimycin A, 3 μg./mg. protein, was added instead of KCN. Mitochondria, 14.6 mg. protein/ml.

| Addition | Reduced NAD (μmoles/g. protein) | Reduced NAD/ Σ NAD | Reduced NADP (μmoles/g. protein) |
|---|---|---|---|
| None | 0.18 | 0.06 | 0.42 |
| ATP | 0.18 | 0.06 | 0.50 |
| Choline | 0.83 | 0.28 | 1.34 |
| Choline, and ATP added after 10 min. incubation | 1.74 | 0.60 | 3.50 |
| Betaine aldehyde | 0.18 | 0.06 | 0.44 |
| Betaine aldehyde and ATP | 0.18 | 0.06 | 0.46 |

Table 5

*The effect of albumin on the reduction of nicotinamide-dinucleotides in the presence of choline*

Conditions as described in Table 3. Albumin, 1.2 mg./ml.; mitochondria, 15.7 mg. protein/ml. Incubation, 10 min. at 37°.

| Addition | Reduced NAD (μmoles/g. protein) | Reduced NAD/ Σ NAD | Reduced NADP (μmoles/g. protein) |
|---|---|---|---|
| None | 0 | 0 | 0.16 |
| Albumin | 0 | 0 | 0.13 |
| Choline | 0.94 | 0.31 | 1.40 |
| Choline and albumin | 1.62 | 0.55 | 3.06 |

experiments were carried out under nitrogen to avoid the possibility of auto-oxidation of cytochrome *b* in the choline oxidase chain.

Albumin, a known coupling agent, had an effect similar to that of ATP (Table 5).

*Reduction of nicotinamide-adenine dinucleotides by sarcosine*

When sarcosine was used as substrate, arsenite was also added to inhibit the oxidation of formaldehyde, the product of sarcosine oxidation. The effect of sarcosine on the reduction of nicotinamide-adenine dinucleotides was similar to that of choline, both in the presence of ATP and albumin (Table 6).

## DISCUSSION

From presented data it can be seen that choline and sarcosine, substrates oxidized without direct participation of nicotinamide-adenine dinucleotide, can reduce this dinucleotide. Similarly as with succinate and glycero-1-phosphate, the reduction is energy-linked and it may occur *via* the reversal of the electron flow through the respiratory chain. Upon the addition of choline the following reactions determine the oxido-reduction state of mitochondrial NAD: energy-linked reduction of NAD by choline and its direct reduction by betaine aldehyde (Fig. 2). Owing to its very low oxido-reduction potential, the aldehyde can reduce mitochondrial NAD to a high extent in the steady-state.

Table 6

*The effect of ATP and albumin on the reduction of nicotinamide-adenine dinucleotides in the presence of sarcosine*

Conditions as described in Table 3. Albumin, 1.2 mg./ml.

| Addition | Reduced NAD (μmoles/g. protein) | Reduced NAD/ Σ NAD | Reduced NADP (μmoles/g. protein) |
|---|---|---|---|
| None | 0.21 | 0.08 | 0.47 |
| ATP | 0.21 | 0.08 | 0.47 |
| Albumin | 0.21 | 0.08 | 0.47 |
| Sarcosine | 0.65 | 0.23 | 1.46 |
| Sarcosine, and ATP added after 10 min. incubation | 0.94 | 0.33 | 1.89 |
| Sarcosine and albumin | 1.17 | 0.40 | 2.23 |

From the thermodynamic considerations it follows that the reverse reaction is determined by the supply of energy. The equilibrium point will depend on the local concentration of substrates, their oxidation products and on the concentration of high-energy compounds. Our results obtained with betaine aldehyde in substrate concentration, do not indicate the shift of the equilibrium towards oxidation of reduced

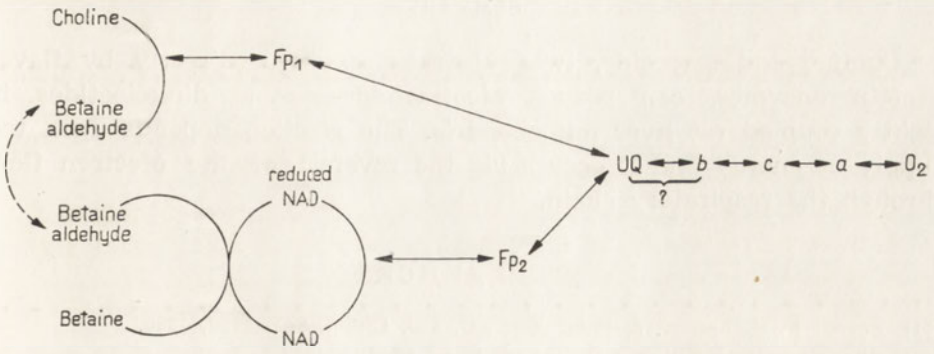


Fig. 2. Outline of choline and betaine aldehyde oxidation in liver mitochondria. $Fp_1$, choline dehydrogenase; $Fp_2$, reduced NAD dehydrogenase.

NAD upon the addition of ATP. This fact, however, does not exclude the possibility that during oxidation of choline in mitochondria the concentration of betaine aldehyde formed can under some circumstances be such that this reaction would be possible.

The use of arsenite, which inhibits the reduction of NAD by betaine aldehyde, creates a convenient system for the study of the reversal of electron flow with choline as substrate; it eliminates the possibility of the reduction of NAD by the formed aldehyde in consequence of competition either for a common with choline oxidative pathway [9], or for a common intermediate of oxidative phosphorylation. The presence of arsenite eliminates also the participation of endogenous substrates in NAD reduction.

The same considerations are valid with respect to sarcosine and the product of its oxidation, formaldehyde.

NADP undergoes similar oxido-reduction changes as NAD, probably due to the action of reduced NAD-NADP transhydrogenase. Taking the values of 3.2 μmoles/g. protein for NAD and 4.8 μmoles/g. protein for NADP in rat liver mitochondria [7], it seems that the equilibrium lies towards reduction of NADP.

According to Kimura *et al.* [4] the choline and succinate oxidase chains are different at the level of cytochrome *b*; the *b* component of choline oxidase chain may become auto-oxidizable under suitable conditions. Both oxidases operate through the respiratory chains, which are interlinked between cytochrome $c_1$ and oxygen. The presented possibility of NAD reduction by choline in the system inhibited by Antimycin A indicates the existence of an intercommunication between NAD and this part of the electron pathway which is different for choline.

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SUMMARY

Choline and sarcosine, two substrates oxidized directly by flavoprotein enzymes, can reduce nicotinamide-adenine dinucleotides in tightly coupled rat liver mitochondria. The reaction depends upon the supply of energy and it occurs *via* the reversal of the electron flow through the respiratory chain.

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REDUKCJA DWUNUKLEOTYDÓW NIKOTYNAMIDO-ADENINOWYCH W MITOCHONDRIACH PRZEZ CHOLINĘ I SARKOZYNĘ ZALEŻNA OD DOSTARCZENIA ENERGII

Streszczenie

Cholina i sarkozyna, substraty utleniane bezpośrednio przez flawoproteinowe enzymy, mogą redukować dwunukleotydy nikotynamido-adeninowe w skojarzonych mitochondriach wątroby szczura. Reakcja ta zależy od dostarczenia energii i zachodzi poprzez odwrócenie kierunku przepływu elektronów.

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*Note added in proof*: At the time this work was submitted for publication, a paper by G. Bianchi & G. F. Azzone dealing with pathways of choline oxidation in rat liver mitochondria, appeared in *J. Biol. Chem.* **239**, 3947, 1964.

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# PREPARATION AND PROPERTIES OF THE ARGINASE INHIBITOR FROM SUNFLOWER SEEDS

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Some new enzyme inhibitors of animal and plant origin have been recently described. Birk *et al.* [1] have isolated from soya beans a trypsin inhibitor of proteinlike nature. Lindberg [3] has partially purified an inhibitor of pancreatic deoxyribonuclease from calf spleen. Philpot & Stanier [5] have described a thermostable ribonuclease inhibitor of low molecular weight, obtained from leaf extracts of *Ligustrum vulgaris*. In our laboratory three new inhibitors of plant origin were found: an arginase inhibitor from sunflower seeds [6], an inhibitor of urease from poplar leaves [7, 9] and an inhibitor of ornithine carbamoyltransferase from carrot leaves [8].

In this paper is presented the partial purification of the arginase inhibitor from sunflower seeds, and some of its properties.

## MATERIALS AND METHODS

*Preparation of the inhibitor.* Kernels from sunflower seeds, 100 g., were finely ground in a porcelain mortar and suspended in 1 liter of distilled water. Glacial acetic acid, 10 ml., was slowly added, the suspension thoroughly mixed for about 1 hr. and centrifuged at 4 000 r.p.m. for several minutes. The supernatant solution covered with a layer of liquid fat, extracted from the seeds, was filtered through folded filter paper. The clear yellowish supernatant was then evaporated to dryness on a boiling water bath and the dry brown residue was dissolved in the cold in 50 ml. of methanol. The precipitate was centrifuged off and washed again with additional 50 ml. of methanol. After repeated centrifugation, the precipitate was discarded and the combined supernatants evaporated to dryness on a water bath, care being taken to remove most of the acetic acid. The dry residue was dissolved in 30 ml. of methanol, and 30 ml. of chloroform were slowly added under constant stirring. A viscous light brown matter which

separated on addition of the chloroform, was discarded. The methanol--chloroform mixture was carefully evaporated to dryness, the residue taken up in 10 ml. of distilled water, and to the clear solution 50 ml. of 2% barium hydroxide solution were added. The solution of barium hydroxide was added in one lot; a bulky bright yellow precipitate was formed which was immediately centrifuged for several minutes at 4000 r.p.m. The supernatant was then discarded and the precipitate suspended in 5 ml. of distilled water. Then 1 N-$H_2SO_4$ was added carefully to pH about 5, the precipitate dissolving on stirring, and the resulting barium sulphate was centrifuged off. The clear supernatant was concentrated to about 3 ml. and put on a Sephadex G-50 (coarse) column (2.3 cm. × 24 cm.). Distilled water was used as eluent with a flow rate of about one drop per 8 - 10 seconds. The dark brown fraction emerging as the first effluent was discarded and fractions were collected from the moment when one drop of the effluent gave a greenish-yellow test with 2 ml. of 5% NaOH. The first collected fraction of about 40 - 45 ml. contained the bulk of impurities with very little active material. The second fraction of 50 ml. had a light straw--yellow colour and contained almost all of the inhibitor. This fraction was evaporated *in vacuo* at room temperature and the dry material adhering to the walls of the vessel was removed with a stainless steel spatula. A dry mustard-coloured powder was obtained, with a yield of 60 to 80 mg. per 100 g. of sunflower seed kernels.

*Determination of arginase inhibition.* Aliquots of the inhibitor dissolved in 0.5 ml. of water were pipetted into small test tubes, followed by 0.5 ml. of a 1% suspension of acetone powder from 7-day-old plants of bitter lupin in maleate buffer, pH 7.0, as a source of arginase [4]. To this 0.5 ml. of a 0.5 M-solution of arginine brought to pH 10 with 0.2 N-HCl were added, and the mixture was incubated for 2 hr. at 38°. After incubation, 1 ml. of 0.2 N-HCl and 2 ml. of 0.25% urease (Nutr. Biochem. Corp.) solution in 0.15 M-phosphate buffer, pH 6.6, were added and again incubated for 30 min. at 38°; 1 ml. samples in quadruplicates were then distilled off, using the Conway technique [2]. For the estimation of arginase activity, the same procedure was employed, except that the inhibitor solution was replaced by 0.5 ml. of distilled water.

## RESULTS AND DISCUSSION

Figure 1 demonstrates the effect of the inhibitor concentration on arginase activity. Distinct inhibition was observed already at a concentration of 0.5 μg. of inhibitor per 1 ml. of the assay mixture. At 40 μg. the inhibition amounted to over 80% and no further inhibition could be obtained at higher concentrations of the inhibitor. Preincubation of the

inhibitor with the enzyme at neutral pH, caused no changes in the rate of inhibition as compared with samples that had not been preincubated.

In order to establish the nature of inhibition, experiments were conducted with various concentrations of the enzyme and of the substrate. Fig. 2 shows the effect of different concentrations of the inhibitor at

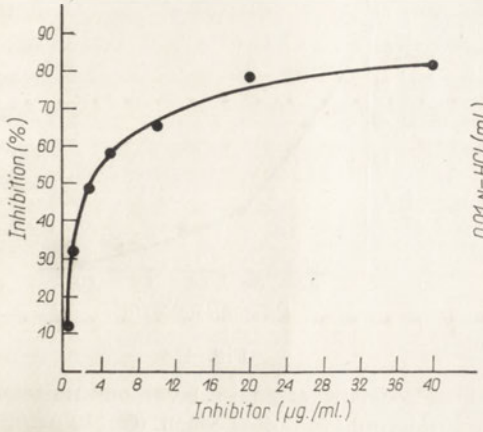


Fig. 1

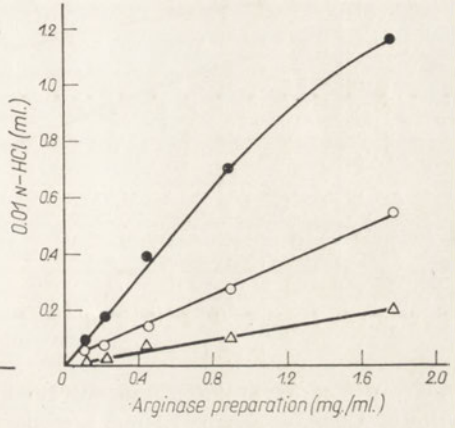


Fig. 2

Fig. 1. Inhibition of arginase activity as a function of inhibitor concentration. Arginase concn., 1.1 mg. acetone powder/ml.; arginine concn., 56 μmoles/ml.

Fig. 2. Effect of arginase concentration at constant substrate level on inhibition activity: (●), without inhibitor; (○), with 5 μg./ml. inhibitor; (△), with 20 μg./ml. inhibitor. Arginine concn., 56 μmoles/ml. Activity expressed in ml. 0.01 N-HCl, used in the Conway technique.

constant levels of substrate, when increasing amounts of the enzyme were added. The inhibition was proportional to the amount of the enzyme used. With decreasing amounts of the enzyme at the studied levels of the inhibitor, a corresponding rise in the rate of inhibition could be observed.

At constant enzyme and inhibitor levels, arginase activity was quite markedly released by growing concentrations of the substrate (Fig. 3). A two-fold increase of arginine caused a slight drop of inhibition activity, but a four-fold increase of the substrate caused a notable loss. As can be seen, this release of arginase activity was not proportional to the quantities of substrate and was furthermore not large enough to suggest competitive inhibition. Evidence reported later in this paper suggested that this release was due to partial destruction of the inhibitor at the alkaline pH values at which arginase is normally determined, additionally enhanced by the large excess of arginine present.

It has already been reported that L-cysteine reactivated arginase that had been inactivated by the inhibitor [6]. As can be seen from Table 1,

other reducing agents such as mercaptoethanol and reduced glutathione may also restore the original arginase activity that had been blocked by the inhibitor from sunflower seeds.

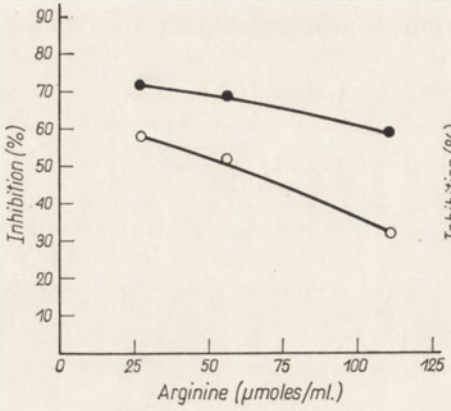


Fig. 3

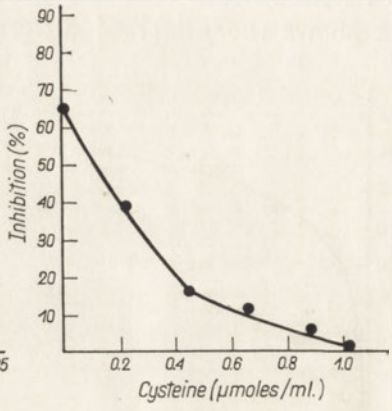


Fig. 4

Fig. 3. Effect of arginine concentration at constant arginase level on inhibition activity. Arginase concn., 1.1 mg. acetone powder/ml.; inhibitor concn. (●), 10 μg./ml.; (○), 2.5 μg./ml.

Fig. 4. Effect of L-cysteine on the inhibition of arginase by the inhibitor from sunflower seeds. Arginase concn., 1.1 mg. acetone powder/ml.; arginine concn., 56 μmoles/ml.; inhibitor concn., 10 μg./ml.

Table 1

*Effect of reducing agents on reactivation of arginase*

Conditions: Arginase concn., 1.1 mg. acetone powder/ml.; arginine concn., 56 μmoles/ml.; inhibitor concn., 4.7 μg./ml.; concn. of the respective reducing agent, 10 μmoles/ml.

| Addition | Arginase activity (%) |
|---|---|
| None | 100 |
| Inhibitor | 43 |
| Inhibitor and cysteine | 100 |
| Inhibitor and glutathione | 100 |
| Inhibitor and mercaptoethanol | 98 |

Figure 4 illustrates the reactivation by cysteine of the inhibited arginase; 0.22 μmoles of cysteine caused about 88% reactivation of arginase inhibited by 10 μg. of the inhibitor. We believe that cysteine titrations will be very helpful in the elucidation of the mechanism of arginase inhibition, once the inhibitor has been obtained in a chemically pure state.

*General properties of the inhibitor.* Our purest inhibitor preparation was only 23 times as active as the crude extract. Nevertheless it was possible to arrive at certain conclusions with respect to the nature of the inhibitor, the mechanism of its action and some of its properties.

The difficulties in purification of the inhibitor were primarily due to lack of a rapid and relatively exact method for the determination of the inhibitor. The purity of the substance could only be measured by the time-consuming assay of arginase inhibition. The additional difficulty consisted in the high sensitivity of the partially purified inhibitor to air oxidation. So for instance recycling of the purified material on Sephadex resulted in greatly decreased yield of the pure fraction with an additional small decline of its final activity as compared with the starting material. Filtration on Sephadex column in acid media, under nitrogen or with $H_2S$-containing water remained without effect. Also attempts to obtain purification by paper chromatography have largely failed. Various solvents were tried, such as benzene - acetic acid (9 : 1 and 1 : 1), isoamylalcohol - acetic acid (1 : 1), methanol - acetic acid (1 : 1), phenol - acetic acid (1 : 19), dioxan - acetic acid (1 : 1), cyclohexanol - acetic acid in various proportions. Paper electrophoresis was also unsuccessful. Only chromatography and rechromatography in butanol - acetic acid - - water (4 : 1 : 2) gave preparations of the inhibitor comparable to those obtained by the use of Sephadex, but the final yield was considerably lower.

Similarly column chromatography gave no satisfactory results. Amberlite IRC-50, DEAE-Sephadex, CM-Sephadex and activated silica gel caused total or partial losses of the inhibitor, without increasing the activity in any of the collected fractions. Chromatography on Cellite and cellulose were also unsuccessful. Sephadex G-25 and G-75 were less effective than Sephadex G-50.

As mentioned before, the purest obtained inhibitor preparations were relatively unstable in dilute neutral solutions. As can be seen from Table 2, the inhibitor was destroyed in neutral solutions even at a tem-

Table 2

*Effect of time on the stability of the inhibitor in dilute solutions in acid and neutral media at a temperature of 6 - 8°*

Initial concn. of inhibitor, 1.35 μg./ml. The results express the recovery, determined by measuring the inhibition of arginase activity, the quantity of the inhibitor, in μg./ml., being calculated from Fig. 1.

| Inhibitor recovered | Time of storage (days) | | |
|---|---|---|---|
| | 14 | 20 | 34 |
| In 0.01 N-HCl | 1.35 | 1.25 | 1.32 |
| In neutral solution | 0.35 | 0.25 | 0.15 |

perature of 6 - 8°, and after 14 days only 26% of its initial activity could be recovered. In acid solutions the inhibitor was stable and no changes were observed after 34 days at 6 - 8°.

For short periods of time (15 min.) the inhibitor was stable both in neutral and acid solutions even at a temperature of 100°. In alkaline solution the activity of the inhibitor dropped rapidly and heating for 1 min. in 0.1 N-NaOH caused a 70% loss of initial activity (Table 3).

Table 3

*Effect of heating at 100° on the stability of the inhibitor*

Initial concn. of inhibitor, 7.8 μg./ml. The amount of inhibitor recovered was calculated as for Table 2.

| Time of heating (min.) | Inhibitor recovered (μg./ml.) | | Recovery in 0.1 N-NaOH (%) |
|---|---|---|---|
| | in neutral solution | in 0.1 N-NaOH | |
| 1 | 7.5 | 2.1 | 27 |
| 2 | 7.7 | 2.1 | 27 |
| 5 | 7.5 | 1.0 | 13 |
| 10 | 7.7 | 0.95 | 12 |
| 15 | 7.8 | 0.8 | 10 |

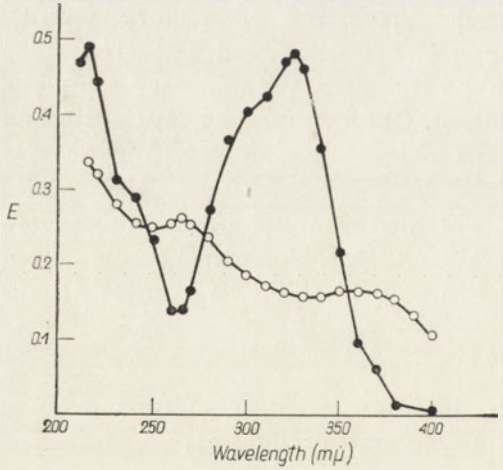


Fig. 5. Ultraviolet spectrum of the inhibitor from sunflower seeds, (●), in neutral solution; (○), in alkaline solution, pH 12. Inhibitor concn., 10 μg./ml.

The ultraviolet absorption spectrum (Fig. 5) of the inhibitor showed absence of protein and nucleic acids. In neutral and acid media the spectra were identical. However, in alkaline solution at pH 12 already after 30 min. the absorption spectrum has changed irreversibly, showing destruction of the inhibitor. The broad peak in alkaline solution in conjunction with positive phenol tests, suggest that the inhibitor may be

a compound of polyphenol nature. No attempt was made to interpret the UV spectrum, as the degree of purity of the inhibitor is not yet known.

The inhibitor is a substance of low molecular weight, as was shown by its behaviour on the Sephadex column. It was well retained by the grains of the gel and emerged last on continued elution. Furthermore, the inhibitor dialysed easily through semipermeable membranes (Table 4). After 1.5 hr. dialysis against 2.5 volumes of water, 50% of the inhibitor was dialysed away, and after 26 hr. only 5% of the inhibitor remained in the dialysing bag.

Table 4

*Effect of dialysis of the inhibitor at a temperature of 6 - 8°*

Inhibitor solution, 8 ml., was dialysed against 20 ml. of water, under constant stirring of the external fluid, which was renewed at times indicated in the Table. The amount of inhibitor was calculated as for Table 2. After 26 hr. of dialysis, 4.23 mg. of the inhibitor remained in the dialysing bag.

| Successive change of water | Time of dialysis (hr.) | Inhibitor in the external fluid (mg.) |
|---|---|---|
| 1 | 1.5 (0 - 1.5) | 43.8 |
| 2 | 1.5 (1.5 - 3) | 14.3 |
| 3 | 1.5 (3 - 4.5) | 15.0 |
| 4 | 1.5 (4.5 - 6) | 6.8 |
| 5 | 1.0 (6 - 7) | 3.2 |
| 6 | 19 (7 - 26) | 4.6 |

In conclusion it must be mentioned that standard optimal conditions for determination of arginase activity were detrimental to the stability of the inhibitor. Arginase is normally determined at pH 10 in presence of a large excess of arginine. These conditions caused noticeable destruction of the inhibitor, particularly when it was present in very low concentrations. In the future it will, therefore, be essential to relinquish the advantage of arginase determinations under conditions of maximum activity, and accept lower rates of the reaction by lowering the pH value of the medium and by decreasing the concentration of the substrate. In this way conditions of relative stability for the inhibitor will be obtained and precise studies on the mechanism of inhibition will be facilitated.

SUMMARY

1. The arginase inhibitor from sunflower seeds has been purified about 23 times over the activity of crude extracts.

2. The action of the inhibitor is due to direct binding with the

enzyme. L-Cysteine, mercaptoethanol and glutathione overcome the inhibition.

3. The inhibitor is a low-molecular compound as shown by Sephadex-gel ultrafiltration and by dialysis. It is stable in acid solution, relatively stable in neutral solution and is destroyed in alkaline solution.

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## PRZYGOTOWANIE I WŁASNOŚCI INHIBITORA ARGINAZY Z NASION SŁONECZNIKA

### Streszczenie

1. Otrzymano preparat inhibitora arginazy oczyszczony około 23 razy w porównaniu z aktywnością surowych ekstraktów.

2. Aktywność inhibitora polega na związaniu się z enzymem. L-Cysteina, merkaptoetanol i glutation reaktywują arginazę zahamowaną przez inhibitora.

3. Inhibitor jest związkiem niskocząsteczkowym, jak wynika z ultrafiltracji na Sephadex'ie i dializy. Jest on trwały w środowisku kwaśnym, względnie trwały w środowisku obojętnym i ulega zniszczeniu w środowisku alkalicznym.

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## RECENZJE KSIĄŻEK

ADVANCES IN ENZYME REGULATION (G. Weber, ed.), vol. I. Pergamon Press, London 1963; str. 420, cena £ 5. Vol. II, Pergamon Press, London 1964; str. 405, cena £ 5.

Nowe to wydawnictwo seryjne przynosi pełną publikację wykładów i dyskusji z dorocznych sympozjów odbywanych z początkiem października w Indianopolis, U.S.A.

Tom I obejmuje 4 sesje pierwszego sympozjum z r. 1962, poświęcone kolejno: biochemii regulacji aktywności i syntezie enzymów (przewodniczący W. E. Knox); fizjologii i regulacji hormonalnej (przewodniczący J. W. Wilson); patologii i działaniu leków (przewodniczący V. R. Potter) oraz regulacji aktywności enzymatycznej w nowotworach (przewodniczący S. Weinhouse). Poza tym specjalny wykład o glukoneogenezie nerkowej wygłosił Sir H. A. Krebs. Na każdą sesję składało się 4 - 6 wykładów oraz ogólna dyskusja, ewentualnie z podsumowaniem przewodniczącego lub jego referatem uzupełniającym. Większość tematów dotyczyła enzymów wątroby normalnej oraz hepatoma.

Tom II przynosi materiały z sympozjum z r. 1963. Tematem pierwszej sesji, której przewodniczył Sir H. A. Krebs, była enzymatyczna regulacja glukoneogenezy. Specjalny wykład sympozjum i w tym roku dotyczył glukoneogenezy i przygotowany był przez Krebsa i współpracowników. Druga sesja pod przewodnictwem J. W. Wilsona traktowała o glukoneogenezie w różnych stanach fizjologicznych i patologicznych. Trzecia sesja pod przewodnictwem C. F. Cori'ego poświęcona była regulacji aktywności glukokinazy. V. R. Potter przewodniczył czwartej sesji, dotyczącej regulacji aktywności i syntezy enzymów wątroby drogą sprzężenia zwrotnego („feedback"). W sesji piątej (przewodniczący W. E. Knox) omawiano regulację poprzez zmianę stężeń kofaktorów, a w ostatniej, szóstej (przewodniczący S. Weinhouse) regulację enzymów w hepatoma.

Zarówno tematy poszczególnych sesji obu sympozjów, jak i osoby przewodniczących charakteryzują to nowe wydawnictwo jako cenne źródło informacji z pierwszej ręki na temat regulacji czynności enzymatycznej komórki, a więc i regulacji metabolizmu w ogóle. Ważnym uzupełnieniem są wykazy literatury dołączone do każdego wykładu.

Nowe to wydawnictwo musimy uznać za wielkie ułatwienie dla biochemików pracujących nad enzymami oraz nad zagadnieniami regulacji metabolizmu. Poza tym tomy te będą interesującą lekturą dla ogółu biochemików, fizjologów i lekarzy, których uwagę w coraz większym stopniu przyciągają zagadnienia organizacji życia na poziomie molekularnym.

*Józef Heller*

D. A. Hall. ELASTOLYSIS AND AGEING. Charles C. Thomas Publ., Springfield (Ill.) 1964; str. XIII + 160; cena $ 6.75.

Wiele monografii poświęconych jest biochemii tkanki łącznej. Dotyczą one jednak głównie substancji podstawowej oraz włókien kolagenowych, a włóknom elastycznym poświęca się zwykle bardzo mało miejsca. Przyczyną tego stanu jest z jednej strony stosunkowo mała zawartość tych włókien w tkance łącznej, z drugiej zaś strony wiadomości o ich budowie i własnościach były do niedawna niewystarczające, aby je można było połączyć w logiczną całość. W ostatnich dziesiątkach lat ukazał się szereg prac wskazujących na duże znaczenie włókien elastycznych w strukturze tkanki łącznej normalnej oraz w stanach patologicznych, np. w arteriosklerozie.

Recenzowana książka jest monografią, która wyczerpująco omawia biochemię tkanki elastycznej i w ten sposób uzupełnia piśmiennictwo poświęcone tkance łącznej. Autor przytacza około 300 pozycji literatury, w tym kilkadziesiąt pozycji własnych i swoich współpracowników. Pozycje własne dotyczą szeregu zagadnień, zaczynając od struktury włókien elastycznych, poprzez badania nad enzymami biorącymi udział w rozkładzie tkanki elastycznej, kończąc na zmianach zachodzących w stanach patologicznych.

Książka składa się z pięciu rozdziałów. W rozdziale pierwszym krótko omówiono terminologię używaną w dalszej części pracy. Jest to konieczne, ponieważ różni autorzy używają różnych nazw dla tych samych substratów lub enzymów.

Rozdział drugi poświęcony jest biochemii tkanki elastycznej. Szeroko tu omówiono morfologię włókien elastycznych łącznie z ich ultrastrukturą, metody izolowania, sposoby oznaczania oraz skład aminokwasowy. Omówiono też udział lipidów i węglowodanów w budowie włókien elastycznych. Na końcu rozdziału przedstawiono zmiany jakościowe i ilościowe zachodzące podczas starzenia się.

Rozdział trzeci, najobszerniejszy, poświęcony jest enzymom rozkładającym tkankę elastyczną, a więc głównie elastazie i elastolipoproteinazie. Przedstawiono tam rozmieszczenie tych enzymów w tkankach, sposoby izolowania (podano szczegółowo przepis na oczyszczanie elastazy stosowany przez Autora), omówiono także szeroko własności chemiczne i fizykochemiczne tych enzymów, oraz sposoby oznaczania aktywności. Na końcu tego rozdziału przedstawiono w ciekawych schematach proponowany mechanizm działania elastazy i elastolipoproteinazy, który dobrze tłumaczy z jednej strony udział jonów wapnia i lipopolisacharydów w budowie włókien elastycznych, a z drugiej strony współdziałanie elastazy i elastolipoproteinazy w rozkładzie tych białek.

Rozdział czwarty poświęcony jest czynnikom hamującym elastolizę. Omówiono tutaj czynniki drobnocząsteczkowe (EDTA) oraz czynniki wielkocząsteczkowe, termolabilne zawarte w surowicy krwi, omówiono też dotychczasowe dane doświadczalne dotyczące inhibitorów elastazy zawartych w ścianie naczyniowej.

W rozdziale piątym (ostatnim) przedstawiono zmiany zachodzące w tkance elastycznej podczas starzenia się oraz w arteriosklerozie. Z przedstawionych badań wynika, że zmiany te dotyczą głównie aktywności enzymów rozkładających włókna elastyczne, co odbija się na zawartości tych włókien w tkance łącznej. Zmiany te łączą się ze zmianami w innych elementach tkanki łącznej, tak że na podstawie dotychczasowych badań trudno jeszcze wyrobić sobie szerszy pogląd na to zagadnienie.

Chociaż niejednokrotnie doświadczenia są jeszcze niekompletne, a czasami i kontrowersyjne, przejrzysty i przystępny sposób podawania niekiedy zawiłych zagadnień czyni książkę dostępną wszystkim badaczom, którzy interesują się biochemią i patologią tkanki łącznej.

*Wiktor Rzeczycki*

CHEMICAL AND BIOLOGICAL ASPECTS OF PYRIDOXAL CATALYSIS. Proceedings of a Symposium of the International Union of Biochemistry, Rome (E. E. Snell, P. M. Fasella, A. E. Braunstein & A. Rossi Fanelli, eds.) Pergamon Press, Oxford - London - New Jork - Paris 1963; str. 599, cena £ 7.

Znaczenie witaminy $B_6$ jako związku kluczowego w przemianach białkowych jest ogólnie znane. Wielkim osiągnięciem ostatnich lat jest poznanie mechanizmu reakcji katalizowanych przez pirydoksal. Badania te otworzyły nowe perspektywy w zakresie zrozumienia czynności enzymów, przedmiotu o zasadniczym znaczeniu ze względów teoretycznych jak i praktycznych dla wszystkich nauk biologicznych.

Konieczność zorganizowania zebrania poświęconego wyłącznie roli pirydoksalu w procesach chemicznych i biologicznych wyłoniła się w czasie V Międzynarodowego Kongresu Biochemii w Moskwie w 1961 r. Jako miejsce spotkania wybrano Rzym i w październiku 1962 r. odbyło się Sympozjum zorganizowane przez Międzynarodową Unię Biochemiczną. Na sympozjum złożyło się 46 referatów wygłoszonych przez najwybitniejszych znawców tego przedmiotu; przeważnie były to referaty dotyczące ostatnio wykonanych prac. Całość wydano w 30-tym tomie serii sympozjalnej Unii. Po każdym referacie zamieszczono dyskusję uzupełniającą omawiane zagadnienia.

Sympozjum otwiera referat E. E. Snella z Berkeley, odkrywcy mechanizmu nieenzymatycznej transaminacji. Autor podał ogólny przegląd reakcji nieenzymatycznych katalizowanych przez pirydoksal oraz przedstawił proponowane schematy ich przebiegu. Treść siedmiu następnych referatów dotyczyła badań nad rolą pirydoksalu i jego pochodnych w nieenzymatycznej transaminacji. A. E. Martell z Chicago, C. Cennamo z Modeny oraz B. Pullman z Paryża omówili w swych referatach mechanizm tworzenia i hydrolizy zasad Schiffa. Nad wyjaśnieniem mechanizmu tych reakcji pracowali również T. C. Bruice i R. M. Topping z Nowego Jorku, którzy oprócz analogów pirydoksalu używali układów zawierających imidazol i jon imidazjolowy w badaniach nad transaminacją kwasu $\alpha$-aminofenylooctowego. F. Olivo, C. S. Rossi i N. Siliprandi z Instytutu Biochemii w Padwie przedstawili swoje badania nad nieenzymatyczną transaminacją kwasu $\gamma$-aminomasłowego.

Dalsze referaty były poświęcone roli pirydoksalu w układach biologicznych. Kilka z nich dotyczyło najbardziej zbadanej aminotransferazy kwas glutaminowy - kwas szczawiooctowy. I tak I. W. Sizer i W. T. Jenkins z U.S.A. przedstawili otrzymanie oczyszczonego enzymu w formie pirydoksalowej i pirydoksaminowej, potwierdzając tym teorię mechanizmu Snella i Braunsteina. C. Turano i współpracownicy z Rzymu podali skład aminokwasowy tego enzymu. O. L. Polyanovsky i Yu. M. Torchinsky ze Związku Radzieckiego omówili rolę grup -SH w jego działaniu. Badacze japońscy Y. Morino i H. Wada podali różnice pomiędzy oczyszczonym enzymem wyizolowanym z frakcji mitochondrialnej wątroby i wyizolowanym z supernatantu. Barbara Banks z Londynu ze współpracownikami przedstawiła badania kinetyczne nad aminotransferazą wyizolowaną z mięśnia sercowego świni.

Kilka referatów poświęcono dekarboksylacji aminokwasów. $\beta$-Dekarboksylazą kwasu asparaginowego zajmowali się Jeanne Cattanéo i J. C. Senez z Marsylii oraz A. Meister i współpracownicy z Bostonu (U.S.A.). Omówiono również dekarboksylazę leucyny (H. K. King z Liverpoolu) i aminokwasów aromatycznych (S. Udenfriend z Bethesda), zaś badania nad hamowaniem dekarboksylazy przedstawił S. R. Mardashev z Moskwy.

Oprócz transaminacji i dekarboksylacji są jeszcze inne reakcje biochemiczne aminokwasów katalizowane przez enzymy wymagające do swego działania fosfopirydoksalu. Rola koenzymu w tych reakcjach nie została jeszcze wyjaśniona. Ba-

dacze włoscy D. Cavallini i współpracownicy, F. Chapeville i P. Fromageot z Francji oraz M. Flavin z Bethesda przedstawili badania nad enzymami pirydoksalowymi katalizującymi przemianę siarki w aminokwasach siarkowych. O. Hayaishi, M. Gefter i H. Weissbach z Bethesda badali dehydrazę treoninową z *Cl. tetanomorphum*. K. T. Yasunobu i H. Yamada omówili rolę pirydoksalu i miedzi jako grupy prostetycznej w oksydazie monoaminowej z osocza krwi. Interesujący referat R. Kapeller-Adler dotyczył histaminazy, flawoproteidu wymagającego do swego działania fosforanu pirydoksalu. Badania E. H. Fischera i współpracowników (Washington) dotyczyły roli fosfopirydoksalu w strukturze cząsteczki fosforylazy mięśniowej.

Oddzielną grupę referatów poświęcono zagadnieniom tego typu jak wpływ hormonów tarczycy na czynność enzymów pirydoksalowych, zreferowany przez F. Chatagner i B. Jollés-Bergeret, oraz rola pirydoksalu w transporcie aminokwasów w komórkach nowotworu Ehrlicha, podana przez H. Christensena. Badacze radzieccy przedstawili trzy referaty dotyczące inhibitorów pirydoksalowych enzymów a badacze z Tokio swoje badania nad działaniem toksopirymidyny. E. E. Snell ze współpracownikami podał wyniki badań nad rozpadem witaminy $B_6$ przy udziale bakteryjnych układów enzymatycznych.

Sympozjum zakończył referat A. E. Braunsteina, odkrywcy procesu transaminacji. Celem referatu było dokonanie ogólnego przeglądu reakcji enzymatycznych zależnych od pirydoksalu. Zadanie to nie było łatwe, ponieważ, jak powiedział Braunstein, „wielu biochemików uważa, że łatwiej jest sporządzić listę reakcji zachodzących w przemianie aminokwasów bez udziału fosfopirydoksalu, aniżeli reakcji zachodzących z jego udziałem". Braunstein zbierając dotychczasowe wiadomości podzielił reakcje enzymatyczne katalizowane przez pirydoksal na cztery zasadnicze typy. Do trzech pierwszych zaklasyfikował reakcje enzymatyczne prowadzące do odszczepienia i podstawienia przy $\alpha$-C, $\beta$-C i $\gamma$-C, do czwartego typu zaszeregował nietypowe układy pirydoksalowe. W ten sposób sporządzona szczegółowa klasyfikacja stanowi doskonały przegląd i uporządkowanie zagadnień związanych z metaboliczną rolą pirydoksalu.

*Krystyna Bełżecka*

A. I. Oparin, THE CHEMICAL ORIGIN OF LIFE (Translated from the Russian by Ann Synge), Charles C. Thomas Publ., Springfield (Ill.) 1964; str. 124 +XXVII, cena $ 6,75.

Omawiana książka jest dalszym tomem z serii „American Lectures in Living Chemistry"; zawiera ona poglądy Oparina na chemiczne pochodzenie życia na ziemi, przedstawione w sposób jasny i ciekawy. Jest to lektura tym ciekawsza, że A. I. Oparin jest jednym z pierwszych biochemików, którzy dążyli do laboratoryjnego stworzenia takich warunków, w jakich prawdopodobnie powstawało prymitywne życie, i był organizatorem pierwszego Sympozjum poświęconego pochodzeniu życia na ziemi, które odbyło się w Moskwie w r. 1957.

Słusznie pisze Autor na wstępie, że od wielu setek lat trzy wielkie zagadnienia przyciągają umysły ludzkie. Jest to problem istoty zjawiska życia, jego pochodzenia i jego rozprzestrzenienia we wszechświecie. Logiczny wydaje się wniosek, że łatwiej byłoby zrozumieć istotę życia, gdybyśmy znali dokładnie drogi jego powstawania na ziemi; łatwiej byłoby także wówczas wykazać i zrozumieć ewentualne życie na innych planetach, do których zapewne człowiek wkrótce dotrze.

W pięciu rozdziałach swojej książki, Autor, posługując się faktami z zakresu geologii, paleontologii i biochemii porównawczej, przedstawia swoją teorię powsta-

nia życia na ziemi. We wprowadzeniu omawia on ogólne założenia tej teorii i wymienia trzy stadia rozwoju substancji organicznych, które, jego zdaniem, musiały mieć miejsce przed pojawieniem się życia na ziemi. Następnie omawia kolejno początkowe stadia ewolucji związków węgla, formowanie się „pierwotnej pożywki" (primaeval broth), powstawanie hipotetycznych koacerwatów i protobiontów, które prawdopodobnie mogły istnieć jeszcze wówczas, kiedy atmosfera ziemska miała charakter redukujący, a więc przed pojawieniem się w niej tlenu. Dalszy rozdział poświęcony jest rozważaniom na temat sposobu, w jaki mogły się rozwinąć z protobiontów najprymitywniejsze organizmy jednokomórkowe i wreszcie zwierzęta wielokomórkowe.

W ostatnim rozdziale Autor zbiera wnioski z teoretycznych rozważań i faktów przedstawionych w poprzednich rozdziałach, dając szereg ciekawych i obrazowych porównań. Szczególnie przemawiające wydaje się zobrazowanie wieku naszej planety i czasu istnienia życia na niej, przez porównanie do książek: „... jeżeli chcielibyśmy przedstawić kompletną historię Ziemi, moglibyśmy napisać dziesięć tomów po 500 stron każdy, tak ażeby każda strona obejmowała milion lat" (str. 99). Następnie Autor zastanawia się, ile z tych tomów potrafimy dzisiaj odczytać i w którym z nich pojawiło się życie. Z przedstawionych rozważań wynika, że nie jest to łatwe do ustalenia, a nawet ostatnie tomy historii Ziemi są bardzo trudno czytelne.

Toteż ze stron książki wyraźnie przebija przekonanie Autora, że jego teoria powstawania życia na ziemi z konieczności opiera się w znacznie większym stopniu na hipotetycznych założeniach niż na stwierdzonych faktach. O jednym z najważniejszych dla teorii przypuszczeń dotyczącym prymitywnych układów, o wiele mniej złożonych aniżeli jakiekolwiek dzisiaj żyjące ustroje, ale takich, które już można za żywe uważać, Autor mówi: „Te układy muszą posiadać niezależny, „celowo" zorganizowany metabolizm, skierowany ku ich stałemu zachowaniu, wzrostowi i samoreprodukcji w warunkach zewnętrznego środowiska zbliżonych do „pierwotnej pożywki", to jest prostego wodnego roztworu różnych substancji organicznych i nieorganicznych" (str. 108).

Książka wydana jest bardzo starannie i wydrukowana na znakomitym papierze. Nie ustrzeżono się jednak przed kilkoma błędami drukarskimi, np. na stronie 4 dwukrotnie podano jako symbol ołowiu P zamiast Pb. Nie zmniejsza to oczywiście w niczym wysokiej wartości książki jako lektury dla wszystkich, którzy interesują się zjawiskiem życia.

*Mariusz Żydowo*

LIPID TRANSPCRT. Proceedings of an International Symposium. (H. C. Meng, J. G. Coniglio, V. S. Lequire, G. V. Mann & J. M. Merrill, eds.) Charles C. Thomas Publ. Springfield (Ill.) 1964; str. XIII + 226; cena $ 10.50.

Książka stanowi zbiór referatów wygłoszonych w dniach 10 i 11 października 1963 r. w Nashville (Tennessee, U.S.A.) na Sympozjum poświęconym transportowi lipidów. Jest ona cennym uzupełnieniem Sympozjum na temat błon plazmatycznych, które odbyło się w Nowym Jorku w grudniu 1961 r. (*Circulation* **26**, 983, 1962).

Problemy poruszone na Sympozjum dotyczą absorpcji lipidów w jelicie oraz transportu tłuszczów we krwi, tkance tłuszczowej i wątrobie. Udział biochemików, fizjologów i morfologów zapewnił wielostronne naświetlenie omawianych zagadnień. Referaty, przygotowane przez specjalistów poszczególnych dziedzin, sumują w formie skondensowanej doświadczenia własne oraz ważniejsze zdobycze innych pracowni. Celowość dialogu chemików z morfologami uwidoczniła się szczególnie

przy omawianiu absorpcji trójglicerydów w jelicie. Mimo szeregu jeszcze niejasności obraz zarysowany przez B. Borgströma oraz przez S. L. Palay'a i J. P. Revela stanowi logiczną całość.

Pozostałe problemy obejmują transport lipidów oraz regulację ich gospodarki w głównych narządach i tkankach. Poruszono także sprawy związane z budową błon plazmatycznych i mechanizmem przepuszczalności. Referenci i specjalnie zaproszeni dyskutanci podjęli trudne zadanie powiązania danych biochemicznych i biofizycznych z morfologicznymi. Wiele pytań pozostało bez odpowiedzi, szereg hipotez budzi zastrzeżenia — mimo to, podjęcie dyskusji wydaje się być na czasie. Dyskusja zajmuje wiele miejsca i stanowi ważną część omawianej książki.

O lipoproteidach, które grają decydującą rolę zarówno w budowie błon półprzepuszczalnych, jak i w transporcie lipidów, pisze J. L. Oncley zajmujący się tymi zagadnieniami od wielu lat. Lipoproteidy występują niemal we wszystkich tkankach i posiadają w swej cząsteczce grupy wysoce polarne i niepolarne. Dzięki temu mogą one, jak sugeruje J. F. Danielli, otwierać lub zamykać „pory" zmieniając swoje ustawienie na pograniczu faz. Mimo pokaźnej liczby publikacji na temat lipoproteidów (w latach 1957 - 1962 ukazało się ich blisko 1300) wiadomości o ich strukturze są skąpe. Dotyczy to szczególnie lipoproteidów komórkowych.

Książka daje przegląd aktualnego stanu wiedzy o transporcie lipidów i związanych z nim problemów. Czytelnik łatwo zorientuje się w kierunkach badań najbliższych lat i to zarówno ubiegłych, jak i nadchodzących. Dzięki Sympozjum zarysowało się bowiem wiele konkretnych pytań, na które odpowiedź staje się paląca. Fizjolodzy, biochemicy i morfolodzy zainteresowani przemianą i transportem lipidów znajdą tu wiele interesującego materiału. Dla klinicystów zajmujących się chorobami wynikłymi z zaburzeń przemiany lipidów lub ich transportu, książka może okazać się także bardzo pożyteczna.

*Ryszard Niemiro*

C. H. Stuart-Harris and L. Dickinson, THE BACKGROUND TO CHEMOTHERAPY OF VIRUS DISEASES. Charles C. Thomas Publ., Springfield (Ill.) 1964; str. X + 175, cena $ 10.50.

Książka posiada siedem rozdziałów traktujących o chemicznym składzie wirusów i mechanizmie ich namnażania, o patologii infekcji wirusowych, o interferonie — jego produkcji i biologicznym znaczeniu, o doświadczalnych badaniach w zakresie chemoterapii zakażeń wirusowych i stosowaniu środków chemicznych w lecznictwie.

Najpierw Autorzy książki wprowadzają czytelnika w ogólne zagadnienia składu chemicznego wirusów, niektórych ich własności fizykochemicznych i biologicznych. W odrębnej tablicy zestawiono najważniejsze grupy wirusów, sklasyfikowane na podstawie rodzaju posiadanego kwasu nukleinowego i rozmiarów cząsteczki. Dalej następuje omówienie poszczególnych etapów cyklu rozwojowego wirusów w komórkach żywiciela, lokalizacja w komórce, własności serologiczne wirusów oraz zmiany metaboliczne komórki podczas poszczególnych stadiów reprodukcji różnych wirusów. Interesującym jest rozdział poświęcony reakcji obronnej komórki zakażonej wirusem. Wyrazem tej reakcji jest synteza substancji białkowej zwanej interferonem o własnościach inhibitora procesów metabolicznych komórki, powstającego w wyniku wtargnięcia obcego kwasu nukleinowego. Zagadnienie mechanizmu powstawania interferonu, jak i jego funkcja biologiczna, nie są jeszcze zupełnie jasne i Autorzy książki krytycznie oceniają możliwości praktycznego

zastosowania tej substancji w leczeniu infekcji wirusowej. Stosunkowo szeroko potraktowano wykorzystanie różnych związków chemicznych w procesie hamowania rozwoju wirusa w komórce. Szczegółowo omówione zostały antymetabolity biosyntezy kwasów nukleinowych i białka, środki z grupy tiosemikarbazonów oraz produkty różnych drobnoustrojów wstrzymujące rozwój niektórych wirusów. Wszystkie te substancje i sposoby ich dawkowania zebrane są w przejrzystym zestawieniu.

Książka Stuarta-Harrisa i Dickinsona będzie interesować nie tylko wirusologa czy biochemika zajmującego się wirusami, lecz również patologa i klinicystę, którzy pragną poznać nowe drogi eksperymentalnej wirusologii i możliwości leczenia schorzeń wirusowych u ludzi. Piśmiennictwo (ok. 500 pozycji), zebrane do roku 1963 włącznie, obejmuje znaczną część publikacji ostatnich kilku lat związanych z problematyką współczesnej wirusologii.

*Włodzimierz Ostrowski*

G. B. Ansell and J. N. Hawthorne, PHOSPHOLIPIDS. CHEMISTRY, METABOLISM AND FUNCTION. Elsevier Publ. Comp., Amsterdam - London - New York 1964; str. 437; cena Dfl. 55—, sh. 110, DM 61.50.

Książka Ansella i Hawthorne'a ukazuje się w kilkanaście lat po znakomitej monografii Wittcoffa o fosfolipidach wydanej w roku 1951. Miarą postępu wiedzy w tej dziedzinie może być fakt, że ponad 80% prac, cytowanych przez Ansella i Hawthorne'a — to pozycje, które ukazały się po 1950 roku i nie były omawiane przez Wittcoffa. Każda z tych dwóch monografii daje podsumowanie dorobku i obrazuje aktualny stan badań, toteż porównanie obydwu pozwala ocenić osiągnięcia w dziedzinie wiedzy o fosfolipidach w ciągu tych stosunkowo niewielu lat, które dzielą daty tych publikacji.

Książkę Ansella i Hawthorne'a można by podzielić na dwie części, z których jedna omawia strukturę i metabolizm fosfolipidów, a druga zajmuje się raczej ich biologiczną rolą w żywym organizmie, głównie zresztą w organizmie zwierzęcym.

Nie ulega wątpliwości, że nasza wiedza o strukturze fosfolipidów wzbogaciła się ogromnie w ostatnich czasach. Nowoczesne metody umożliwiły zarówno otrzymywanie poszczególnych substancji w stanie czystym, jak i ich chemiczną syntezę. Autorzy szczegółowo i krytycznie omawiają nowe metody analizy fosfolipidów i, od lat pracując w tej dziedzinie, nie szczędzą praktycznych wskazówek, niekiedy bardzo cennych. Zdaniem Autorów, dokładne scharakteryzowanie wszystkich składników fosfolipidowych tkanek i komórek jest kwestią najbliższej przyszłości.

W kilku rozdziałach Autorzy omawiają zagadnienia biosyntezy i katabolizmu fosfolipidów w organizmie zwierzęcym. W tej dziedzinie postęp jest rzeczywiście ogromny, jeśli wziąć pod uwagę, że jeszcze dziesięć lat temu praktycznie nic nie było wiadomo o szlakach syntezy fosfolipidów w żywym ustroju. Obecnie skomplikowane te procesy są stosunkowo dobrze poznane i nawet w szczegółach wyjaśnione, co jest w dużej mierze zasługą pięknych prac Kennedy'ego i jego współpracowników. Podobnie wiele zdziałano w kierunku wyjaśnienia enzymatycznej degradacji fosfolipidów w ustroju i ich utleniania.

Wiele miejsca poświęcają Autorzy krytycznemu omówieniu prac, dotyczących rozmieszczenia, składu i metabolizmu fosfolipidów. Na uwagę zwłaszcza zasługuje obszerny rozdział o fosfolipidach w układzie nerwowym, przynoszący mnóstwo cennych informacji. Uzupełnia tę część książki szereg zbiorczych tabel, o dużej wartości dla czytelnika interesującego się tą dziedziną biochemii.

Tak więc wspólna praca wielu badaczy doprowadziła do stosunkowo dokładnego scharakteryzowania składników fosfolipidowych w komórkach i do wyjaśnienia głównych szlaków ich przemian. Pytanie jednak, które od lat pasjonuje biochemika i fizjologa, dotyczy biologicznej roli tych związków, ich funkcji w żywym organizmie. Sama struktura fosfolipidów, połączenie w jednej cząsteczce grup polarnych i apolarnych, zdaje się predestynować te związki do pełnienia najrozmaitszych funkcji, zarówno w poszczególnej komórce, jak w tkance czy narządzie. Tym też zagadnieniom poświęcona jest większa część monografii Ansella i Hawthorne'a, którzy na podstawie wielu setek prac starają się zobrazować aktualny stan badań i krytycznie je ocenić. Nie silą się oni na wyciąganie jednoznacznych wniosków; przy obecnym stanie wiedzy niewiele jest pytań z tej dziedziny, na które można by definitywnie odpowiedzieć.

Udział fosfolipidów w procesie chłonienia tłuszczu postulowano już od bardzo dawna. Dopiero jednak poznanie szlaku biosyntezy glicerydów i stwierdzenie, że intermediatem reakcji jest kwas fosfatydowy, pozwoliło zrozumieć zdanie Sinclaira sprzed 25 lat, mianowicie, że resynteza glicerydów w śluzówce jelitowej zachodzi poprzez stadium fosfolipidów. Nie wyjaśniło to jednak wszystkich problemów i ani rola fosfolipidów, ani, co dziwniejsze, sam proces absorpcji tłuszczu w jelicie nie zostały jeszcze dotąd wyjaśnione (nawiasem mówiąc, to ostatnie zagadnienie — chłonienie tłuszczu — jest wciąż przedmiotem tak ożywionej dyskusji i osobistego nieraz „zaangażowania", że Autorzy nie zdobyli się na obiektywizm w przedstawieniu tej sprawy).

Fosfolipidy są stale występującym i nieodzownym składnikiem wszelkich membran biologicznych. Obecność hydrofilnych i hydrofobowych grup w cząsteczce sprawia, że są one doskonale przystosowane do tego, aby stanowić integralną część błony oddzielającej jedno środowisko wodne od drugiego. Ta ich lokalizacja dała podnietę do badań nad ich możliwym udziałem w czynnym transporcie jonów przez błonę. Badania dotyczyły „pompy sodowo-potasowej" w erytrocytach, wydzielania NaCl przez gruczoły solne niektórych ptaków, metabolizmu kationów w tkance nerwowej, wydzielania niektórych hormonów itd., i choć w chwili obecnej nie można jeszcze powiedzieć, czy i jaki istnieje związek pomiędzy aktywnym transportem substancji przez błony a metabolizmem niektórych fosfolipidów, to jednak zagadnienie to jest wciąż żywe i niewątpliwie zasługuje na uwagę.

Fosfolipidy, będąc integralnym składnikiem błon, stanowią znaczną część suchej substancji mitochondriów i mikrosomów. Udział fosfolipidów w łańcuchu transportu elektronów zdaje się już obecnie nie ulegać wątpliwości, głównie dzięki świetnym pracom Greena i jego szkoły. Przekonywające są też dowody na to, że fosfolipidy są niezbędne dla aktywności enzymatycznej cząstek sub-mitochondrialnych. Na czym jednak rola ich polega — trudno jeszcze obecnie powiedzieć.

Znacznie bardziej zagadkowe jest znaczenie obfitego występowania fosfolipidów w mikrosomach, które niektórym autorom nasunęło przypuszczenie o ich roli w procesie syntezy białka. Prowadzone są również prace doszukujące się związku pomiędzy metabolizmem fosfolipidów a procesami mitozy w jądrze komórkowym.

Zastanawiającym, a nawet paradoksalnym, może wydawać się fakt, że w dziedzinie od lat niezwykle intensywnie badanej (i bogato subsydiowanej), dotyczącej mianowicie zaburzeń w układzie krążenia, nauka dotychczas nie potrafiła odpowiedzieć na pytanie, jaki jest związek pomiędzy metabolizmem lipidów a patologią miażdżycy. Tomy już zapisano na ten temat, ale z powodzi tysięcy danych, częstokroć ze sobą sprzecznych, trudno jest wyciągnąć jednoznaczne wnioski.

Autorzy monografii nie starają się bynajmniej ukrywać faktu, że w wielu dziedzinach podsumowują raczej stan naszej niewiedzy. Dają bogaty przegląd prac,

krytycznie oceniają ich wartość dowodową, starają się podać jak najwięcej informacji i nie narzucać swojego poglądu.

Monografia Ansella i Hawthorne'a obszernie omawia zagadnienia, które przed 15 laty, gdy ukazała się publikacja Wittcoffa, nie były jeszcze nawet sformułowane. Pytania, które nauka stawia obecnie, są bez porównania śmielsze, bardziej skomplikowane i głębiej sięgające. Fakt, że zostały sformułowane, pozwala jednak mieć nadzieję, że prędzej czy później uda się znaleźć właściwą odpowiedź. I ten optymistyczny wniosek można by wyciągnąć z książki, której każdy niemal rozdział kończy się znakiem zapytania.

*Paulina Włodawer*

PTERIDINE CHEMISTRY. Proceedings of the third International Symposium held at the Institut für Organische Chemie der Technischen Hochschule Stuttgart, September 1962. (W. Pfleiderer & E. C. Taylor, eds.) Symposium Publications Division, Pergamon, Press, Oxford - London - Edinburgh - New York - Paris - Frankfurt 1964; str. XX + 535; cena £ 5.

Dnia 20 czerwca 1889 roku na posiedzeniu Towarzystwa Chemicznego w Londynie pewien młody student medycyny, późniejszy laureat nagrody Nobla, F. Gowland Hopkins doniósł o wyizolowaniu ze skrzydeł motyli z rodziny Pieridae bezpostaciowego produktu o zabarwieniu ochry. Produkt ten dawał intensywną reakcję mureksydową, co sugerowało jego pokrewieństwo z kwasem moczowym. Kilka lat później ukazała się praca F. G. Hopkinsa pod nieszablonowym tytułem: "The pigment of the Pieridae: a contribution to the study of excretory substances which function in ornament".

Minęło dalszych trzydzieści lat zanim C. Schöpf, asystent Henryka Wielanda, otrzymał sól sodową leukopteryny, barwnika ze skrzydeł motyla *Pieris brassicae*. Po wielu próbach, pewnego dnia pod koniec 1925 roku, stwierdził on wreszcie, że żółte kryształki jego preparatu różnią się nie tylko barwą od krystalicznego moczanu, lecz że zawierają dwukrotnie mniej sodu. Pełen radości, mimo późnej godziny zadzwonił Schöpf do swego szefa. W odpowiedzi usłyszał jedynie krótkie "So so" — i więcej nigdy już nie niepokoił Wielanda wieczornymi telefonami.

Strukturę barwników, tak długo mylonych z kwasem moczowym, ustalił ostatecznie R. Purrmann, który w 1940/41 r. przeprowadził również chemiczną syntezę leukopteryny, ksantopteryny i izoksantopteryny.

Były to początki chemii pterydyn. Żywą relację z tego okresu badań nad pterydynami stanowił referat prof. dr C. Schöpfa inaugurujący III Międzynarodowe Sympozjum na temat Chemii Pterydyn, które odbyło się we wrześniu 1962 r. w Stuttgarcie. W Sympozjum wzięło udział 52 badaczy z kilku czołowych ośrodków zajmujących się chemią i biochemią pterydyn. Wśród uczestników byli badacze tej miary, co A. Albert, J. M. Buchanan, L. Jaenicke, S. Kaufman, M. Viscontini, H. C. S. Wood.

Wygłoszono 36 referatów, z których blisko połowa dotyczyła syntezy chemicznej pochodnych pterydynowych. Szereg z nich, np. 2,4,7-trójamino-6-arylopterydyny oraz 4,7-dwuamino-2,6-dwuarylopterydyny (J. Weinstock i D. Wiebelhaus; I. J. Pachter; T. S. Osdene) wykazują działanie diuretyczne; 2,4,7-trójamino-6-arylopterydyny mają również własności czynników antyfolianowych, te zaś to potencjalne leki przeciwnowotworowe. Wydaje się, że spośród poliaminopterydyn antagonistycznie w stosunku do pochodnych folianu działają analogi posiadające, podobnie jak aminopteryna, co najmniej dwie grupy aminowe podstawione w pozycji 2 i 4 układu pterydynowego. Sądzi się (T. S. Osdene, E. C. Taylor), że obecność grup amino-

wych w tych pozycjach niezbędna jest dla związania antymetabolitu z enzymem. Wykazano ponadto, że dwuaminowe pochodne częściowo zdeazowanych pterydyn zachowują własności antagonistów folianu. Przypuszcza się przy tym, że warunkiem koniecznym i wystarczającym dla zachowania własności antyfolianowych jest obecność obydwu atomów azotu w pierścieniu pirymidynowym 2-4 dwuaminodeazopterydyny. Może to sugerować, że właśnie te dwa atomy grają ważną rolę w łączeniu się pochodnych folianu z białkami (O. D. Bird, V. Oakes, K. Undheim, H. N. Rydon).

W wielu referatach omówiono różne techniki rozdziału i identyfikacji pterydyn (J. E. Fildes; H. S. Forrest i S. Nawa; H. N. Guttman; J. Komeda; H. Rembold i L. Bushmann; R. Tschesche, B. Hess, I. Ziegler, H. Machleidt; M. Viscontini). Między innymi dyskutowano strukturę niektórych pterydyn, jak drozopteryny i sepiapteryny, występujących u Drosophyla (M. Viscontini; H. S. Forest i S. Nawa).

Badania nad syntezą i strukturą pterydynoglikozydów dały podstawę do wysunięcia pod dyskusję nowego schematu ilustrującego prawdopodobny tok przemian puryn w pterydyny (W. Pfleiderer, R. Lohrmann, F. Reiser, D. Söll).

Zjawisko kowalencyjnej hydratacji niektórych wiązań C = N układu pterydynowego zaobserwowane po raz pierwszy w 1951 r. w laboratorium A. Alberta, było przedmiotem jego referatu. Mechanizm kowalencyjnej hydratacji wyjaśnia Albert następująco: obecność kilku podwójnych wiązań C = N w układzie pterydynowym może powodować takie przesunięcia elektronów, że w wyniku jedno z wiązań podwójnych zostaje silnie spolaryzowane i w konsekwencji atom azotu może przyłączać kowalencyjnie czynniki nukleofilne. Resonans powodujący stabilizację tego stanu umożliwia, według Alberta, przyłączenie niezbyt silnie nukleofilnej cząsteczki wody. Wydaje się, że znaczenie kowalencyjnej hydratacji w procesach biochemicznych polega na ułatwieniu takich na przykład oksydacji enzymatycznych jak utlenianie hypoksantyny do ksantyny. Reakcje przyłączania odczynników nukleofilnych do dwuhydropterydyn i prawdopodobne znaczenie takich procesów w przemianach biochemicznych były treścią referatu A. C. S. Wooda i współpracowników (T. Rowan i A. Stuart).

Transformacje pterydyn podczas enzymatycznej hydroksymetylacji fenyloalaniny przedstawił na podstawie badań własnych S. Kauffmann. Udział pterydyn w procesie hydroksylacji fenyloalaniny stanowi pierwszy i jak dotąd jedyny znany przykład koenzymatycznej roli pterydyn niezwiązanych. Można jednak przewidywać, iż związki te czynne są również w innych enzymatycznych procesach oksydacji i redukcji.

Koenzymatyczna rola tetrahydrofolianu w metabolizmie fragmentów jednowęglowych oraz przypuszczalne mechanizmy enzymatycznych oksydacji i redukcji biegnących przy udziale tetrahydrofolianu były przedmiotem referatu F. M. Huennekensa i K. G. Scrimgeora. Rolę *N*-5-metylotetrahydrofolianu w enzymatycznej syntezie grup metylowych metioniny omówiono w referacie J. M. Buchanana, A. R. Larrabee, S. Rosenthala i R. E. Cathou. Problemy biosyntezy pterydynopyrofosforanu (L. Jaenicke), biopteryny, kwasu foliowego (A. Wacker, E. Lochmann i S. Kirschfeld) oraz ryboflawiny (G. W. E. Plaut) były również omawiane podczas Sympozjum.

Tom zawierający zbiór referatów sympozjalnych wraz z dyskusją daje wgląd w najbardziej aktualne zagadnienia chemii pterydyn. Dzięki zaś udziałowi wybitnych badaczy, osobiście od lat zaangażowanych w pracy eksperymentalnej w tej dziedzinie, problemy poruszane są ze znawstwem, co ułatwia czytelnikowi zorientowanie się w ich bardziej ogólnych aspektach i powiązaniach. Monografia Pteridine chemistry jest jednak raczej przeznaczona dla grona specjalistów, nie ograniczającego się jednak do chemików syntetyków i biochemików, lecz obejmującego również farmakologów.

*Zofia M. Zielińska*

T. L. V. Ulbricht, PURINES, PYRIMIDINES AND NUCLEOTIDES and the Chemistry of Nucleic Acids. Pergamon Press, Oxford - London - Edinburgh - Paris - Frankfurt; The Macmillan Company, New York, 1964; str. 1 + 79; cena 12s 6d.

Recenzowana książka stanowi 25-ty tom serii krótkich monografii z różnych dziedzin chemii organicznej, opracowanych przez najwybitniejszych znawców pod redakcją Sir Roberta Robinsona. Książka Ulbrichta znakomicie spełnia postawione Autorowi zadanie: podaje w krótkiej, niemal encyklopedycznej, lecz wyczerpującej formie podstawy chemii puryn i pirymidyn oraz ich pochodnych. Tekst jest uzupełniony dużą liczbą wzorów (253) oraz schematami, co sprawia, że jest jasny i zrozumiały nawet dla czytelnika nie znającego zagadnienia. Odnośniki do piśmiennictwa są starannie opracowane, uwzględniają najnowsze pozycje i często są opatrzone krótką wskazówką lub informacją.

W pierwszym rozdziale dotyczącym chemii puryn i pirymidyn Autor uwzględnia reakcje podstawienia przez odczynniki elektro- i nukleofilne, przekształcenia w obrębie cząsteczki oraz reakcje addycji, redukcji, utlenienia, acylacji i alkilacji. Osobny rozdział poświęcony jest syntezie puryn i pirymidyn. W następnych trzech kolejnych rozdziałach omówiono strukturę, syntezę i biosyntezę nukleozydów, nukleotydów oraz kwasów nukleinowych.

Chemia puryn i pirymidyn oraz tworzonych przez nie połączeń, wśród których wiele wykazuje działanie farmakologiczne, jest zwykle zbyt pobieżnie ujmowana nie tylko w podręcznikach chemii organicznej, lecz również w podręcznikach związków heterocyklicznych. Dlatego książka Ulbrichta w okresie obecnego wielkiego zainteresowania rolą kwasów nukleinowych w żywych organizmach stanowi bardzo cenne źródło wszechstronnych, podstawowych informacji chemicznych o tych związkach i ich składnikach.

*Konstancja Raczyńska-Bojanowska*

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Б. ЗАГАЛЯК и Е. ПАВЕЛКЕВИЧ

## СИНТЕЗ И СВОЙСТВА АНАЛОГОВ КОЕНЗИМА $B_{12}$ МЕТИЛИРОВАННЫХ В АДЕНОЗИЛОВОЙ ЧАСТИ МОЛЕКУЛЫ

Резюме

Описывается синтез и свойства двух аналогов коензима $B_{12}$, содержащих вместо аденозина соответственно 1-метиладенозин и $N^6$-метиладенозин. Исследовалось влияние этих аналогов на энзиматическое превращение глицерола в гидроксипропионевый альдегид, а также этиленгликола в уксусный альдегид и 1,2-пропандиола в пропионовый альдегид. Оба аналога оказались конкурентными ингибиторами реакции глицерола. Аналог содержащий 1-метиладенозин оказался также неактивным в обмене диолов, тогда как аналог с $N^6$-метиладенозином был катализатором этих реакций. Константы диссоциации комплексов апофермента с аналогами и с коэнзимом $B_{12}$ являются числами того же порядка ($K'_m$ $10^{-7}$ м). Комплекс апофермента с коэнзимом $B_{12}$ более активен, чем комплекс апофермента с аналогом, содержащим $N^6$-метиладенозин. Константа Михаэлиса для реакции превращения 1,2-пропандиола в пропионовый альдегид в присутствии коэнзима $B_{12}$ составляет $3.1 \times 10^{-4}$ м, а в присутствии аналога с $N^6$-метиладенозином $1.1 \times 10^{-3}$ м. Обсуждается зависимость коэнзиматической активности коэнзима $B_{12}$ от его химического строения.

В. ВИСЬНЕВСКИ

## РАЗДЕЛЕНИЕ БЕЛКОВ ПРОПИОНОВОКИСЛЫХ БАКТЕРИЙ НА ЦЕЛЛЮЛОЗНЫХ ИОНООБМЕННИКАХ

Резюме

Разработан метод разделения белков пропионовокислых вактерий на колонках с DEAE-целлюлозой применяя элюцию буферными растворами со ступенчатым увеличением ионной силы при постоянном pH. Белки штаммов Propionibacterium shermani, P. petersoni, P. freudenreichi и P. arabinosum были разделены на 9 фракций, в которых определялась активность $\beta$-галактозидазы и щелочной фосфатазы. $\beta$-галактозидаза не была найдена в штаммах культивируемых на средах с глюкозой. Этот фермент был индуцирован при росте бактерий на средах, содержащих лактозу. Распределение фосфатазы в хроматограммах белков P arabinosum отличалось от распредения в других штаммах.

А. ТАЙЛЁР и К. ТАЙЛЁР

## РЕЦЕПТОРНЫЕ СВОЙСТВА ПОЛИСАХАРИДОВ Vi ВЫДЕЛЕННЫХ РАЗЛИЧНЫМИ МЕТОДАМИ

Резюме

Из бактерий Escherichia coli 5396/38 выделен значительно очищенный препарат полисахарида Vi методом хроматографии на оболочках эритроцитов адсорбированных на целите. Этот препарат (Vi-T) сравнивался с двумя другими препаратами полисахарида Vi, полученными из того же штамма, а именно с препаратом полученным Вебстером и сотр. (Vi-W) методом химического фракционирования и с препаратом, полученным Жерви и сотр. (Vi-J), методом препаративного электрофореза.

Препараты Vi-J и Vi-T обладали рецерторной активностью по отношению к бактериофагу Vi II, тогда как препарат Vi-W не обладал этой активностью. Бактериофаг Vi II при инкубации с препаратами Vi-J и Vi-T уничтожает их рецепторную активность.

М. ХИЛЛЯР и В. ЖЕЧИЦКИ

## ВЛИЯНИЕ МАКРОКАТИОНОВ И МАКРОАНИОНОВ НА МИТОХОНДРИИ

Резюме

Установлено, что основные белки оказывают тормозящее действие на спонтанное и индуцированное набухание митохондриев. Это влияние можно устранить добавлением макроанионов таких, как например: ганглиозид или муцин. Ганглиозид и муцин сами стимулируют набухание.

Влияние основных белков зависит от связывания их митохондриями. Результаты обсуждаются на основании предварительно опубликованных результатов.

Т. БОРКОВСКИ, Г. БЕРБЕЦЬ и Г. БЖУШКЕВИЧ

## ИЗМЕНЕНИЯ ХИМИЧЕСКОГО СОСТАВА ИЗОЛИРОВАННЫХ КЛЕТОЧНЫХ ЯДЕР ИЗ МОЗГА И ПЕЧЕНИ КРЫСЫ

Резюме

Изолированные клеточные ядра мозга и печени инкубировались в изотоническом растворе сахарозы с глюкозой. При инкубации фосфат меченый $^{32}P$ включался в ядерную RNA даже в тех случаях, в которых одновременно наблюдалось уменьшение содержания общего RNA на 50% и увеличение содержания свободных нуклеотидов.

П. МАШТАЛЕЖ, З. ВЕЧОРЭК и М. КОХМАН

## ПОТРЕБЛЕНИЕ ФОСФОРА СВЯЗАННОГО С УГЛЕРОДОМ МИКРООРГАНИЗМАМИ


### Резюме

1. Установлено, что E. coli 307 и E. freundi растут на минеральной среде содержащей этилфосфорную кислоту или 2,3-дигидроксипропилфосфорную кислоту вместо ортофосфата.

2. Показано, что M. phlei не растет на средах, содержащих вместо ортофосфата этилфосфорную кислоту, но растет на средах с 2,3-дигидроксипропилфосфорной кислотой.

3. При росте на 2,3-дигидроксипропилфосфорной кислоте образуют кислотолабильные фосфорные соединения.


К. ТАЙЛЁР

## ИССЛЕДОВАНИЯ НАД ПОТЕРЕЙ РЕЦЕПТОРНОЙ АКТИВНОСТИ ПОЛИСАХАРИДА Vi ПРИ ИНКУБАЦИИ С Vi-ФАГОМ II


### Резюме

Установлено, что потеря рецепторной активности полисахарида Vi под влиянием фага Vi II может протекать в 0,1 м-ацетате аммония. Исследовалось влияние температуры и pH на скорость этой реакции. Эта реакция тормозится EDTA, Тогда как *n*-хлормеркуробензойная кислота и *N*-этилмалеимид не влияют на эту реакцию.

После инкубации с полисахаридом сохраняется как инфекционность фага, так и его способность уничтожать рецепторную активность новой порции полисахарида.


Г. МИХАЛЭК-МОРИККА

## СРАВНИТЕЛЬНЫЕ ИССЛЕДОВАНИЯ НАД ОБМЕНОМ ТИРОЗИНА У РАЗНЫХ ВИДОВ ЖИВОТНЫХ


### Резюме

1. Установлено, что в печени голубя, ящерицы, судака и щуки аэробный катаболизм тирозина протекает таким же путем, как и у млекопитающих, т. е. с образованием фумарата и ацетоацетата.

2. У лягушки не удается обнаружить одного фермента из ряда реакций катаболизма тирозина, а именно оксидазы п-гидроксипирувата. У лягушки имеется, повидимому, в этом месте метаболическая блокада.

3. У улитки была найдена лишь трансаминаза тирозина. У этих организмов тирозин разлагается, повидимому, иным путем .

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## ВОССТАНОВЛЕНИЕ НИКОТИНАМИД-АДЕНИНОВЫХ ДИНУКЛЕОТИДОВ ХОЛИНОМ И САРКОЗИНОМ В МИТОХОНДРИЯХ, ЗАВИСЯЩЕЕ ОТ ДОСТАВКИ ЭНЕРГИИ


Резюме

Холин и саркозин, субстраты окисляемые непосредственно флавопротеиновыми ферментами могут восстанавливать никотинамид-адениноные динуклеотиды в митохондриях с ненарушенным сопряжением из печени крысы. Эта реакция зависит от доставки энергии и происходит путем обратного направления потока электронов.


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## ПОЛУЧЕНИЕ И СВОЙСТВА ИНГИБИТОРА АРГИНАЗЫ ИЗ СЕМЯН ПОДСОЛНЕЧНИКА


Резюме

1. Получен препарат ингибитора аргиназы очищенный приблизительно в 23 раза по сравнению с активностью исходных экстрактов.

2. Активность ингибитора проявляется в его связывании с ферментом. L-цистеин, меркаптоэтанол и глютатион реактивируют аргиназную активность заторможенную действием ингибитора.

3. Ингибитор является низкомолекулярным соединением, на что указывают разультаты ультрафильтрации на желе „Sephadex" и диализа. Ингибитор устойчив в кислой среде, относительно устойчив в нейтральной среде и подвергается разрушению в щелочной среде.