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ON THE ENZYMIC FORMATION AND THE ISOLATION OF POLYPHOSPHATES OF ADENINE DEOXYRIBOSIDES*

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INTRODUCTION

In 1954 SABLE *et al.*[1] found that enzymes from muscle catalyzed the transfer of phosphate from ATP*** to deoxy-AMP. They found, likewise, that a suspension of liver mitochondria catalyzed the formation of acid-labile phosphate in the presence of deoxy-AMP. Evidence for the formation of the deoxyribose analogs of ADP and ATP under these conditions was obtained. Recently, LIEBERMAN *et al.*[2] have found that an enzyme from yeast catalyzes the transfer of phosphate from ATP to deoxy-AMP. The oxidative phosphorylation of thymidine monophosphate and deoxycytidine monophosphate by the "cytoplasmic" fraction of liver homogenate has been demonstrated by HECHT *et al.*[3]. The products were isolated and identified as di- and tri-

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*** The following abbreviations are used: AMP, ADP and ATP for adenosine mono-, di- and triphosphate; deoxy-AMP, deoxy-ADP and deoxy-ATP for deoxyadenosine mono-, di- and triphosphate; deoxy-GMP for deoxyguanosine monophosphate; deoxy-CMP for deoxycytidine monophosphate; TMP for thymidine monophosphate, Tris-buffer for tris(hydroxymethyl)aminomethane-HCl buffer and P for phosphate.

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phosphates of thymidine and deoxycytidine. We have found that aqueous extracts of both red bone marrow and of muscle contain enzymes which catalyze the phosphorylation of both deoxy-AMP and deoxy-GMP. The products of the deoxy-AMP phosphorylation have been isolated by column chromatography. The analyses of the compounds obtained suggest that they are the deoxyribose analogs of ADP and ATP. A preliminary note on this work has been published[4].

MATERIALS

ATP was a product of the Sigma Chemical Company (St. Louis, Mo., USA), and the deoxynucleotide monophosphates were obtained from the California Foundation for Biochemical Research (Los Angeles, Calif., USA). All deoxynucleotides, both commercial and rechromatographed, were found to give a positive reaction for pentose, corresponding to the presence of about 4 % of pentose. This is in agreement with the reported[5] unspecificity of the orcinol reaction for pentose. For cellulose powder columns, Whatman's standard grade cellulose powder was used. It was treated with a solution of 8-hydroxyquinoline in acetone and washed with water until the effluent was free of ultraviolet absorption. An aqueous suspension of this powder was used to pack the columns. The charcoal used for adsorption was Norit which had been washed with 50% ethanol adjusted to pH 9, until the effluent was free of ultraviolet absorption, then with 0.1 *N* HCl and with water. Acid-washed Whatman No. 1 filter paper was used for paper chromatography.

Myokinase was prepared according to COLOWICK AND KALCKAR[6]. Red bone marrow extract from rabbits was prepared by extracting the bone marrow twice with one volume of ice-cold water.

Pentose was determined according to MEJBAUM[7], and orthophosphate according to FISKE AND SUBBAROW[8]. Acid-labile phosphate was determined as orthophosphate after hydrolysis at 100°C for 7 min in 1 *N* H_2SO_4, and total phosphate after ashing in sulfuric acid – nitric acid mixture. Deoxyribose was determined with diphenylamine[5]. This assay could not be employed in the presence of formic acid.

METHODS

For the study of the metabolism of deoxymononucleotides, it was found that two-dimensional paper chromatography was suitable, since complete separation of these compounds from ribonucleosides and ribonucleotides could be obtained. The solvents were the ethanol – ammonium acetate mixture, pH 7.5, of PALADINI AND LELOIR[9], and a borate-containing solvent developed by PLESNER[10]. This solvent consisted of 70 % ethanol and 30 % of a one molar ammonium acetate solution adjusted to pH 9 with ammonia and saturated with sodium tetraborate. After the ethanol is mixed with the salt solutions, the excess borate should be allowed to crystallize out before use. Fig. 1a shows the paper chromatographic separation of deoxy-AMP from AMP, ADP and ATP with these two solvents. The chromatograms were developed on photographic paper with "Mineralight". In the aliquots of the incubation mixtures to be chromatographed, the enzyme reactions were stopped by addition of perchloric acid to a final concentration of 5 %, and, after neutralization enough Norit was added to adsorb the purine or pyrimidine compounds present. The charcoal was collected by centrifugation, washed once with water, and eluted with 50 % alcohol adjusted to pH 9 with ammonia. The eluate was taken to dryness under vacuum. The residue was dissolved in a small volume of water and applied to the paper.

For the preparative separation of a ribonucleotide from a deoxyribonucleotide it was found that chromatography on a cellulose powder column was suitable. The nucleotides to be separated were adsorbed on Norit which was then collected by centrifugation and transferred to the cellulose powder column, on top of which it should form an even layer. A few ml of 96 % ethanol were run into the column which was then eluted with a mixture of equal volumes of 96 % ethanol and a 1 *M* solution of ammonium acetate saturated with sodium tetraborate and adjusted to pH 9 with ammonia*. This solvent has two functions. (1) It elutes the nucleotides from the charcoal**. (2) It separates a deoxynucleotide chromatographically from its ribose analog on the cellulose powder column. The latter property of the solvent is due to the presence of sodium tetraborate.

* It is important that this solvent should not come in contact with rubber or polyvinyl chloride tubing, since it dissolves ultraviolet-absorbing compounds from these materials.

** PLESNER[11] has found the optimal concentration of alcohol for elution of nucleotides from Norit to be 50 %.

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It appears that the complex formed between borate and a ribose compound moves much more slowly in the solvent than does a corresponding deoxyribose compound. The configuration of deoxyribose prevents it from forming a complex with borate.

RESULTS

Paper chromatographic evidence

When incubated with ATP extracts of red bone marrow and muscle were found to contain enzymes which catalyze the formation of new purine deoxyribose derivatives from deoxy-AMP. Evidence for these reactions was detected by the appearance of two additional ultraviolet-absorbing spots on paper chromatograms. The new spots gave a positive reaction for deoxyribose when sprayed with cysteine-sulfuric acid[12]. The position of the spots suggests that they consist of polyphosphates of deoxynucleosides. This indicated that the reaction might be a myokinase-like reaction between ATP and deoxy-AMP. This was further supported by the finding that the reactions leading to the formation of the additional deoxyribose compounds were also catalyzed by myokinase of muscle. The position of these compounds on paper chromatograms is seen in Fig. 1b. Similar results were obtained when deoxy-GMP was used instead of deoxy-AMP. In this case, however, the reaction proceeded at a slower rate. No evidence for an enzyme reaction between ATP and deoxy-CMP or TMP was obtained with either bone marrow extract or muscle extract.

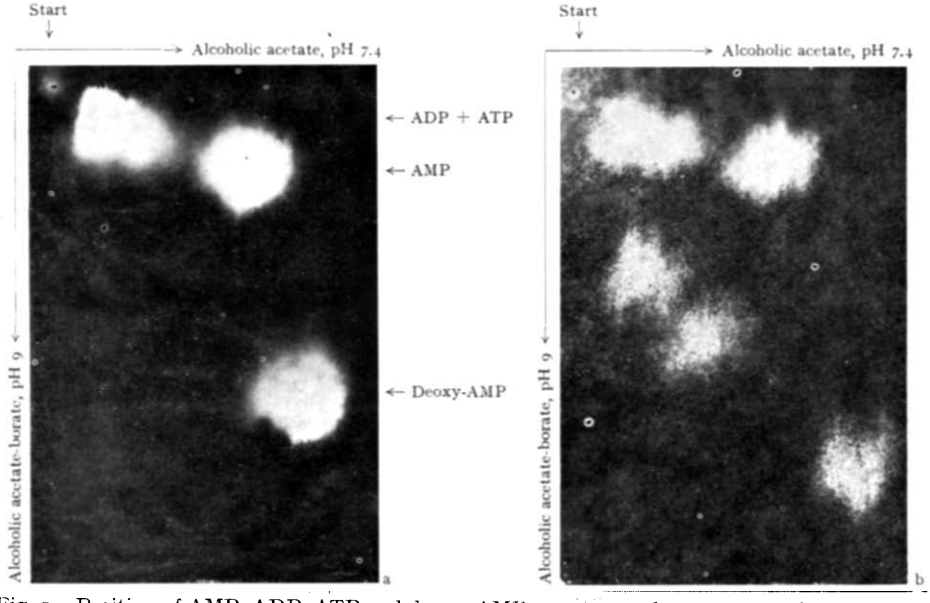

Fig. 1a. Position of AMP, ADP, ATP and deoxy-AMP on a paper chromatogram developed in the first dimension with PALADINI AND LELOIR's neutral solvent[9], and in the second dimension with PLESNER's solvent[10]. The spots indicate ultraviolet-absorbing material.

Fig. 1b. Ultraviolet spots on a paper chromatogram of an incubation mixture of ATP (1 μmole), deoxy-AMP (0.65 μmoles), myokinase (3.4 mg of protein) and $MgCl_2$ (1 μmcle) in 350 μl of Tris-buffer (0.03 *M*, pH 7.5,). The chromatogram was developed as described for Fig. 1a.

Isolation of adenine deoxyriboside polyphosphates

Isolation on a preparative scale of the products of the reaction between ATP and

deoxy-AMP was accomplished by column chromatography. The procedure was typically as follows: The incubation mixture contained deoxy-AMP (120 μmoles), ATP (235 μmoles), $MgCl_2$ (150 μmoles), 2 ml of myokinase (14 mg protein), and was 0.05 *M* with regard to Tris-buffer. The pH of the mixture was 7.3, and the final volume was 14.5 ml. To allow full equilibration of the enzyme-catalyzed reactions, the mixture was incubated for 8 hours at room temperature.

After incubation the mixture was immersed in boiling water for two minutes and cooled. The coagulated protein was removed by centrifugation, and the supernatant was adjusted to pH 8 with ammonia and slowly passed through a column (3.8 cm^2 × 10 cm) of Dowex-1, X-10, 200–400 mesh, in the formate form. After one resin bed volume of water was passed slowly through, gradient elution of the column was carried out with ammonium formate, pH 5, using the technique of HURLBERT *et al.*[13]. The effluent was analyzed for absorption of ultraviolet light (260 mμ) and for the presence of pentose. Three separate ultraviolet-absorbing peaks were obtained, and they were found to coincide with three peaks of ribose components. The ratios of ribose to adenine (using the molar extinction coefficient at 260 mμ of 15,000) were approximately 0.7 in the three peaks. The molar ratio in the three peaks between

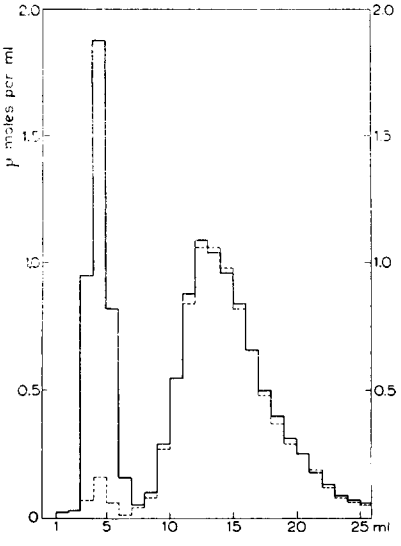

Fig. 2. Rechromatography of the third ultraviolet-absorbing peak of the Dowex chromatogram of an incubation mixture of ATP, deoxy-AMP and myokinase (see text). To the pooled fractions containing the third ultraviolet-absorbing peak (about 50 μmoles adenine derivative) 250 mg of Norit was added. After a few minutes the Norit was collected by centrifugation and transferred as a suspension to a column of cellulose powder (0.8 cm^2 × 13 cm). Excess aqueous solution was displaced by applying slight air pressure. The charcoal, which should form an even layer on top of the cellulose powder, was covered with a piece of filter paper, and 3 ml of 96% alcohol was passed slowly into the column by applying slight air pressure. This column was then eluted with a mixture of equal volumes of 96% alcohol and 1 *M* ammonium acetate solution saturated with sodium tetraborate. The rate of elution was about 6 ml per hour. Abscissa: ml of effluent. Ordinate: μmoles per ml: —— adenine; ···· pentose.

Fig. 3. Paper chromatogram developed with PLESNER's solvent[10].

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acid-labile phosphate and adenine was 0, 1.1 and 2.0, respectively. This suggests that the peaks contain mono-, di- and triphosphates, respectively, of both adenosine and, presumably, of deoxyadenosine. The fractions of each peak were pooled and rechromatographed separately on columns of cellulose powder as described under METHODS. In Fig. 2 is shown the result of such a chromatogram. The first ultraviolet peak contained only small amounts of pentose, while the second contained almost equimolar amounts of pentose and adenine. The fractions containing the first ultraviolet peak were pooled, and after the alcohol had been removed under vacuum the nucleotide was freed from salts by treatment with Norit. Table I shows the analysis of compounds obtained in this way from the second and the third peak of the Dowex chromatogram. It can be seen that the ratios of adenine: deoxyribose: total phosphate: labile phosphate indicate that the compounds may be di- and triphosphates, respectively, of adenine deoxyriboside. However, the two compounds give in addition positive reactions for pentose corresponding to a content of 11% of pentose per adenine molecule. On paper chromatograms each of the two compounds moved as single spots in the solvent of PLESNER[10] (see Fig. 3).

TABLE I

ANALYSIS OF ISOLATED COMPOUNDS

	Molar amounts relative to adenine			
	Deoxyribose	*Labile P*	*Total P*	*Ribose*
"deoxy-ADP"	1.00	1.03	1.97	0.11
"deoxy-ATP"	1.03	1.90	3.06	0.11

DISCUSSION

The analysis of the deoxyribose compounds formed in the incubation mixtures of ATP, deoxy-AMP and myokinase suggests that they are adenine deoxyriboside dif phosphate and -triphosphate, respectively. It is therefore assumed that the myokinase-preparation catalyzes the following reactions:

$$\text{ATP} + \text{deoxy-AMP} \rightleftharpoons \text{ADP} + \text{deoxy-ADP}$$
$$2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$$
$$2\ \text{deoxy-ADP} \rightleftharpoons \text{deoxy-AMP} + \text{deoxy-ATP}$$

The possibility also exists that a nucleoside diphosphate kinase catalyzing the reaction:

$$\text{ATP} + \text{deoxy-ADP} \rightleftharpoons \text{ADP} + \text{deoxy-ATP}$$

is present in the enzyme preparation used.

The finding that the positive pentose reaction given by deoxy-ADP and deoxy-ATP was appreciably higher (about three times) than that of deoxy-AMP might be due either to a contaminating impurity in the preparation or to the additional phosphate molecules in the compounds, the presence of which might possibly further decrease the specificity of the pentose assay. In the case of the first possibility, the pentose could be a ribonucleotide which moved with the deoxyribose compounds on the cellulose powder column due to incomplete formation a of borate complex. However, rechromatography of the isolated compounds resulted in one ultraviolet

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peak coincident with a pentose peak, and the analysis of the compounds was found to be unchanged. The compound could also be a pentose with a configuration which prevents it from forming a complex with borate, and which either was present as impurity in the ATP, or was formed during incubation with myokinase. However, when an incubation mixture of ATP, AMP and myokinase was chromatographed as described above, no pentose-reacting peak appeared in the cellulose powder chromatogram at the positions where deoxy-ADP or deoxy-ATP would have appeared. Finally, a stable complex between a ribose compound and the deoxyriboside polyphosphates might have been formed, a possibility which has not been excluded experimentally.

The possibility also exists that the pentose reaction of the compounds isolated is due to unspecificity of the assay. In this case an interaction between the terminal phosphates and the deoxyribose of deoxy-ADP and deoxy-ATP may occur before the phosphates are split off by the strong acid of the reagent mixture, since the presence of free phosphate had no effect on the reaction of deoxy-AMP with the pentose reagents. Although this possibility might seem unlikely, it is interesting that hydrolysis of the deoxy-ADP or deoxy-ATP in 1 N H_2SO_4 prior to the assay, was found to lower the intensity of the pentose reaction (see Fig. 4). Also, it has been shown[14] that rate of color development with this assay for pentose depends very much on the presence of phosphate at carbon No. 5 of the sugar molecule. In Fig. 4 are shown the spectra obtained with different compounds in the orcinol assay. It can be seen that all compounds tested had the same absorption maximum, and that the unspecific absorption between 520 mμ and 600 mμ is relatively higher for all of the deoxyribose compounds than for AMP. It is further seen that after hydrolysis of deoxy-ADP in 1 N H_2SO_4 the absorption decreases to about 70% of the original value. No such effect of acid hydrolysis was obtained with deoxy-AMP or AMP. When deoxy-ADP and deoxy-ATP were assayed with the method of VON EULER AND HAHN[15] for pentose, they were found to give readings which were two to three times as high as those given wih deoxy-AMP.

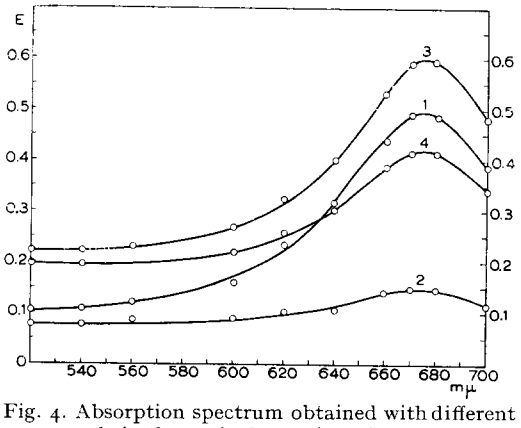

Fig. 4. Absorption spectrum obtained with different compounds in the orcinol reaction. Curve 1: 0.046 μmoles of AMP. Curve 2: 0.3 μmoles of deoxy-AMP. Curve 3: 0.3 μmoles of deoxy-ADP. Curve 4: 0.3 μmoles of acid-hydrolyzed (7 minutes at 100°C in 1 N H_2SO_4) deoxy-ADP.

Work is in progress on the isolation and characterization of the products of the enzyme reaction between deoxy-GMP and ATP.

SUMMARY

By means of paper chromatography it has been shown that extracts of red bone marrow and preparations of myokinase contain enzymes which catalyze the formation of two additional deoxyribose compounds from mixtures of either deoxy-AMP and ATP or deoxy-GMP and ATP.

Two of the products of the reaction between deoxy-AMP and ATP have been prepared in mg quantities by column chromatography. The analysis of the compounds obtained suggest that they are the deoxyribose analogs of ATP and ADP.

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LA FORMATION ENZYMATIQUE DE L'ACIDE CYSTÉINESULFINIQUE Á PARTIR DE SULFITE PAR L'EMBRYON DE VEAU

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Un précédent travail[1] a montré que la poudre acétonique de rein de lapin est capable d'assurer, par voie enzymatique, la condensation de sulfite radioactif et d'une molécule organique, sous forme d'acide cystéinesulfinique. Par la suite, on a observé le même phénomène *in vivo*, chez le lapin[2]. Dans ce dernier cas, on a isolé après administration de sulfite marqué non seulement de l'acide cystéinesulfinique ^{35}S, mais aussi de la cystine ^{35}S et de la taurine ^{35}S.

Nous avons repris l'examen de cette condensation du sulfite ^{35}S en présence de foie d'embryon de veau, *in vitro*. La plus forte activité spécifique du sulfite ^{35}S employé a permis l'addition d'acide cystéinesulfinique comme entraîneur. On peut alors en préparer un dérivé, l'acide dinitrophénylcystéique, qui confirme l'identité du produit isolé et en mesurer la quantité. On observe aussi, à la fin de l'incubation, la présence de composés organiques soufrés non aminés et qui ne sont pas des esters de l'acide sulfurique.

En ce qui concerne la nature de la molécule organique susceptible, chez l'animal supérieur, de fixer le sulfite pour donner ensuite plus ou moins directement l'acide cystéinesulfinique, nous avions admis précédemment[1] que l'acide pyruvique puisse jouer un tel rôle, par analogie avec son aptitude à fixer le CO_2 pour donner l'acide oxalacétique. D'autre part, ROBERTS et coll.[3] ont montré que chez *E. coli*, la sérine

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