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The Renewal of Phosphate in Acid-Soluble Nucleotides in the Liver and the Brain

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Presumably the rate at which phosphate in different nucleotides * is renewed *in vivo* depends on the function of the compounds. In quantitative determination of the acid-soluble phosphorus compounds in a tissue, fractionation based on differences in the solubilities of barium, mercury or silver salts [1–3] of these compounds is usually employed. Generally this method is not sufficiently accurate if the specific activity of several compounds in the same extract is to be determined exactly, inasmuch as it is impossible to obtain the different compounds in pure enough form. This is particularly true if the concentrations of the substances to be investigated are low. The experimental errors become greatest in determination of the specific activity of a compound with a low turnover rate. The admixture of only very small amounts of a compound with a rapid turnover results in completely erroneous values. Kalckar [4] has described a method of determining different phosphate esters and their labeling degree with the aid of specific enzymes. Use of this method is restricted, however, by the fact that only certain phosphorus compounds are suitable and that as a rule purified enzymes are required.

By means of paper chromatography it is possible to separate certain phoshorus compounds such as nucleotides in a nucleic acid hydrolysate [5, 6], dinucleotides [7], and some carbohydrate esters [8]. With this method Hummel and Lindberg [7] have been able to show that phosphate bound to FAD has a high turnover rate in a respiring liver homogenate.

* In this report the following abbreviations are used:
ATP for adenosine triphosphoric acid;
ADP for adenosine diphosphoric acid;
AMP for adenosine-5-phosphate;
DPN for diphosphopyridine nucleotide;
FAD for flavin adenine dinucleotide;
NMN for nicotinamide mononucleotide.

In the following experiments the specific activity of phosphate was determined in the acid-soluble nucleotides of the brain and the liver following chromatographic purification.

METHODS

Material. — The following compounds were used as test substances: adenine, Hoffman—La Roche, Basel; adenosine, Lemke Co., N. Y; AMP, synthetic *; adenosine-3-phosphate, a Schwartz product; ATP prepared from rabbit musculature [9] and from the Sigma Chemical Co., St. Louis; ADP, Sigma Chemical Co.; DPN prepared according to Williamson and Green [10]; hexokinase prepared according to Kunitz and McDonald [11]; potato adenyl pyrophosphatase (apyrase) prepared according to Kalckar [12].

*Choice of solvent system***. — The most suitable eluating substance for separating the adenine compounds occurring in a biological extract has been found to be the following mixture: 66 % isobutyric acid (purified before use by fractionated distillation), 33 % water, and 1 % concentrated aqueous ammonia, (v/v). The pH value of the solvent system is 3.8. During the c. 8 hours for which the chromatogram should run at a temperature of 20° C, no hydrolysis of ATP or ADP takes place. Several buffered systems with pH values between five and seven were tested, but they were unsuitable, as separation of the desired compounds could not be obtained.

Chromatographic method. — For the most part the procedure described by Consden, Gordon, and Martin [13] was followed: *i. e.*, chromatography in closed glass vessels in accordance with the downward-running principle. The temperature was kept as constant as possible. Munktell's No. OB filter paper was used. Three times a volume of 8 μl was applied to the paper with a micropipette, and after each application the spot was dried under an infrared lamp. In this manner up to 200 μg of dissolved substance can be applied to the same chromatogram. When the front of the solution was 5 cm from the lower edge of the paper, chromatography was concluded.

Demonstration of different components. — After the papers were removed from the chromatographic chamber, they were dried in warm air at a temperature of 70° C, rinsed in ether to remove the isobutyric acid, and dried again. The purines were localized by absorption of the light from a Mineralight fluorescent lamp with high intensity at 2400—2600 Å. They were also localized with uranyl acetate [14]. The distribution of radioactive phosphate on the chromatogram was determined according to Lindberg and Hummel [15]. Radioactivity measurements were carried out on a Scaler 64 Electronic Counter (A/S Brüel and Kjaer, Copenhagen, Denmark) which gave 3×10^8 impulses/min. per millicurie P^{32}.

Extraction of different components. — Spots with purine, radioactivity, or both were cut out and thereafter extracted twice in 5 ml of water, each time for four hours. This extraction was by no means quantitative, but was used because the admixture of foreign substances was avoided. The isobutyric acid remaining in the water solution was removed by shaking the solution with an equal volume of ether three times. The ether was removed

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** During the preparation of the manuscript the same method has been published by Magasanik, B., Vischer, E., Doninger, R., Elson, D., and Chargaff, E. *J. Biol., Chem.* **186** (1950) 37, for separation and estimation of ribonucleotides in a nucleic acid hydrolysate.

from the water phase by aeration, after which the solution was evaporated to the volume desired. As a rule the spots from five to ten chromatograms were extracted simultaneously.

Preparation of solution suitable for chromatographic separation of phosphate-labeled nucleotides in the liver and brain. — White rats, weighing c. 100 g, were injected with 0.3 millicuries of radioactive phosphate as Na_2HPO_4 containing a negligable amount of phosphate. In liver experiments the injections were carried out intraperitoneally and in brain experiments subarachnoidally [16]. After the desired period the animals were drowned in solid carbon dioxide chilled acetone. Prepared brain or liver [17] was pulverized in a mortar chilled with solid carbon dioxide. The pulverized tissue was extracted with 12 % (w/v) trichloroacetic acid (TCA). The ice cold TCA extracts were neutralized to pH 7.0 with sodium hydroxide, whereafter they were precipitated with Hg^{++} at pH 4.0. Complete precipitation was obtained after the sample had been stored in the refrigerator for 12 hours. The precipitate was washed with 1 % (w/v) mercury acetate, pH 4.0, suspended in 2 % (w/v) TCA, and decomposed with hydrogen sulphide. The mercury sulphide was filtered off and the hydrogen sulphide removed by aeration. As the solution. obtained still contained impurities it was reprecipitated with Hg^{++} at pH 4.0. The precipitate was treated as before. The final solution obtained contained nucleotides almost exclusively. There was no FAD as this compound was quantitatively adsorbed by the mercury sulphide. The bulk of the TCA in the solution was removed by shaking it three times with ether. The ether was removed from the water phase by aeration The solution obtained was directly applied to chromatograms.

Splitting of labeled DPN into two mononucleotides. — Four brains from rats, which were given subarachnoidal injections of radioactive phosphate [16] two hours before being sacrificed, were extracted with 12 % (w/v) TCA. The extracts were precipitated with mercury acetate at pH 4.0 and the precipitate decomposed with hydrogen sulphide following suspension in 2 % (w/v) TCA. The mercury sulphide was filtered off, and the hydrogen sulphide removed by aeration. The solution obtained contained most of the DPN originally present in the brains. As carrier, 15 mg unlabeled DPN was added to the solution, whereafter it was precipitated with lead acetate at pH 4.5. DPN was then purified by repeated shaking up in *p*-cresol [18]. The purified DPN was obtained in a water solution without other phosphorus compounds. Cresol was removed by four shakings of the solution with ether. The solution was incubated with washed granules from rabbit kidneys, whereupon DPN according to Kornberg and Lindberg [19] was split into AMP and NMN. After fixation in 10 % (w/v) TCA, the NMN was purified [19].

Determinations. — The total phosphate was determined according to Martin and Doty [20] following combustion in sulphuric acid with hydrogen peroxide. The specific activity (*i.e.*, the number of impulses per μg P) was determined in accordance with a method previously [21] described. The adenylic acid was determined in accordance with Kalckar's method [22] and the DPN and NMN fluorimetrically with quinine sulphate as standard [23].

RESULTS

The chromatogram with pure substances and their R_f *values.* — Table 1 shows the R_f values of the different adenine compounds in the solvent system employed. Separation was good and the different compounds appeared in well delimited spots.

Table 1. R_f values of different adenine compounds. — A solution containing the compounds listed in the table was applied to a strip of filter paper and chromatographed in a mixture of water, ammonia, and isobutyric acid. (cf. Methods).

Compound	R_f Values
ATP	0.12
ADP	0.25
AMP	0.41
Adenosine-3-Phosphate	0.51
Adenosine	0.80
Adenine	0.90

Apparently ATP was not hydrolyzed when the chromatogram ran; a fresh ATP preparation gave only one spot. ATP which had been stored as the solid Ba-salt at room temperature for two months gave two or three different components. In addition to ATP there was always one spot with the same R_f value as ADP and usually another with the R_f value of AMP. Earlier investigations have also indicated that ATP is split if it is stored or irradiated. Bailey [24] has found that on storage inorganic pyrophosphate splits off from ATP, and according to Carter [25] adenine is formed following ultraviolet irradiation.

Separation of nucleotides from the liver. — Fig. 1 shows the separation of acid-soluble compounds precipitable with Hg at pH 4.0 occurring in the liver. The spots marked on the figure indicate the area with absorption of ultraviolet light. From the amount of activity registered on the same figure in different parts of the chromatogram it appears that all the components giving absorption of ultraviolet light are bound to labeling phosphate.

On the chromatogram there are seven different peaks of activity given by the following compounds.

The compound giving the first peak has not been identified since the compound was not obtained in sufficiently pure form because of its low R_f value. Presumably it is also hydrolyzed during chromatography. The substance has high absorption in ultraviolet light, maximum at 2600 Å, and is precipitable as Ba-salt at pH 8.0. Probably one or more phosphate groups of this compound are renewed at a rate of the same order of magnitude as the acid-labile phosphate of ATP. The compound may be identical with an unknown compound which, according to Bailey [24], exists in all ATP preparations.

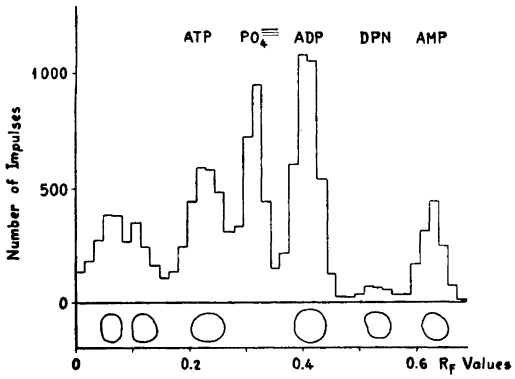

Fig. 1. The migration patterns of phosphorus compounds, insoluble at pH 4.0 as Hg-salts, in a TCA extract of rat liver, after chromatography with the water-ammonia-isobutyric acid mixture described in the text. The spots indicate the areas with absorption of ultraviolet light. The P^{32} values are expressed as impulses per minute. The number of impulses was determined for each ½ cm of the strip of filter paper. The radioactive phosphate was injected 60 minutes before the animal was sacrificed.

It was also impossible to identify definitely the compound which gave the second activity peak. The substance was labeled at a relatively low level. The greater part of the radioactivity found in this spot came from the extended first spot. The compound absorbed ultraviolet light and gave a positive ninhydrin reaction. The Ba-salt was soluble at pH 8.0. The compound might be an aminophosphate, possibly ethanolamine phosphate, which has been isolated from the intestines [26].

ATP gave the third activity peak. If the solution was incubated with hexokinase before chromatography this spot disappeared.

Orthophosphate gave the next peak. This was the only phosphorus compound in the sample which failed to absorb ultraviolet light. Radioactive orthophosphate added to an unlabeled sample before chromatography appeared at this place.

The following activity peak was given by ADP. This peak increased in height following incubation of the sample with hexokinase.

The two last activity peaks were given by DPN and AMP. To separate these two compounds it is necessary that the sample solution is completely free of inorganic salts and that the isobutyric acid used is freshly distilled.

No compounds with higher R_f values containing radioactivity or with absorption of ultraviolet light were observed. Thus, there was no demonstrable amount of adenosine-3-phosphate.

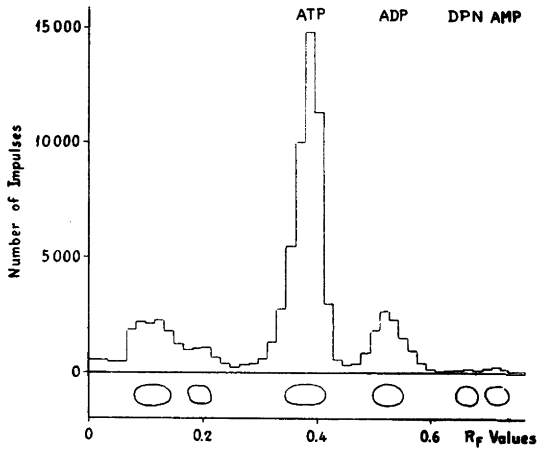

Fig. 2. The migration patterns of phosphate compounds, insoluble at pH 4.0 as Hg-salts, in a TCA extract of rat brain, after chromatography in the same solvent system as in the experiment with liver. The spots indicate the areas with absorption of ultraviolet light. The values are determined and expressed as in Fig. 1. The radioactive phosphate was injected 45 minutes before the animals were sacrificed.

Following incubation with apyrase both the ATP and the ADP peaks disappeared, the former before the latter, being in agreement with the findings of Kalckar [27] that the terminal phosphate group in ATP is earlier split off than the second. Adenosine appeared in agreement with previous investigations [24, 28], according to which apyrase preparations always contain non-specific phosphatase.

Separation of nucleotides from the brain. — Fig. 2 shows a chromatogram of a solution prepared from the brain in the same way as that from the liver. Orthophosphate failed to appear on this chromatogram, which is in accordance with Kerr's observation [29] that orthophosphate in a TCA extract from the brain is not precipitated as mercury salt at pH 4.0. Furthermore there are the same compounds as in the Hg-insoluble fraction of a liver extract. The proportionate amounts of radioactivity in ATP and ADP are wholly different, however. In the liver a larger amount is bound to ADP than to ATP. This is in agreement with other investigations; in the liver the quantity of ADP is considerably greater than the quantity of ATP [30, 31], while in the brain the greater part of the adenylic polyphosphate is ATP [29].

The specific activity of phosphate in different acid-soluble nucleotides — Tables 2 and 3 demonstrate the relative specific activities of the phosphate in AMP and DPN in relation to the specific activity of acid-labile ADP phosphate in

Table 2. The specific activity of the phosphate in adenine compounds from the livers of rats. — Substances purified by means of paper chromatography were extracted, after which the specific activity of the phosphate was determined. See Methods.

Time in minutes after injection of P^{32}	60		90 [c]
Compound	Specific activity	Relative specific activity [b]	Relative specific activity [b]
ADP	270		
AMP	130	32	38
DPN	61	15	17
Labile P of ADP [a]	410		

a) Calculated from the values for total ADP-P and AMP-P.
b) Specific activity of the labile P of ADP = 100.
c) Mean of two determinations.

the liver and brain, assuming that the acid-stable phosphate in ADP has the same specific activity as the phosphate in AMP. The specific activity of AMP's phosphate calculated in this way is the same as reported earlier [32] for the acid-stable phosphate in ATP in relation to the acid-labile. Thus AMP's phosphate is renewed at the same rate as the acid-stable phosphate in ATP. The tables also show that the mean value of the specific activity of DPN's two phosphates is half that of AMP's both in the liver and the brain.

Table 3. The specific activity of the phosphate in adenine compounds from the brains of rats. — Purification and determination as in the liver experiments.

Compound	Specific activity [b]	Relative specific activity [c]
ADP	2 400	
AMP	810	20
DPN	400	10
Labile P of ADP [a]	4 000	

a) Calculated as in the liver experiments.
b) 45 minutes after injection of P^{32}.
c) Specific activity of the labile P of ADP = 100.

Table 4. Labeling degree of the phosphate in DPN bound to adenosine and nicotinamide ribose. — Following the addition of unlabeled DPN as carrier, DPN with radioactive phosphate was purified from a TCA extract of four rat brains. The purified DPN was split into two mononucleotides with washed granules from rabbit kidneys. The NMN formed was partially purified. The animals were sacrificed two hours after the subarachnoidal injection of radioactive phosphate. The values are expressed in micromoles.

	NMN [a]	AMP [a]	Number of impulses per micromol P	Number of impulses per micromol AMP-P
Before purification of NMN	21.6	21.6 [b]	71	141
After partial purification of NMN	18.3	8.4	43	138

a) Free or as DPN.
b) Not determined.

Table 4 shows that the activity in DPN is found exclusively in the phosphate group which is bound to adenosine. Thus the phosphate bound to nicotinamide ribose has not been renewed. The phosphate in DPN which is bound to adenosine, therefore has both in the liver and the brain the same specific activity as the phosphate of AMP and the acid-stable phosphate of ATP.

DISCUSSION

The adenosine-bound phosphate of DPN is renewed *in vivo* although at so slow a rate that this phosphate group cannot directly enter into the phosphate-transferring system.

During the formation of two mononucleotides DPN can be split in two ways.

$$\text{DPN} + \text{pyrophosphate} \rightleftharpoons \text{NMN} + \text{ATP} \quad (1)$$

$$\text{DPN} \rightharpoonup \text{NMN} + \text{AMP} \quad (2)$$

The first of these reactions, described by Kornberg [33], is reversible and is catalyzed by an enzyme found in liver and yeast. The other, described by Kornberg and Lindberg [19], is an irreversible pyrophosphorylysis and is catalyzed by an enzyme purified from potatoes [34].

According to Schrecker and Kornberg [35] FAD can be split according to the following reaction.

$$\text{FAD} + \text{pyrophosphate} \rightleftharpoons \text{flavin mononucleotide} + \text{ATP} \quad (3)$$

Hummel and Lindberg [7] have reported, that a component, containing FAD and having a very high specific activity, can be isolated chromatographically from a liver homogenate which has been incubated under aerobic conditions and in the presence of radioactive orthophosphate. AMP and the flavin mononucleotide were not labeled at all, thus the FAD molecule cannot have obtained its high specific activity according to Reaction (3).

Investigations in this laboratory * have shown, that the highly labeled phosphate bound to FAD is considerably more acid-labile than the phosphate in the FAD molecule itself. Hence the flavin adenine dinucleotide isolated is probably a larger molecule than FAD; possibly an intermediary in Reaction (3). The high specific activity indicates that such a compound is renewed so rapidly that it can function as phosphate transferer in oxidative phosphorylation. Transferal of phosphate taking place in this way must be associated with a very rapid splitting and resynthesis of FAD.

The turned over phosphate in DPN has the same specific activity as the acid-stable phosphate in ATP, for which reason the splitting and resynthesis of DPN must take place more rapidly than the turnover of the phosphate of AMP. The speed of these reactions may be of the same order of magnitude as the renewal of one or more phosphate group in the FAD compound [7]. Since any transfer of phosphate through FAD in accordance with Reaction (3) must result in reversible splitting of the molecule, the rapid splitting and rebuilding of DPN may also be caused by a transferal of phosphate. Cross and associates [36] formulated the hypothesis that coenzyme pyrophosphate is formed primarily in oxidative phosphorylation. From the same laboratory there has been reported conjugation of DPN [37] and of an extremely labile phosphate fraction [38] to the oxidative enzyme complex in washed granules.

SUMMARY

1. A chromatographic method of purifying acid-soluble adenine nucleotides has been developed.

2. The phosphate of AMP is renewed in the liver and brain at the same rate as the acid-stable phosphate in ADP and ATP. In DPN the phosphate bound to adenosine is renewed at the same rate as the phosphate in AMP; the other phosphate is not labeled during short experiments. Thus DPN must be split and resynthesized at a rapid rate. The possibility that DPN transfers phosphate through its splitting is discussed.

* Ernster, L., Lindberg, O., and Zetterström, R. Unpublished experiment.

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