QUANTITATIVE DETERMINATION OF ADENOSINE TRI- AND DIPHOSPHATES

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Quantitative determination of adenosine triphosphate is one of the commonest and most necessary methods of biochemical investigation in laboratory practice. In recent years it has also become necessary to determine the specific activity (Sp. A) of adenosine triphosphate (ATP) phosphorus, as a measure of the metabolic potential in the cell. Many investigations have involved the determination of easily hydrolyzable tissue phosphorus by simple hydrolysis in 1 N HCl at 100° for 7 or 10 minutes. The values found generally reflect the quantitative content of ATP fairly accurately, but such analysis frequently distorts the true fractional distribution of phosphorus, especially if other easily hydrolyzable compounds are present. Moreover, the simple hydrolysis method does not allow of the determination of the specific activity of the ATP phosphorus. Precipitation of ATP by mercury or chromatographic determination cannot always be used, and are not always sufficiently accurate. Enzymatic methods for determination of ATP are also not very convenient.

Thus, there is no sufficiently reliable and accurate method, suitable for a wide range of laboratories, for determination of adenosine triphosphate. In this connection it is necessary to note the principle of the quantitative method for determination of ATP, originally put forward by Fiske [3] and applied in practice by Crane and Lipmann [2]. The method is based on adsorption of nucleotides on activated carbon and subsequent elution of the adsorbed ATP, ADP, and adenylic acid by aqueous pyridine, or removal of ATP and ADP labile phosphorus from the carbon by hydrolysis in 1 N HCl for 10 minutes at 100°.

Analysis of tissues for ATP and ADP by adsorption on carbon has been used for 2 years in the Biochemical Laboratory of the Leningrad Institute of Blood Transfusion. During this period its specificity has been confirmed, the possibility of its use for analysis of complex mixture of phosphorus-containing compounds has been established, and the optimum analytical conditions have been determined. As the results of detailed tests of the method of determination of ATP and ADP by adsorption on carbon, it may be described as a simple, rapid, and exact method, accessible and suitable for a wide range of laboratories.

Ba salts of ATP prepared by the Szent-Gyorgyi method, and Na salt preparations produced by the Ivanovo Meat Combine, were used in the investigations. ADP was obtained by enzymatic hydrolysis of ATP (by myosin). Adenylic acid was prepared by Kerr's method [5], cozymase by the Williamson and Green method [7], fructose-6-phosphate and fructose-1,6-diphosphate by the method of Neuberg et al. [6], and glucose-1-phosphate by the method of Sumner and Somers [1].

The most important factors influencing the results of quantitative determinations of ATP and ADP by adsorption on carbon are the pH of the medium during adsorption, the relative amount of adsorbent, and the procedure for the subsequent removal of extraneous impurities from the carbon with the adsorbed nucleotides. The optimum acidity for quantitative adsorption of ATP and ADP is pH 0.4-0.6. Accordingly, the best concentration of trichloroacetic acid in experiments with trichloroacetic acid centrifugates is 4-5%. Table 1 gives data on the influence of acidity of the medium on the degree of adsorption of pure ATP and ADP by carbon.

Some deviation from the optimum acidity, especially a decrease (down to 1%) has no significant influence on the results. Even in a neutral medium up to 70% ATP is adsorbed. This relative independence of adsorption on the pH of the medium is especially important if the experimental conditions are such that strong acidification must be avoided.

TABLE 1

Effect of Acidity of the Medium on the Degree of Adsorption

Substance	γ of labile P from data for 10-min hydrolysis in 1N HCl at 100°	Found			γ of phosphorus at a final trichloroacetic acid concentration (%)			
		0	1	4	4.5	5	8	9
ATP	185	128	—	—	180	—	—	140
ATP	125	—	115	119	—	—	100	—
ATP	60	—	—	—	55	—	—	45
ATP	50	—	44	47	47	46	—	—
ADP	50	—	—	45	45	—	—	—
ADP	50	—	—	45	—	46	—	—

The relative proportions of the amounts of carbon and adsorbed material are very important in relation to the accuracy of determinations of ATP and ADP. With the ordinary domestic specimens of active carbon (AG-2 and others) practically complete adsorption of ATP can be obtained by taking 1 mg of carbon per 1γ of easily hydrolyzable phosphorus. Since the amount of ATP in various cells and tissues rarely exceeds 200-300 γ of labile phosphorus per 1 g raw weight, 200-250 mg of carbon should be taken per each gram of cells or tissues. Table 2 gives the results of one of the experiments illustrating the effect of the quantitative proportions of the adsorbent and adsorbate on the degree of adsorption.

TABLE 2

Effect of the Proportions of Adsorbent and Adsorbate on the Degree of Adsorption

Carbon taken, in mg	ATP taken, in γ of phosphorus after 10-minute hydrolysis	Labile phosphorus found by the adsorption method	
		γ	% yield
100	45	43	95.5
100	90	87	96.4
100	180	165	90.2
100	360	270	75.0
100	540	350	64.8

Tests for specificity of the adsorption provided a very important criterion of the value of the method. The possibility of adsorption by the carbon from solution of other phosphorus compounds, capable of liberating orthophosphate after 10 minutes of hydrolysis in 1 N HCl at 100° was first examined. It was found that glucose-1-phosphate, fructose-1,6-diphosphate, and inorganic pyrophosphate are not adsorbed to any practical extent by carbon in the experimental conditions, and do not influence the yield of ATP. Diphosphopyridine nucleotide (DPN) is adsorbed by carbon. Our DPN preparation (≈ 60% pure) contained 17 γ of phosphorus, split off by 10 minutes of hydrolysis in 1 N HCl at 100°, per 1 mg dry weight. Carbon treatment gave 13.8 γ of 10-minute phosphorus per 1 mg DPN. However, the amounts of DPN in cells are small; for example, erythrocytes of the rabbit contain about 100γ per ml [4]. It is therefore evident that adsorption of DPN together with adenine

nucleotides by the carbon should not have any practical effect on the determination of ATP and ADP. Treatment of 1 ml of erythrocytes, containing 0.1 mg DPN, with carbon, would liberate little more than 1 γ of phosphorus.

An advantage of the method for isolation and determination of ATP by adsorption on carbon is the ease with which inorganic phosphorus can be removed, especially in experiments with radioactive phosphorus, from carbon containing adsorbed ATP and ADP. At P^{32} concentrations up to 0.5 μC per 1 ml of experimental mixture, twofold washing with water is enough to remove inorganic phosphorus from carbon containing ATP and ADP. With higher activities and in certain special cases it is necessary, before the washing with water, to wash the carbon once or twice with a mixture (1:1) of 0.05 M KH_2PO_4 solution and trichloroacetic acid solution. In one experiment 5 ml of a mixture containing nonradioactive ATP, corresponding to 200 γ of labile phosphorus, and 500 γ of orthophosphate with an activity of 1.0 μC was treated. After the usual procedure, 190 γ of easily hydrolyzable phosphorus was found on the carbon. The inorganic phosphorus was practically not determined, and the radioactivity of samples obtained by 10-minute hydrolysis of the carbon in 1 N HCl at 100° was 360 pulses calculated for the whole hydrolyzate (i.e., the whole original sample).

The possibility of determining ATP in complex mixtures of phosphorus compounds was also examined. A definite quantity of ATP was added to a mixture of orthophosphate, inorganic pyrophosphate, glucose-1-phosphate, fructose-1,6-diphosphate, fructose-6-phosphate, DPN, and adenylic acid (Table 3).

TABLE 3

Determination of ATP in a Mixture of Phosphorus Compounds

Composition of mixture in γ of phosphorus*		Increase in orthophosphate after 10 minutes of hydrolysis in 1N HCl at 100°, γ	Amount of easily hydrolyzable phosphorus
Inorganic orthophosphate	200	122	6
Inorganic pyrophosphate	50		
Glucose-1-phosphate	50		
Fructose-6-phosphate	50		
Fructose-1,6-diphosphate	50		
DPN	18		
Adenylic acid	20		
Ditto + ATP	180	302	175

* In γ of easily hydrolyzable phosphorus for ATP, glucose-1-phosphate, and pyrophosphate.

Table 3 shows that ATP is effectively differentiated from the other easily hydrolyzable phosphorus compounds.

The following standard procedure for analysis of cells and tissues for ATP, ADP, and inorganic phosphorus may be recommended. 3-4 ml of trichloroacetic acid centrifugate at a trichloroacetic acid concentration of 4-5%, and containing a quantity of cells corresponding to 1 g raw weight, is placed in the centrifuge tube with 250 mg of finely ground activated carbon (a weighing inaccuracy of 10-20 mg is permissible) and the mixture is thoroughly stirred with a glass rod. The suspension is left for 10 minutes with periodic stirring. For better sedimentation, 0.2 ml of alcohol (redistilled) is poured in a layer on top of the mixture, and the carbon particles which rise up the moist walls of the tube are carefully pushed into the alcohol layer with the rod. The suspension is centrifuged at 2000-3000 rpm for 10-15 minutes, and the liquid (liquid A) is decanted off. The carbon with the adsorbed ATP and ADP is washed twice with 5 ml of distilled water. The precipitate is carefully

stirred in the water each time by a rod, and a layer of 0.2 ml of alcohol is applied before centrifuging. The wash liquids are rejected. In special cases the carbon is washed with the mixture described above before the water washing. An equal volume of 2 N HCl and 2-3 ml of 1 N HCl is added to the carbon, and the samples are hydrolyzed for 10 minutes at 100°. The labile phosphorus of ATP and ADP is split off by hydrolysis and passes into solution. After hydrolysis, the samples are brought up to their original volume with water, or their new volume is noted. The samples are centrifuged this time without addition of alcohol, and the liquid is taken for photometric and radioactivity determinations. In parallel with the experimental samples, a portion of carbon is treated with the corresponding amount of 4 or 5% solution of trichloroacetic acid (according to the concentration of acid in the test samples). This sample is used as the reactive sample in experiments with the universal photometer or photoelectrocolorimeter.

The liquid A contains other organic phosphorus compounds, in addition to orthophosphate. Mineral phosphorus is precipitated in it by the usual methods. The precipitate contains orthophosphate, and the liquid contains hexose and triose phosphates and other organic phosphorus compounds. Since the inorganic phosphorus is precipitated in a solution already free from ATP and ADP, contamination of the precipitate is slight and in a number of cases reprecipitation is not necessary.

Adsorption of ATP and ADP on carbon may serve as the basis for a relatively simple, rapid, and fairly accurate method for determination of the contents and specific radioactivities of these compounds.

SUMMARY

The specificity and conditions of the application of the determination method of adenosine tri- and adenosine diphosphates by means of nucleotides adsorption on coal (method developed by Crane and Lipmann) have been studied. It has been stated that the presence in samples, besides ATP and ADP, of fructose-1, 6-diphosphate, fructose-6-phosphate, glucose-1-phosphate, inorganic pyrophosphate, orthophosphate, adenylic acid and DPN does not interfere with the determination of ATP and ADP. Completeness of ATP adsorption depends upon the pH of the medium, as well as upon the ratio between the adsorbed substance and the adsorbent. Acidity corresponding to 4-5% trichloracetic acid and the ratio 100 mg of coal per 100 γ of readily hydrolyzed phosphorus of ATP are the most favorable conditions. This method is simple, specific, and accurate.

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