ACTA CHEMICA SCANDINAVICA 26 (1972) 1166–1170

Determination of Inorganic Phosphate in Rat Liver

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Three methods for determination of inorganic phosphate in liver tissue by means of complexing with molybdate and reduction of the extracted complex to molybdenum blue are compared, the Wahler and Wollenberger method, the Martin and Doty method and a modification of the latter. The Martin and Doty method was found to give higher values than the two other mehods in which lower concentrations of acid and molybdate are employed.

It is concluded that methods for estimation of inorganic phosphate should be used, which, like the Wahler and Wollenberger procedure, employ concentrations of acid and molybdate as low as compatible with formation of the phosphomolybdate complex. If these precautions are not taken, hydrolysis of labile phosphate compounds may invalidate the analysis.

The increasing interest in phosphorylation processes has accentuated the need for determination of inorganic phosphate in different tissues. Nearly all methods used for such analyses are based on the principle that phosphomolybdic acid by reduction is converted to molybdenum blue. However, some of these procedures may lack specificity, particularly owing to danger of hydrolysis of labile organophosphate compounds.

Most recent investigators have used Martin and Doty's method [1] for determination of inorganic phosphate in tissues. Wahler and Wollenberger [2] found, however, that this method caused some hydrolysis of labile phosphate compounds. This was denied by Ernster *et al.*[3] who claimed that creatine phosphate in their modification of Martin and Doty's method [4] was not hydrolyzed.

As determinations of inorganic phosphate in rat liver in our hands gave lower results with the method described by Wahler and Wollenberger than with that of Martin and Doty we decided to investigate this discrepancy.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing about 200 g, fed *ad libitum*, were used for all experiments. Animals were anesthesized with pentobarbital (100 mg/kg). The abdomen was opened and a biopsy weighing about 250 mg was taken *in situ* with a freeze-clamp,

precooled in liquid nitrogen. The sample was immediately transferred to a weighed 10 ml centrifuge tube containing 1.5 ml ice-cold $HClO_4$ (3 N). The accurate weight of the sample was determined by reweighing. A second biopsy was taken from another lobe of the liver. About two minutes passed between removal of the first and the second biopsy. No ligation of liver lobes was performed after the first biopsy, but partial hemostasis was achieved with Spongostan® (a gelatine sponge with hemostatic effect).

The samples were homogenized at 0°C in the centrifuge tube with a teflon pestle precooled in ice. After centrifugation (10 min at 10 000 *g* and 0°C) 1.25 ml supernatant was neutralized in the cold with KOH-triethanolamine-hydrochloride buffer to pH 7.0. This mixture was centrifuged again and the clear supernatant was used for all phosphate determinations.

Inorganic phosphate was determined on each sample according to the methods described below.

1. The Martin and Doty method as described by Ernster

Reagents. 1, H_2SO_4, 5 M; *2,* H_2SO_4, 0.5 M; *3,* ammonium molybdate, 10 %; *4,* isobutanol-benzene mixture, 1:1 (vol/vol); *5,* $SnCl_2.2H_2O$, 10 % in concentrated HCl; *6,* working solutions of $SnCl_2$ were made by dilution of reagent 5 with H_2SO_4, 0.5 M (reagent 2) (1:200); *7,* H_2SO_4, 3.2 % (vol/vol) in absolute ethanol; *8,* phosphate stock solutions, 10 mM.

Working standards were made from reagent 8 by diluting to 1.0, 2.0, and 5.0 mM, respectively.

Procedure. 0.200 ml of the deproteinized neutralized tissue extract is placed in a test tube. To this is added 1.300 ml H_2O, 0.250 ml of reagent 1, 2.50 ml of reagent 4, and 0.250 ml of reagent 3. The mixture is shaken for 15 sec. After separation of the phases 1.000 ml of the organic phase is transferred to another tube and 1.500 ml of reagent 7 is added, followed by 0.250 ml $SnCl_2$-solution (reagent 6). The tube is shaken and allowed to stand for 15 min. Extinction is read against H_2O at 625 nm. Reagent blank is made by substituting 0.200 ml tissue extract with H_2O. Standards are made by adding 0.050 ml of working standards instead of 0.200 ml tissue extract. The entire procedure is performed at room temperature.

2. The modified Martin and Doty method

This method differs from the one mentioned above in two ways. First, the concentration of acid and molybdate used in the extraction procedure is lowered to the same level as in the method of Wahler and Wollenberger (*vide infra*). This is achieved by substituting reagent 1 in the method described above with H_2SO_4, 0.5 M, and reagent 3 with molybdate, 1 %. Second, the temperature is kept at 0°C as in Wahler and Wollenberger's method. The rest of the procedure is identical with that described by Ernster.

3. The Wahler and Wollenberger method

Reagents. 1, Na_2MoO_4, 0.005 M; *2,* perchloric acid, 5 % (5 ml 70 % PCA diluted to 100 ml with H_2O); *3,* ethylene glycol monomethyl ether, *p.a.*; *4,* isopropyl acetate, *p.a.*; *5,* $SnCl_2.2H_2O$, *p.a.*; *6,* phosphate stock solution, 10 mM.

Working standards (100 μM, 50 μM, and 25 μM) were made from reagent 6 just prior to use by diluting with reagent 2.

Procedure. In a test tube 1.500 ml of reagent 1, 2.000 ml of reagent 4, 0.100 ml of deproteinized tissue extract and 0.500 ml of reagent 2 are mixed. All components are precooled in ice. The tube is capped tightly and shaken vigorously for 30 sec and left on ice for separation of the phases. The rest of the procedure takes place at room temperature.

To 1.000 ml of the supernatant organic phase 1.000 ml of reagent 3 is added followed by 200 mg of finely powdered $SnCl_2$ (reagent 5).

After at least 15 min the extinction is read against ethylene glycol monomethyl ether as reference at 710 nm. Reagent blank and standards are made by substituting the sample with 0.500 ml reagent 2 or 0.500 ml working standard, respectively.

Phosphate determination on creatine phosphate

Creatine phosphate, 5 mM, was subjected to phosphate determination according to all three methods. In procedures 1 and 2, 0.050 ml was treated as phosphate standard. In the method of Wahler and Wollenberger the solution was diluted 1:20 and treated as sample.

RESULTS AND DISCUSSION

The individual values found for inorganic phosphate in six different rat livers are given in Table 1. It is seen that Martin and Doty's method as modified by us and the Wahler and Wollenberger method give identical results. However, the original method of Martin and Doty as described by Ernster gives results which are 70 % higher than the results of the other methods. Consequently recovery experiments were performed with all three methods.

Table 1. The concentration of inorganic phosphate in rat liver. Results are expressed in μmol/g wet wt. of liver.

Expt.	Method of Martin and Doty		Modified method of Martin and Doty		Method of Wahler and Wollenberger	
	1st biopsy	2nd biopsy	1st biopsy	2nd biopsy	1st biopsy	2nd biopsy
141270	4.36	5.89	2.44	3.14	2.83	3.42
161270	4.35	4.92	2.66	3.17	2.59	2.79
060171	4.35	5.63	2.14	2.52	2.47	3.08
080171	3.98	4.34	2.42	2.67	2.14	2.46
130171	3.79	4.53	2.63	3.17	2.49	2.86
150171	3.61	4.31	2.01	2.45	2.01	2.40
Mean	4.07	4.94	2.38	2.85	2.42	2.84
±S.E.	0.13	0.28	0.11	0.14	0.12	0.16

In the Wahler and Wollenberger procedure this was done by substituting 0.500 ml of perchloric acid in the sample assay by 0.500 ml of a 25 μM standard phosphate solution in perchloric acid. In the two other methods 0.050 ml of a 1.00 mM solution were added to the sample assay. As seen in Table 2 the recovery is about 100 % in all three methods. These recovery experiments only verify that the slope of the standard curve is the same with tissue extracts as with aqueous solutions of the substance to be determined. Still the intercepts with the axes might be different. To check this, recovery experiments were also made by diluting the samples 1:1 prior to the assay. As seen in Table 2 there is good agreement between the concentration found in the sample and after dilution. From this it can be concluded that the intercepts with the

Table 2. Determination of inorganic phosphate in the rat liver. Recovery experiments.

Method	Conc. in sample	Conc. added to sample	Conc. measured	Recovery (%)	Conc. in diluted sample (1:1)
Wahler and Wollen-berger	53 μM	25 μM	74 μM	95	29 μM
	53	50	110	107	–
	57	25	75	92	30
	118	25	142	99	63
	92	25	120	103	49
Martin and Doty	2.45 mM	1.00 mM	3.60 mM	104	1.25 mM
Modified method of Martin and Doty	0.60 mM	1.00 mM	1.75 mM	109	0.25 mM
	0.65	1.00	1.75	106	0.30
	2.00	1.00	3.05	102	1.10
	0.80	1.00	1.88	104	0.45
	0.95	1.00	2.15	110	0.45

axes in all three methods are identical for curves from tissue extracts and from aqueous standard solutions.

The reason for the higher values for inorganic phosphate with Martin and Doty's method could be that some of the phosphate in labile organic compounds is liberated during the assay. Therefore we estimated to which extent a 5 mM creatine phosphate solution assayed as phosphate gives false values. As seen in Table 3 creatine phosphate undergoes a considerable hydrolysis during the assay according to Martin and Doty, while this is not the case in Wahler and Wollenberger's method. The cause of this hydrolysis in the method of Martin and Doty is certainly partly the high concentration of acid and molybdate used and partly that the assay is performed at room temperature. As shown by Yanagita [5] the final concentration of H_2SO_4 and molybdate could be lowered to 0.1 N and 1.6×10^{-3} M, respectively, without disturbance of the quantitative formation of phosphomolybdate. In the original method of Martin and Doty the final concentration of H_2SO_4 and molybdate is 1.0 N and 10.7×10^{-3} M.

From the above mentioned we must conclude, that both the Wahler and Wollenberger method and the method described by us are more reliable than

Table 3. Determination of creatine phosphate as inorganic phosphate by different methods. The concentration of the creatine phosphate was 5 mM.

Expt.	Method of Martin and Doty (mM)	Modified method of Martin and Doty (mM)	Method of Wahler and Wollenberger (mM)
031270	2.35	0.30	0.25
081270	2.05	0.20	0.30
030271	1.98	0.35	0.20
040271	2.25	0.35	0.30

the Martin and Doty method. As the method described by Wahler and Wollenberger is easier to handle, requiring fewer prepared reagents, we would recommend it as a standard procedure.

In most literature concerned with the level of inorganic phosphate in liver, a concentration range of $4-6$ mM is considered to be normal.[6,7] Very often this is based upon measurements by the Martin and Doty procedure.

Schultz *et al.* [8] employing an enzymatic method performed at neutral pH without molybdate, obtained values of about 2.8 mM, which is only a little higher than the value we found. The small difference can probably be explained by the fact that Schultz *et al.* submerge the whole animal in liquid N_2, instead of freezeclamping the liver tissue, and consequently the cooling takes somewhat longer time. This will imply a risk of anoxia with resulting hydrolysis of labile phosphate compounds. This is in accordance with our observation that the second biopsy always shows somewhat higher phosphate concentration than the first one taken from the same animal (Table 1). This indicates that reliable results can only be expected when freeze-clamping is performed from the liver *in situ* and with the least possible damage to the animal before taking the sample. Any procedure leading to even very short ischemia or anoxia or to delayed cooling of the sample must be avoided.

Deodhar and Mistry [9] utilize a method by Parvin and Smith [10] which is not based upon formation of molybdophosphate but molybdovanadophosphate. They report values of about 2 mM.

The original paper by Wahler and Wollenberger reports no normal values for liver tissues, but in our hands the method gives a mean value of 2.42 ± 0.12 μmol/g liver. In the light of the literature and considering the recovery experiments and the creatine phosphate experiment, this seems to be rather close to the true value.

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Received June 8, 1971.