12. Kemp, T., *Acta. Path. Microbiol. Scand.*, 1933, Suppl. 16, 189.

13. Snell, G. D., *Anat. Rec.*, 1930, v47, 316.

14. Smith, P. E., MacDowell, E. C., *ibid.*, 1930, v46, 249.

15. ———, *ibid.*, 1931, v50, 85.

16. Carsner, R. L., Rennels, E. G., *Science*, 1960, v131, 829.

17. Kemp, T., Marx, L., *Acta. Path. Microbiol. Scand.*, 1936, v13, 512; 1937, v14, 197 (suppl. 32).

18. Møllenback, C. J., *Acta. Path. Microbiol. Scand.*, 1940, v18, 169.

19. Grunnet, J., *ibid.*, 1942, v19, 37.

20. Bartels, E. D., *ibid.*, 1941, v18, 20.

21. Francis, T., *ibid.*, 1944, v21, 929; 1945, v22, 138.

22. Evans, H. M., Simpson, M. E., *Am. J. Physiol.*, 1931, v98, 511.

23. Evans, H. M., Simpson, M. E., Meyer K., Reichert, F. L., Memoirs of Univ. of California, Univ. of California Press, Berkeley, 1933, v11, 422.

24. Geschwind, I. I., Alfert, M., Schooley, C., *Biol. Bull.*, 1960, v118, 66.

25. Nielsen, E. L., *Acta. Path. Microbiol. Scand.*, 1953, v32, 316.

26. Marlowe, T. J., *J. Animal Sci.*, 1960, v19, 810.

27. Foley, C. W., Heidenreich, C. J., Lasley, J. F., *J. Hered.*, 1960, v51, 278.

28. Mirand, E. A., Osborn, C. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 746.

29. Stein, I., Stein, R. O., Beller, M. L., *Living Bone in Health and Disease*, 1955, p132, J. B. Lippincott Co., Philadelphia and Montreal.

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Enzymatic Estimation of Phosphocreatine.* (27097)

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The most widely used method for estimation of phosphocreatine (PC)† in tissues is that of Fiske and Subbarow(1). In this method, inorganic phosphate (Pi) is precipitated from a neutralized trichloracetic or perchloric acid extract by alkalinized calcium chloride, and the PC in the filtrate is determined as Pi after hydrolysis with acid molybdate at room temperature for 30 minutes. The criticisms of this method have been reviewed by Ennor and Morrison(2). In our experience, the main disadvantage of this method is that relatively large tissue samples are required. If small organs are under study, a micro-method is required for a complete analysis of all phosphorus compounds and other metabolites. The method described here meets such a need. It makes use of the enzyme creatine-phosphokinase (CPK), first crystallized by Kuby, Noda and Lardy(3) and now available commercially. Furthermore, the CPK-catalysed reaction is coupled with the enzymatic reactions utilized for estimation of ATP. It is thus possible to determine ATP and PC in the same sample of muscle extract corresponding to as little as 2 to 5 mg of fresh muscle.

Experimental. Principle of method. In the presence of excess 3-phosphoglyceric acid (3-PGA), limiting amounts of ATP can be determined according to the reactions:

1. 3-phosphoglyceric acid + ATP $\xrightarrow{\text{3-phosphoglyceric kinase (PGK)}}$ 1,3-diphosphoglyceric acid + ADP

2. 1,3-diphosphoglyceric acid + DPNH + H^+ $\xrightarrow{\text{glyceraldehyde phosphate dehydrogenase (GAPDH)}}$ glyceraldehyde phosphate + DPN^+ + H_3PO_4

The decrease in DPNH and thus of the optical density (ΔE) at 340 mμ is a measure

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† Abbreviations: Phosphocreatine (PC), inorganic phosphate (Pi), Adenosine-5′, tri- and diphosphate (ATP, ADP), Creatine Phosphokinase (CPK), 3-Phosphoglyceric kinase (PGK), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), reduced glutathione (GSH), Phosphoglyceric acid (PGA), reduced diphosphopyridine nucleotide (DPNH).

of the amount of ATP present.

If phosphocreatine is present in the extract, addition of creatine phosphokinase gives rise to reaction 3.

3. Phosphocreatine + ADP $\xrightarrow{CPK}$ creatine + ATP

ATP formed in this reaction follows reactions 1 and 2 and the second decrease in optical density corresponds to amount of PC present.

The assay of ATP based on reactions 1 and 2 has been described by Bücher(4). (See also "Biochemica" Boehringer 1960). However, if PC is to be determined along with ATP the concentrations of the reactants have to be modified as described below.

Enzymes and reagents. All reagents are dissolved in glass-redistilled water, DPNH in Tra-EDTA buffer. The enzymes CPK, PGK and GAPDH were purchased from C. F. Boehringer & Soehne, Mannheim-Waldhof, Germany. Prof. H. A. Lardy also kindly supplied us with a sample of CPK. PGK and GAPDH are supplied as suspensions in ammonium sulphate solution, and are stable for months if kept in the refrigerator. CPK is obtained as a lyophilized powder. It is suspended in 2M ammonium sulfate and keeps for at least a month under refrigeration.

Tra-EDTA buffer pH 7.6 (Tra 0.1M, EDTA, 0.01M) 9.3 g triethanolamine · HCl (Boehringer) and 1.86 g ethylenediamine tetraacetate $Na_2 \cdot 2H_2O$ (Merck-Darmstadt) were dissolved in glass-redistilled water. The pH was adjusted to 7.6 with NaOH and the solution diluted to 500 ml.

0.5M $MgSO_4$

0.01M DPNH Na_2 (Boehringer)

0.1M 3-phosphoglyceric acid, tricyclohexylammonium salt · $3H_2O$. (Boehringer)

0.05M reduced glutathione (GSH) freshly neutralized with 1N NaOH to pH7.

0.01M ADP $Na_3 \cdot H_2O$ (Boehringer)

Phosphocreatine Potassium Salt: The natural as well as synthetic PC Ca salt was prepared in this laboratory(1,5,6). The potassium salt was prepared by shaking the calcium salt with a suspension of Dowex 50, K form.

Procedure. The preparation of extracts from frozen tissues has been described(7), except that here perchloric acid was used for deproteinization instead of trichloracetic acid. 9.2 ml of 0.65 N $HClO_4$ is used per gram frozen tissue in order to correct for the tissue water content and end with a 1:10 dilution. After filtration the extract is neutralized in the cold with KOH, using per 1 ml extract 5 µl of 1% phenolphthalein in 50% alcohol as indicator. After 10 min. chilling in ice water the $KClO_4$ is centrifuged off. CO_2 gas is now blown over the surface of the perchlorate-free extract until decolorization. The extract so prepared may be stored for a few hours in the refrigerator before use. If PC alone is to be analysed the extract may be stored refrigerated overnight. The volume of the sample (0.02-0.1 ml) is so chosen that total decrease in optical density (ΔE) at 340 mµ due to ATP and PC should not exceed 0.4.

Into a semimicro-cuvette (light path 1 cm, width 0.4 cm) of a Beckman B spectrophotometer or an equivalent instrument the following reagents are pipetted in this order:

	Final concentration
0.500 ml Tra EDTA	50 mM Tra, 5 mM EDTA
0.015 ml $MgSO_4$	7.5 mM
0.015 ml DPNH	0.15 mM
0.070 ml 3-PGA	7 mM
0.035 ml GSH	1.75 mM
0.01 ml ADP	0.1 mM
Sample to be analysed	
Water to a vol of 0.982	

Stir with a plunger made of polyethylene tubing closed and flattened at one end. After one or more readings are taken, the enzymes for ATP determination are pipetted onto the foot of the plunger: 3 µl of GAPDH containing 50 Bücher units(8) (10 mg protein/ml) and 5 µl of PGK containing 100 Bücher units (2 mg protein/ml). Stir and take readings at 2-minute intervals until there is no further decrease in optical density. This takes up to 10 minutes if ATP is present. Now add 10 µl of CPK containing 120 Bücher units (20 mg lyophilized powder/ml) and read every 2 minutes till the reactions come to an end. This may take from 5-20 minutes. The blank cuvettes contain only Tra EDTA buf-

TABLE I. Comparison of PC Values Obtained by Enzymatic Method and Colorimetric Method of Fiske and Subbarow. All figures refer to mg phosphorus per 100 g fresh tissue, except those for the natural and synthetic phosphocreatine where values indicate mg P/100 ml solution.

	Colorimetric method			Enzymatic method
	PC · P	Inorg. P	Total P	
Natural phosphocreatine	4.57	0	4.77	4.85
Synthetic "	3.73	0	3.85	3.92
Rat skeletal muscle	55.1			57.3
" heart	10.6			11.4
" liver*	16.9			.77
Dog skeletal muscle	51.3			54.0
" liver*	12.9			.74
" diaphragm	45.3			48.3
" diaphragm*	63.4			46.4
" kidney cortex	1.06			.84
" " medulla	.74			.56

fer. Omission of the enzymes in the blank solution introduces no significant error for routine work. The increase in optical density due to their opacity is counteracted by a decrease due to their dilution effect. For very accurate work the enzymes should be added to the blank also and appropriate corrections made for dilution effects. Addition of ADP is superfluous when the tissues contain enough ATP, since ADP is formed in the reactions used to determine ATP. It is advisable to omit ADP at first and let reactions 1 and 2 run. If ΔE for ATP determination is 0.1 or more, no ADP is required for the PC determination. If ΔE (for ATP) is less than 0.1, 5-10 μl of the ADP solution is added. After a steady reading is obtained, CPK is introduced and the ΔE for PC measured. All measurements are taken at a temperature between 25 and 26°C.

Calculations. Example: 6 cc of dog muscle extract (1:10) were diluted to 6.83 by neutralization. Sample = 0.03 ml; ΔE for ATP = 0.09; ΔE for PC = 0.285.

The optical density at 340 mμ of a solution containing 1 micromole of DPNH/ml (light path 1 cm) is taken as 6.22. The terminal ATP phosphorus in mg per 100 g muscle =

$$\frac{0.09}{6.22} \times 31 \times \frac{1000}{0.03} \times \frac{6.83}{6} \times \frac{1}{1000} = 17.0$$

(or 34 mg "Labile Nucleotide Phosphorus")

The PC phosphorus in mg per 100 g muscle =

$$\frac{0.285}{6.22} \times 31 \times \frac{1000}{0.03} \times \frac{6.83}{6} \times \frac{1}{1000} = 54.0$$

Results and discussion. A comparison of the enzymatic method with that of Fiske and Subbarow(1) is illustrated in Table I which contains typical results of analysis of pure phosphocreatine solutions as well as tissue extracts. Duplicate determinations by use of the enzymatic method agree to within 1%, which is rarely attainable with the colorimetric method. However, we have consistently obtained higher values (4-7%) of phosphocreatine with the enzymatic method than with the colorimetric method. This is true of tissue extracts as well as pure PC solutions. We have so far been unable to account for this discrepancy.

Our results establish the validity of the method of Fiske and Subbarow. We have been unable to confirm some of the criticisms directed against the latter method *viz.*: that the inorganic phosphate is not completely precipitated by alkaline calcium chloride or that a significant part of the ATP is hydrolysed during 30-minute contact with acid molybdate at room temperature. We wish, however, to call attention to 2 possible pitfalls in using the Fiske and Subbarow method. First, the extract should be just neutral to phenolphthalein before precipitating inorganic phosphate with calcium chloride. If the solution is too alkaline, calcium hydroxide is also precipitated and carries with it a significant amount of phosphocreatine. Second, if the solution contains too much inor-

ganic phosphate, a voluminous calcium phosphate precipitate adsorbs small amounts of phosphocreatine. This latter problem does not arise during routine analysis of tissues, but only when large amounts of inorganic phosphate are added to the sample. It also occurs during the synthetic preparation of PC.

There is one condition, however, where the method of Fiske and Subbarow fails, *i.e.*, when the tissues to be analysed contain more than 2% glycogen. Under these conditions the inorganic phosphate is not precipitated by calcium chloride and one obtains "false positives" for PC. The values obtained for inorganic phosphate by the Fiske and Subbarow scheme are almost zero. This finding has been reported for liver by Flock, Bollmann and Mann(9) and by Sacks(10). We have repeatedly failed to find PC in the liver. Whenever such "false positives" were obtained, we tested for PC in the protein-free extract by preparing a hydrolysis curve according to Fiske and Subbarow(1) and concluded that the apparent PC was only inorganic phosphate. Use of the enzymatic method has confirmed our previous findings as shown in the Table (items marked with asterisks). It can thus be stated that there are no significant amounts of phosphocreatine in dog and rat livers.

The comments concerning the liver apply also to muscle. The table contains a case for a dog diaphragm. Here again the inorganic phosphate value was zero, and the enzymatic estimation is more reliable than the colorimetric method. We assume also that the colorimetric method cannot be used for skeletal muscles containing excessive amounts of glycogen, as in the glycogen storage diseases, or for heart muscle where, owing to diabetes or starvation, there may be accumulation of glycogen.

Summary. An enzymatic micro-method has been described which permits simultaneous determination of adenosine triphosphate and phosphocreatine in extracts corresponding to 2-5 mg of muscle. The method has been found to be essentially in agreement with the colorimetric method of Fiske and Subbarow. Phosphocreatine in tissues containing more than 2% glycogen cannot be estimated by the colorimetric method and the enzymatic procedure must be used.

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1. Fiske, C. H., Subbarow, Y., *J. Biol. Chem.*, 1929, v81, 629.

2. Ennor, A. H., Morrison, J. F., *Physiol. Rev.*, 1958, v38, 631.

3. Kuby, S. A., Noda, L., Lardy, H. A., *J. Biol. Chem.*, 1954, v209, 191.

4. Hohorst, H. G., Kreutz, F. H., Bücher, T., *Biochem. Z.*, 1959, v332, 18.

5. Zeile, K., Fawaz, G., *Z. Physiol. Chem.*, 1938, v256, 193.

6. Fawaz, G., Zeile, K., *ibid.*, 1940, v263, 175.

7. Fawaz, G., Hawa, E. S., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 277.

8. Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., Pfleiderer, G., *Z. f. Naturforschung*, 1953, v8b, 555.

9. Flock, E., Bollman, J. L., Mann, F. C., *J. Biol. Chem.*, 1936, v115, 179.

10. Sacks, J., *J. Biol. Chem.*, 1949, v181, 655.

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