We thank Drs. G. B. Jerzy Glass and W. Mersheimer for initiating these studies and D. N. Teller, J. M. Guidi, and J. B. Wenig for invaluable technical assistance.

1. Chow, B. F., Barrows, L., Ling, C. T., *Arch. Biochem. & Biophys.*, 1951, v34, 151.

2. Hellegers, A., Okuda, K., Nesbitt, Jr., R. E. L., Smith, D. W., Chow, B. F., *Am. J. Clin. Nutr.*, 1957, v5, 327.

3. Rice, E. G., Herndon, J. F., Van Loon, E. J., Greenberg, S. M., *Am. J. Physiol.*, 1958, v193, 513.

4. Glass, G. B. J., Boyd, L. J., Luhby, A. L., *J. Lab. and Clin. Med.*, 1955, v46, 60.

5. Luhby, A. L., Ccoperman, J. M., Donnenfeld, A. M., Herrero, J. M., Teller, D. N., Wenig, J. B., *Proc. Soc. Ped. Res.*, May 1958, p140.

6. ———, *A.M.A. J. Dis. Child.*, 1958, v96, 532.

7. Ross, G. I. M., Mollin, D. L., *Vit. B_{12} and Intrinsic Factor*, F. Enke, Stuttgart, 1957, p437.

8. Boger, W. P., Bayne, G. M., Wright, L. D., Beck, G. D., *New England J. Med.*, 1957, v256, 1085.

9. Izak, G., Rachmilewitz, M., Stein, Y., Berkovici, B., Sadowsky, A., Aronovitch, Y., Grossowicz, N., *A.M.A. Arch. Int. Med.*, 1957, v99, 346.

10. Lust, J. E., Hagerman, D. D., Villee, C. A., *J. Clin. Invest.*, 1954, v33, 38.

Received October 15, 1958. P.S.E.B.M., 1959, v100.

Total, Phospholipid and Labile Phosphorus in Serum and Tissue Employing Chloric Acid and N-Phenyl-P-Phenylenediamine.* (24577)

JESSE F. GOODWIN (Introduced by Alfred Jay Bollet)

Dept. of Medicine, Wayne State University College of Medicine, and Receiving Hospital, Detroit, Mich.

The advantages of chloric acid as a reagent suitable for digestion of serum and serum extracts in the colorimetric estimation of phosphorus have been pointed out(1). Its rapidity, ease of digestion and of handling has resulted in considerable reduction of digestion time. Our purpose is to report an improvement in the chloric acid procedure which provides a less tedious method for color development thus rendering the method more desirable for routine determinations. In most procedures the inorganic phosphate present after digestion is treated with molybdate forming a phosphomolybdate complex. Addition of a suitable reducing agent selectively reduces this complex to yield a blue color. Many reducing agents have been investigated in an effort to find one which will give a stable color in the presence of the neutralized decomposition products of chloric acid digestion. Stannous chloride(2,3,4), 1,2,4-aminonaptholsulfonic acid(5) 2,4-diaminophenol(6), p-methyl aminophenol sulfate(7,8) and ferrous sulfate (9) all proved to be unsatisfactory with chloric acid digests. Hydrazine sulfate(1) has proved a sensitive reductant, but requires heating for color development. Use of p-semidine hydrochloride(10) eliminates this step.

Procedure for phospholipid phosphorus in serum. Chloric acid reagent. 500 g of potassium chlorate in a 3 liter beaker with 900 ml of distilled water was heated until solution was effected. After cooling, 375 ml of 72% perchloric acid was added with constant stirring. Some potassium perchlorate precipitated; the covered beaker was kept in a refrigerator for 12-24 hours, then filtered with suction through Whatman 41H filter paper. The filtrate was an approximately 28% solution of chloric acid. *Standard phosphorus solution stock.* Pure dry mono potassium phosphate, 0.351 g was dissolved in distilled water. Ten ml of 10N sulfuric acid was added and diluted to 1 liter with water. This solution contained 0.08 ml of phosphorus per ml. *Working standard phosphorus solution.* 50 ml of the standard phosphorus stock solution was diluted to 200 ml with water. Each ml contained 0.02 mg of phosphorus. *Molybdate reagent.* 0.024 M ammonium molybdate in

* With aid of grants from Mich. Chapter Arthritis and Rheumatism and Receiving Hospital Research Corp.

10N sulfuric acid. *p-Semidine hydrochloride.*† 50 mg in 100 ml of 1% sodium bisulfite. After vigorous shaking the small amount of insoluble residue was filtered. *Phenolphthalein indicator.* One per cent solution of phenolphthalein in 95% ethanol. *8% sodium hydroxide in water. Alcohol-ether mixture.* Three volumes of 95% reagent ethanol and one volume of reagent grade diethyl ether were mixed. *Extraction.* 1 ml of serum was slowly pipetted into a 25 ml volumetric flask containing approximately 18 ml of alcohol-ether mixture. This was stoppered loosely, placed in a boiling water bath and brought to a boil. Cooled and diluted to the 25 ml mark. The mixture was filtered rapidly to avoid loss of solvent by evaporation. *Digestion.* 5 ml aliquots of the filtrate were evaporated to dryness in a 180 ml electrolytic beaker. Two or 3 acid washed Henger crystals were put into the beaker, along with 5 ml of chloric acid, and 4 drops of 10% NaCl. The beaker was covered with a borosilicate watch glass and digested on a hot plate till white fumes of perchloric acid appeared. At this point the watch glass was removed and the residue heated to incipient dryness. The beakers were removed from the hot plate and the crystalline residue redissolved in 5 ml of water, washing down the walls of the container. The beaker was placed back on the hot plate and boiled for 2-3 minutes without cover and cooled to room temperature. *Color development.* The phosphate containing solution was neutralized with 8% sodium hydroxide, and a drop of 1% phenolphthalein as indicator. After transfer to a 25 ml volumetric cylinder, 2 ml of molybdate reagent, and 6 ml of p-semidine reagent were added and the volume brought to 20 ml with water. The absorbance was measured after 20 minutes at 700 mμ in a Coleman spectrophotometer. *Standards.* One-half, one and 2 ml portions of the working phosphorus standard solution were pipetted into 180 ml electrocytic beakers. Four drops of 10% sodium chloride were added and digestion and color development proceeded as above. Water carried through the determination was used a blank. *Procedure for total serum phosphorus and organic phosphorus.* To 0.2 ml of whole serum or suitable sample containing 10 to 80 γ of phosphorus in a 180 ml electrocytic beaker, 7 ml of chloric acid was added; digestion and color development proceeded as above. *Procedure for labile phosphorus.* The sample to be analyzed (containing 8 to 80 γ of hydrolyzable phosphorus in one ml or less) was deproteinized by adding 5 ml of 5% trichloroacetic acid. After centrifuging at 2500 rpm for 15 minutes, 1 ml of 5N sulfuric acid was added to the supernatant solution. The tubes were heated in a boiling water bath for 5 to 20 minutes, and cooled. To a 5 ml aliquot, in a 25 ml volumetric cylinder, 1.35 ml of 10N H_2SO_4 was added and mixed. Two ml of 0.024M ammonium molybdate and 6 ml of p-semidine reagent was added and the volume brought to 20 ml with water. The absorbance was measured after 20 minutes at 700 mμ in a Coleman Jr. Spectrophotometer.

Results. Serum samples of donors selected at random at the time of blood donations were estimated for phosphorus content by this

TABLE 1. Comparison of Methods for Phospholipid Phosphorus in Blood Serum Using Chloric Acid, 18 Samples.

(mg of phosphorus/100 ml)		
Hydrazine reduction method at 830 mμ (Beckman D.U. spectrophotometer)	p-Semidine reduction method at 700 mμ (Coleman Jr. spectrophotometer)	% diff.
7.2	7.2	.00
10.8	10.9	.93
8.0	8.0	.00
8.2	8.2	.00
10.6	10.6	.00
8.0	8.0	.00
6.8	6.6	−2.94
6.4	6.4	.00
7.9	8.0	1.26
8.9	8.8	−1.11
6.5	6.5	.00
8.3	8.2	−1.32
8.5	8.4	−1.17
8.3	8.5	2.40
10.5	10.3	−1.90
8.3	8.3	.00
9.6	9.5	−1.04
9.4	9.1	−3.20
8.46 ± 1.326*	8.42 ± 1.315*	

* Mean and stand. dev.

† Obtainable from Distillation Products Industries, division of Eastman Kodak Co., Rochester, N. Y. as N-phenyl-p-phenylenediamine hydrochloride, #2043.

TABLE II. Comparison of Methods for Total Phosphorus in Blood Serum Using Chloric Acid, 12 Samples.

(mg of phosphorus/100 ml)		
Hydrazine reduction method at 830 mμ (Beckman D.U. spectrophotometer)	p-Semidine reduction method at 700 mμ (Coleman Jr. spectrophotometer)	% diff.
13.80	14.00	1.45
14.50	14.00	−3.45
13.00	12.80	1.54
10.30	10.35	.48
13.50	13.00	−3.70
12.50	12.50	.00
14.00	13.80	−1.43
13.80	14.40	4.35
12.60	13.10	3.97
12.50	12.50	.00
13.00	13.00	.00
13.60	13.80	1.47
13.09 ± 1.086*	13.10 ± 1.072*	

* Mean and stand. dev.

method. Chloric acid digestion(1) compares well with the Fiske-Subbarow(5) method for phosphorus estimation. Duplicate samples were determined in establishing values using both methods. The results are shown in Tables I and II.

Discussion. The color obtained by p-semidine reduction is stable, although the rapidity of maximal color development in chloric acid digests is retarded when freshly prepared reagent is not used. Freshly prepared solutions of p-semidine gave maximal color development in 8-12 minutes, the 2-day-old reagent in 12 to 14 minutes, and 10-day-old reagent 20-25 minutes. Therefore, whenever possible this reagent should be prepared immediately prior to use. Filtration of the p-semidine reagent after mixture with one per cent sodium bisulfite is not essential, provided the powdered drug is dispersed sufficiently by thorough shaking in a machine such as a Burrell wrist-action shaker. The insoluble particles remaining do not interfere with color development and in most cases the unfiltered reagent remained stable for a longer period. (Instability was indicated by a change in color to yellow.)

During the digestion process it is necessary that all of chloric acid be destroyed, since it alters color development (the blue reduced phosphomolybdate complex changes to greenish blue). This is accomplished by taking the digest almost to dryness followed by boiling the residual salts in a small amount of water. Reproducibility of the standards is enhanced by addition of 1 or 2 drops of 10% sodium chloride. The sodium chloride probably prevents formation of pyrophosphate during the stage when the digest is brought almost to dryness. The naturally occurring salt in blood serum should have a similar protective influence.

The principal advantages of this method are the relative ease of digestion with chloric acid and the ease of color development and stability of color formed by p-semidine reduction of the phosphomolybdate complex. The phosphorus assay described has been used by us to measure hexokinase and phosphoglucomutase activity in tissue extracts.

Summary. A method is described for determination of serum total and organic bound phosphorus using chloric acid digestion and subsequent formation and reduction of a phosphomolybdate complex with p-semidine. The advantages of the method are ease of digestion and color development and stability of color-complex. This method compares well with the Fiske-Subbarow method.

1. Goodwin, J. F., Thibert, R., McCann, D., Boyle, A. J., *Anal. Chem.*, 1958, v30, 1097.

2. Kuttner, T., Cohen, H. R., *J. Biol. Chem.*, 1927, v75, 517.

3. Leichtenstein, V., *ibid.*, 1930, v86, 671.

4. Shinowara, G. Y., Jones, J. B., Reinhart, H. L., *ibid.*, 1942, v142, 921.

5. Fiske, C. H., Subbarow, Y., *ibid.*, 1925, v66, 375.

6. Allen, J. L., *Biochem. J.*, 1940, v34, 858.

7. Gomori, G., *J. Lab. and Clin. Med.*, 1942, v27, 955.

8. Maclay, E., *Am. J. Med. Technol.*, 1951, v17, 265.

9. Taussky, H. H., Sherr, E. J., *J. Biol. Chem.*, 1953, v202, 675.

10. Dryer, R. L., Tammes, A. R., Routh, J. I., *ibid.*, 1957, v225, 177.

Received October 31, 1958. P.S.E.B.M., 1959, v100.