and Silverberg, J. H., *Am. J. Clin. Path.*, 1952, v22, 146.

9. Quick, A. J., and Stefanini, M., *J. Gen. Physiol.*, 1948, v32, 191.

10. Muirhead, E. E., *Surg., Gyn. and Obst.*, 1951, v92, 734.

11. Harrington, J., Minnich, J., Hollingsworth, J. W., and Moore, C. V., *J. Lab. and Clin. Med.*, 1951, v38, 1.

12a. Stefanini, M., and Chatterjea, J. B., *J. Clin. Invest.*, 1951, v30, 676.

12b. Stefanini, M., Chatterjea, J. B., Dameshek, W., Zannos, L., and Perez, E. S., *Blood*, 1952, v7, 53.

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Direct Titrimetric Method for Determination of Carbonates in Small Amounts of Serum.* (19467)

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This paper presents a simple, direct acidimetric titration procedure for the estimation of carbonates, employing small volumes of serum or plasma. A simple method employing small volumes has often posed a critical problem in the management of newborns and prematures. The principle of the new method is: 1) $HCO_3^- + Ba(OH)_2 \rightarrow BaCO_3$ (ppt.); 2) Ash to remove organic material (not necessary for pure inorganic solutions); 3) $BaCO_3 + H_3BO_3$ (hot 10% sol. in excess) $\rightarrow Ba(H_2BO_3)_2$; 4) $Ba(H_2BO_3)_2 + 2HCl \rightarrow BaCl_2 + 2H_3BO_3$; bicarbonate ion is precipitated as the barium salt, by the addition of barium hydroxide directly to the solution to be analyzed (*i.e.*, serum or plasma). The barium carbonate is dissolved in a hot solution of 10% boric acid in a manner similar to that described for dissolving calcium carbonate in the ultramicro estimation of serum calcium(1-3). This solution is diluted and titrated directly with standard hydrochloric acid to the pH of pure boric acid solution of similar strength. This titration represents the amount of carbonate in equivalents. A correction is made for the bicarbonate present in the reagents. The new method has a number of desirable features. The procedure is simple and accurate in spite of the small amounts involved. The direct titration as employed has a sharp end point. Only the stable standard acid is used (in contrast to the hitherto available titrimetric methods where serum carbonate is converted to carbon dioxide gas which is distilled into an excess of barium hydroxide, and the excess is then back titrated)(4,5). It is possible to set up simultaneously any number of determinations and complete all of them in about 1½ to 2 hours, the overall working time (with large numbers of determinations) being distinctly less than in the gasometric procedures of Van Slyke (6). Moreover, the hazard of handling large amounts of mercury is avoided and the cleaning problem is eliminated. The equipment is less expensive, and on the scale described in this paper, the method is not hinged to an ultramicro burette, which was employed, since with 0.1 N standard acid instead of 1.0 N acid one can use the usual type of two milliliter microburette. Conversely, in employing more dilute standard acids, such as 0.1 or 0.01 N, it is possible to modify this method from 0.01 ml to 0.001 ml of sample. The drawback of the new method is that it requires about 1½ hours for a single determination, which prevents it from being used in emergencies. Another drawback is that a modicum of experience must be acquired in determining the reagent blank, which in this

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method is about 2 to 5% of the total titration obtained for a normal blood serum. As regards the time factor, it is likely to be shortened in the future. Preliminary experiments indicate that further simplifications and greater speed are possible using sodium ethylene diamine tetraacetate (SED)(7) to titrate the barium in the precipitate after the barium carbonate is dissolved in dilute hydrochloric acid. It will eliminate the drying and ashing step and the solution of the barium carbonate in hot 10% boric acid, and thus a method requiring an overall time of twenty-five to thirty minutes may become feasible.

Procedure. Reagents. 1) *Saturated barium hydroxide* sol. 60 g of C.P. $Ba(OH)_2 \cdot 8H_2O$ per 1000 ml of distilled water. The solution is filtered and protected in a soda-lime tube. For the method, this solution was placed in a self-filling Machlett burette of 5 ml capacity, calibrated in 0.01 ml and protected with a soda-lime tube. The delivery arm from the upper bulb is filled with glass wool to remove any precipitate that may form. 2) *Washing fluid I.* Equal volumes of 95% alcohol and distilled water are placed in a self-filling Machlett burette of 5 ml capacity, protected with a soda-lime tube. Prior to mixing, the 95% ethyl alcohol is redistilled over barium hydroxide, and the water is boiled to remove dissolved carbon dioxide. 3) *Washing fluid II.* Previously boiled distilled water contained in a self-filling burette, protected with a soda-lime tube, or simply freshly boiled distilled water. 4) *Boric acid sol.* 10 g of recrystallized C.P. H_3BO_3 is dissolved in about 100 ml of distilled water by heating. This solution is supersaturated at room temperature, and consequently it is heated before use until the boric acid has gone into solution. The solution is used while hot. 5) *Indicator sol.* 5 parts of 0.1% Brom Cresol Green are mixed with 1 part of 0.1% Methyl Red. 0.04 ml of this indicator mixture is used for each ml of carbon dioxide free water. 6) *Standard sol.* of sodium carbonate—238.2 mg of Bureau of Standards grade Na_2CO_3 is diluted to 100 ml. 0.1 ml of this solution will evolve 0.05 ml of carbon dioxide (50 μl) measured at standard conditions. 0.1 ml = 2.248 μM. 7. *1.0 N acid* is standardized against the sodium carbonate solution using Brom Cresol Blue. 8. *Anticoagulant*—3 g of ammonium oxalate and 2 g of potassium oxalate are dissolved in 500 ml of distilled water. 0.1 ml is dried and used for each ml of blood. Instead of this mixture, 0.2 mg of heparin per ml of blood can be employed. In most cases, anticoagulant was not employed.

Apparatus. 1. *Microburette*—The Gilmont ultramicro burette was used in this study. The total capacity of this burette is 100 μl. The total volume can be delivered in steps of 1/1000 of the total so that each scale is 0.1 μl and 0.01 μl can be estimated with a fair degree of accuracy. The stirring device of the manufacturer was discarded and in its place a Spinal (Quincke) B-D Yale Luer-Lok was used. The needle point was ground flat and the Luer-Lok end was ground so as to form a round cylinder instead of the normal 4-sided appearance. This modification allows the air stream that is used for agitation during titration to be introduced to the very bottom of the tube. When an air stream is employed a soda-lime tube is introduced to remove the carbon dioxide. Other ultramicro burettes or 2 ml microburettes may be employed. When the smallest calibration of the ultramicro burrette is 0.001 ml the standard acid should be 0.1 N and when the smallest calibration is 0.01 ml, 0.01 N acid should be employed. 2. *Centrifuge tube.* This is a specially designed Pyrex glass tube with a flat bottom described by Sobel and Sobel(1). A standard 5 ml Pyrex conical tube, although less convenient, may also be employed. 3. *Stoppers.* Rubber or cork stoppers which fit the tube tightly are employed. 4. *Pipette.* 0.1 ml pipette was a capillary Mohr pipette graduated to 0.01 ml between the marks. The overall length of the graduated portion is about 140 to 150 mm. Alternately, one may employ one of the ultramicro pipettes described by Kirk(8).

Collection of specimens. 0.5 ml of whole blood is obtained without stasis and is immediately introduced under mineral oil in a 2 to 4 ml test tube. For analysis on serum the blood is permitted to clot and is centrifuged at 2000 rpm for 5 minutes to obtain the clear serum. For obtaining plasma values in these

studies, 0.2 ml of anticoagulant was dried in the tube first and the blood added and tapped to ensure adequate mixing. To eliminate water shifts, heparin may be employed as an anticoagulant. An alternate procedure of collecting specimens from the finger-tip is that described by Sobel, Besman, and Kramer(9). It must be remembered that fingertip values represent mostly arterial blood.

Procedure. 0.1 ml of serum or plasma is measured into a centrifuge tube to which is added 0.9 ml of carbon dioxide free water, and the contents are mixed by gently tapping. (Note: Always keep the centrifuge tube stoppered.) This is followed by the addition of 0.3 ml of saturated barium hydroxide. The contents of the tubes are mixed by gently tapping, and after a precipitate appears, which takes usually less than one minute, 0.5 ml of the alcohol-water washing mixture is added to each tube. The contents of the tube are mixed by gently tapping and centrifuged at 2000 rpm for 10 minutes. The supernatant fluid is decanted or aspirated off. The precipitate is mixed with the little fluid remaining at the bottom of the tube and again washed by adding 1 ml of the alcohol-water mixture and suspending the precipitate in the fluid. The contents of the tubes are centrifuged as above. The washing procedure is repeated, this time with carbon dioxide free water. (This last washing was found to be empirically better than the alcohol-water washing because it removes the last traces of precipitating agent.) After centrifuging, as much of the supernatant fluid is removed as is feasible without disturbing the precipitate, and the tubes are placed in an air oven at 110° to 120°C until dry (about 20 minutes), and in a muffle furnace to incinerate organic matter adsorbed to the precipitate. (This step is not necessary for inorganic solutions.) After removal from the furnace, the tubes are allowed to cool. They are then immersed in a small boiling water bath, and to each tube 0.2 ml of hot 10% boric acid is added. After one to 2 minutes each tube is tapped to assist in the solution of the barium carbonate. The solution containing the dissolved precipitate is diluted with 0.8 ml of distilled water containing the indicator, and allowed to cool to room temperature. It is then ready for titration with an ultramicro burette. The tip of the burette is immersed below the surface, while a gentle stream of nitrogen or air is bubbled through. The solution is titrated back to the pH of a pure boric acid solution of similar strength. A titration blank containing only the boric acid and the indicator solution is used as the color matching tube. The blue to pink end point is very sharp and a sensitivity to 0.00001 ml can be obtained. This corresponds to an ability to detect 0.05 meq/liter of carbon dioxide. Several reagent blanks are run through with each determination. The value is subtracted from the values obtained on the unknown specimen.

TABLE I. Influence of Dilution on Completeness of Precipitation.

Specimen (.1 ml)	H_2O,* ml	Carbonate precipitated, vol %	Van Slyke value, vol %
Serum I	.1	39.1	63.5
	.4	51.4	63.5
	.9	63	63.5
Standard $NaHCO_3$ sol.†	.1	50.1	50.1
	.4	50.4	50.1
	.9	50.7	50.1

* The administration of water was followed by .3 ml of saturated $Ba(OH)_2$ and 1 ml of alcohol-water mixture, and treated as described under Procedure.

† Theoretical = 50 vol %.

TABLE II. Influence of $Ba(OH)_2$ Concentration on Completeness of Precipitation.

Final conc. of $Ba(OH)_2 \cdot 8H_2O$	Authors' method, vol %		
	Serum 1	Serum 2	Na_2CO_3 standard
1	41.2	52	50.1
1.4	72	63.2	50.6
2	72.7	63.9	51.5
2.5	73	64.3	51.7
Value by method of Van Slyke, *et al.*	72.3	63.4	50.2

Calculations. 1 μl of 1 N acid = 0.5 μE of bicarbonate, or 11.13 μl of carbon dioxide. If 0.1 ml of blood serum is used, then meq/L = μl of titration x 5, and volume % = μl of titration x 11.13.

Discussion of procedure and results. As is evident in Table I, preliminary dilution of plasma or serum is necessary for complete precipitation, although this is not the case for

TABLE III. Comparison Between Authors' Titrimetric Method and the Manometric Method of Van Slyke, in 30 Patients.

Diagnosis	Condition of sample	Authors' method CO_2, vol %	Van Slyke CO_2, vol %	% of Van Slyke value
Coronary	Clear	55.3	56.5	98
Post op. hernia	"	60.3	58.4	103
Coronary	"	68.8	66.6	103
Pleurisy	"	62.8	62.8	100
Chronic pancreatitis	"	62	60.7	102
Urethral growth	"	58.5	58	101
Cirrhosis	"	54.7	54.8	100
Malignancy	"	65	68.1	96
Thrombophlebitis	"	65	64.8	100
Possible uremia	"	48.3	48.6	100
Coronary	Chylous	64.5	62.7	103
Vaginal bleeding	Clear	59.5	59.5	100
Cholecystitis	"	55.1	53.5	103
Incomplete abortion	"	58	58.2	100
Coronary	"	62.8	62.5	101
Coronary	"	65.6	65.8	100
Fever of unknown origin	"	62.2	62.4	100
Coronary	"	57.6	57.6	100
Bleeding ulcer	Hemolyzed	59	60.5	98
Coronary	Clear	59.5	61.8	97
Acidosis	"	25.5	25.9	98.5
Undetermined	"	59.3	60	99
"	"	45.5	45.5	100
"	"	41	42	98
"	"	50	49.1	101
"	"	41.8	41.9	100
"	"	51.1	50.7	100
"	"	58.8	59	100
"	"	44.4	44.2	100
"	"	52.2	52.3	100
	Mean	55.80	55.81	100.05
				Std. dev. ± 1.698
				Std. error ± .3100

pure inorganic solutions. The explanation may be that some of the carbonate is protein bound. This point may require further study, and the difference between carbonate that can be precipitated in concentrated serum, as compared to that in diluted serum, may be a measure of some deviation from the normal in clinical conditions.

Conditions of precipitation. In precipitating the barium carbonate, at first an attempt was made to follow the conditions described by Exton *et al.*(10), as the first step in the precipitation of serum carbonate. However, under these conditions the precipitation of barium carbonate was only 60% to 70% complete in the absence of Celite which these authors employed. A systematic investigation, shown in Table II, indicated that when the concentration of $Ba(OH)_2 \cdot 8H_2O$ in the reaction mixture is about 1.4%, the serum carbonate is quantitatively precipitated. This concentration was therefore chosen for the conditions of precipitation, since any further increase would simply increase the reagent blank, and much below this level, the precipitation is incomplete under the conditions presented. It must be pointed out that the concentration of barium hydroxide in Table I is given prior to the addition of the alcohol and water. The addition of the alcohol-water mixture following barium hydroxide was introduced because it was observed that the results with serum are somewhat lower even though in standard solutions precipitation was complete. The alcohol may accomplish a dual purpose, that is, decrease the solubility of barium carbonate as well as assist in the decomposition of protein bound carbonate. The conditions for washing were arrived at empirically, and it is by no means certain that

further investigation may not produce alternate procedures equally satisfactory.

Results. Table III presents a series of analyses on blood serum taken under oil, compared to results on the manometric method of Van Slyke *et al.*(6). The mean result is 100.05% of the Van Slyke value with a standard deviation of ±1.70 and a standard error of 0.31. Thus, it is evident that the present method yields a fairly high degree of precision even though carried out on one-tenth of the amounts that the Van Slyke method employs.

Summary. 1. A direct acidimetric titration method is presented for the estimation of serum carbonate in small volumes of serum or plasma. Serum bicarbonate is precipitated as the barium salt by the addition of barium hydroxide, the precipitated bicarbonate is dissolved in hot boric acid, and the $Ba(H_2BO_3)_2$ in solution is titrated with standard acid to the pH of a pure solution of boric acid with the aid of an ultramicro burette. The titration represents the bicarbonate in equivalents. 2. Various critical points in the method were investigated and are discussed. The probable existence in serum of a protein bound carbonate is indicated. There is close agreement between the values obtained in this method on 0.1 ml of serum and the results obtained by the manometric procedure of Van Slyke, employing 1.0 ml of serum.

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1. Sobel, A. E., and Sobel, B. A., *J. Biol. Chem.*, 1939, v129, 721.

2. Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, 1944, v152, 255.

3. Sobel, A. E., *Ind. Eng. Chem., Anal. Ed.*, 1945, v17, 242.

4. Conway, E. J., *Micro-Diffusion Analysis and Volumetric Error*, 2nd Ed., p189, London, Crosby, Lockwood and Son, 1947.

5. Seligson, D., and Seligson, H., *Anal. Chem.*, 1951, v22, 1877.

6. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry Methods*, Baltimore, Williams and Wilkins Co., 1932.

7. Sobel, A. E., and Hanok, A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 737.

8. Kirk, P. L., *Quantitative Ultramicroanalysis*, New York, John Wiley and Sons, Inc., 1950.

9. Sobel, A. E., Besman, L., and Kramer, B., *Am. J. Dis. Child.*, 1949, v77, 576.

10. Exton, W. G., Schattner, F., and Rose, A. R., *Am. J. Clin. Path.*, 1942, v11, 632.

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Comparative Studies of the β Disturbance of Electrophoretic Patterns in Disease. (19468)

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In electrophoretic patterns of the descending boundary of plasma or serum obtained by the Schlierin scanning technic, the β globulin component often contains a sharp spike extending upward. In an electrophoretic study of the serum from poliomyelitis patients, Kelley and coworkers observed a definite decrease in the length of this β spike(1,2).

In recent studies on poliomyelitis in this laboratory(3), the decrease of the length of the β spike in plasma and serum patterns was compared with those from normal individuals. Since the results, especially those from normal individuals, did not agree with those reported by Kelley *et al.*(2), it was decided to compare the length of the β spike of electrophoretic patterns of plasma and serum from several diseases.