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73. Toshihiro Nishina and Michiya Kimura : Fundamental Studies on Clinical Chemistry. V.*1 A Specific Method for the Determination of Free Cholesterol in Serum.*2

(*Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University**3)

Various procedures have been developed for the determination of cholesterol in serum. The great variety of methods is due to the complications associated with determination of cholesteryl digitonide, the uncertainty concerning the effects of saponification and the lack of stability and sensitivity of the color reaction.

The colorimetric determinations of cholesterol are usually performed by the Liebermann-Burchard reaction[1,2] or the Kiliani reaction.[3] However, these methods are definitely non-specific for free cholesterol alone, and they have been found to affect esterified cholesterols and other steroids.

Djerassi[4] and Tschesche[5] reported that non-conjugated steroid ketones of the type exemplified by cholest-5-en-3-one have been prepared in good yield by oxidation of the corresponding Δ^5-stenols under the Jones' procedure,[6] which consists in rapid addition (2 to 5 min.) of Kiliani's reagent containing sulfuric acid and chromium trioxide to a solution of stenols in acetone.

Previous work*1 in this series has shown that free cholesterol was oxidized in good yield to cholest-4-en-3,6-dione by the modified Jones' procedure.

One of the principles of method described here is based upon this selective oxidation of free cholesterol.

In the field of steroid chemistry, various hydrazine have been used for many years to form derivatives and permit the separation, purification and identification of ketosteroids. Gornall, *et al.*[7] described that 2,4-dinitrophenylhydrazones of ketosteroids in alkaline solution were stable and absorbed at wavelengths of between 425 and 500 mμ. Umberger[8] reported a color reaction for quantitative determination of Δ^4-3-ketosteroids with isonicotinic acid hydrazide. Nakamura, *et al.*[9] suggested that various carbonyl

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*1 Part IV. Yakugaku Zasshi : **84**, 390 (1964).
*2 Studies on new micro determination of cholesterol. (2).
*3 Kita-12-jo, Nishi-5-chome, Sapporo-shi, Hokkaido (仁科甫啓, 木村道也).

1) W. R. Bloor, K. F. Pelkan, D. M. Allen : J. Biol. Chem., **52**, 191 (1922).
2) W. M. Sperry, M. Webb : *Ibid.*, **187**, 97 (1950).
3) A. Zlatkis, B. Zak, A. J. Boyle : J. Lab. Clin. Med., **41**, 486 (1953).
4) C. Djerassi, R. R. Engle, A. Bowers : J. Org. Chem., **21**, 1547 (1956).
5) R. Tschesche, G. Snatzke : Ann. Chem., **636**, 105 (1960).
6) K. Bowden, I. M. Heilbron, E. R. H. Jones, B. C. L. Weedon : J. Chem. Soc., **1946**, 39.
7) A. G. Gornall, M. P. MacDonald : J. Biol. Chem., **201**, 279 (1952).
8) E. J. Umberger : Anal. Chem., **27**, 768 (1955).
9) N. Nakamura, T. Yoshida : Bunseki Kagaku, **11**, 669 (1962).

compounds could condense with *p*-nitrophenylhydrazine to give hydrazones, which were soluble in alcohol and gave red or blue color with dimethylformamide (DMF) and tetraethylammonium hydroxide (TEAH).

It has previously been reported that cholest-4-en-3,6-dione mono-*p*-nitrophenylhydrazone had an absorption maximum at 612 mμ in DMF with sodium hydroxide and had apparent molecular extinction coefficient of about 64,400.*1

Based on these observations, a method for the colorimetric determination of free cholesterol has been developed and applied to the determination of free and total cholesterol in serum.

Reagent and Apparatus

Kiliani's oxidizing reagent : Prepare by dissolving 6.7 g. of CrO_3 in H_2O, adding 5.4 ml. of conc. H_2SO_4 and diluting the mixture to 25.0 ml. with H_2O.

Standard solution of cholesterol : Dissolve 10.0 mg. of cholesterol (m.p. 157~158°), purified by the method of Fieser,[10] with abs. Me_2CO in a 100 ml. volumetric flask. Make to volume with abs. Me_2CO. This stock standard solution contains 0.1 mg./ml. of cholesterol.

p-Nitrophenylhydrazine reagent (PNPH.) : Dissolve 10.0 mg. of *p*-nitrophenylhydrazine (m.p. 157~158) in purified MeOH, which is obtained by boiling with 2,4-dinitrophenylhydrazine and H_2SO_4. Add 1.0 ml. of conc. HCl (35%), make to 100 ml. with MeOH. This reagent should be prepared daily.

Sodium hydroxide solution, $2.0 \pm 0.05N$: Prepare as an aqueous solution from concentrated carbonate-free NaOH solution, removing precipitate by filtration through a glass filter.

Alcoholic potassium hydroxide : Prepare daily, as needed, a mixture of 6 parts of stock solution of 33% KOH with 94 parts of abs. EtOH.

5% Sodium bisulfite : Prepare as an aqueous solution daily. All other reagents and solvents used were of reagent-grade and were used with purification.

Reaction vessels : Test tubes approximately 15×150 mm., fitted with 15/25 standard taper ground-glass stopper were used. These could be readily attached to a reduced pressure still head fitted with 15/25 standard taper joint and a capillary tube for distillation of aliquot of the sample to dryness.

Standard Procedure

Principle

Cholesterol fraction is extracted from serum. Free cholesterol in the extract is oxidized selectively to cholest-4-en-3,6-dione with Kiliani's reagent in Me_2CO. After destroying the excess of oxidizing reagent by $NaHSO_3$ solution, *p*-nitrophenylhydrazine reagent is added to oxidation product and is heated. Its *p*-nitrophenylhydrazine derivative in alkaline solution of DMF produces a stable color with an optical density proportional to the amount of cholesterol present. Total cholesterol in serum is estimated by the same method after hydrolyzing of ester cholesterol in the extracts.

Extraction

Free cholesterol : Place about 2 ml. of Me_2CO-EtOH (1:1) in a 5 ml. volumetric flask and add 0.2 ml. of serum slowly in such a manner that it runs down the wall of the flask and forms a layer under the solvent. As soon as the pipette is withdrawn, mix the contents thoroughly by a swirling motion. A fine divided precipitate should result. Bring the solvent just to a boil on water bath with agitation to prevent bumpling and then cool the flask. Add MeOH-EtOH to mark, mix thoroughly and filter into a small test tube. Aliquots of the clear filtrate are pipetted at once to avoid evaporation. Dissolve the residue in 3 ml. of Me_2CO.

Total cholesterol : Transfer 0.1 ml. of well-mixed serum or plasma to the bottom of a digestion tube. Add 5 ml. of freshly prepared alcoholic KOH and mix by swirling. Place the tubes in a 65° bath for 60 min. with loose stoppers. Remove the tubes and cool to room temperature. Add 10 ml. of hexane with a pipette, replace the stoppers, and mix vigorously by repeated inverting for 1 min. When the hexane layer separates, remove exactly 5.0 ml. of it to the bottom of test tube with pipette and evaporate hexane under the reduced pressure. Smell at tube for the last trace of solvent and cool to room temperature. Add 3 ml. of Me_2CO to test tube.

Colorimetric Procedure

Cool Me_2CO solution in an ice water bath to bring the temperature of solution to 0°. Add 0.1 ml. of the Kiliani's reagent and stand at 0° for 30 min. Destroy the excess of oxidant by addition of 2 ml.

10) L. F. Fieser : Org. Syntheses, 35, 43 (1955).

of a freshly prepared 5% solution of $NaHSO_3$, add 8 ml. of CCl_4, place stopper, shake throughly, draw the aqueous layer with a capillary pipette and discard. Repeat the process of washing with 2 ml. of distilled H_2O three times and remove the solvent at the vacuum.

Add 2 ml. of dry CCl_4 and evaporate again on water bath under the reduced pressure of 10 to 15 mm. Hg for the removal of the last trace of Me_2CO.

Add 3 ml. of PNPH. reagent to each test tube, and mix well. Place the tube in a constant level water bath (4~5 cm.), maintained at $60\pm1°$ for 90 min. Remove the tube and cool at room temperature for several minutes. Add 1.0 ml. of 2*N* NaOH with gentle shaking and then add 10.0 ml. of DMF. Transfer the content of the tube to colorimeter-cuvette and read the per cent transmission or absorbance at 575 mμ against the reagent blank, which is obtained from 3.0 ml. of Me_2CO by the exactly same procedure described above.

Results and Discussion

Oxidation Reaction

The Kiliani's reagent, which contained sulfuric acid and chromium trioxide was used as an oxidizing reagent. The procedure of the oxidation of cholesterol were carried out at the temperatures varying from 0° to the room temperature in acetone solution. The development of the oxidation of 0.1 mg. of cholesterol at 0° and at room temperature (16°) is shown in Fig. 1. At 0°, the intensity reached its maximum value from 15 minutes through 45 minutes, whereas at room temperature its maximum value was obtained after 5 minutes, then it began to diminish immediately. The temperature of the oxidation was highly critical. It was necessary to conduct the oxidation at 0° in order to inhibit further oxidation of ketosteroid. More amounts of oxidizing reagent also exhibited results similar to curve II in Fig. 1. For practical purpose, 30 minutes at 0° with 0.1 ml. of the Kiliani's reagent was selected as the most convenient to allow the oxidation proceed.

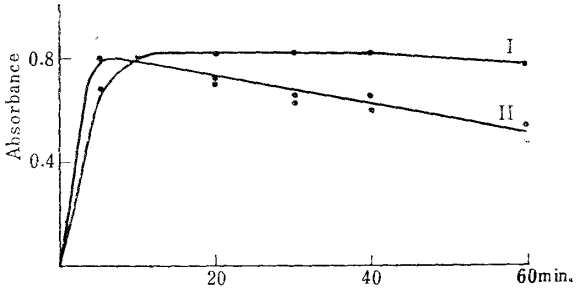

Fig. 1. Effect of Oxidation Reaction on Absorbance at 575 mμ from 0.10 mg. of Cholesterol by the Standard Procedure

I : 0°
II : Room temperature (16°)

Reduction of Excess Oxidizing Agent and Solvent for the Extraction of Oxidation Product

The excess oxidizing reagent should be destroyed because it oxidizes the PNPH reagent. Among various reducing agents investigated for this purpose, 2.0 ml. of 5% sodium bisulfite gave rapid and effective reduction.

Ketosteroids was stable to sodium bisulfite for several hours. Solvents for the extraction studied were ether, chloroform, carbon tetrachloride, hexane, and water-immiscible alcohols. Carbon tetrachloride was found satisfactory as a solvent for extraction of ketosteroid formed, which was more soluble in carbon tetrachloride than in other solvents and was quantitatively extracted from the aqueous phase. As the acid content in the oxidizing reagent described below affected on the formation of hydrazone, the carbon tetrachloride layer was washed with 2.0 ml. of water three times.

Formation of Hydrazone

p-Nitrophenylhydrazine concentration : Fig. 2 shows that the highest absorbance was obtained when the concentration of PNPH was between 0.1 and 0.2 mg. per ml., although the reagent blank increased in color. It was consequently found that a concentration of 0.1 mg. of the reagent per ml. in acidic methanol was most convenient and satisfactory.

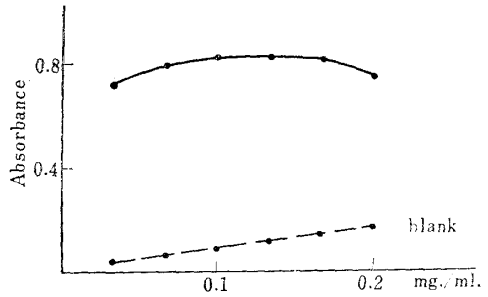

Fig. 2. Effect of Concentration of *p*-Nitrophenylhydrazine Reagent on Absorbance at 575 mμ from 0.10 mg. of Cholesterol

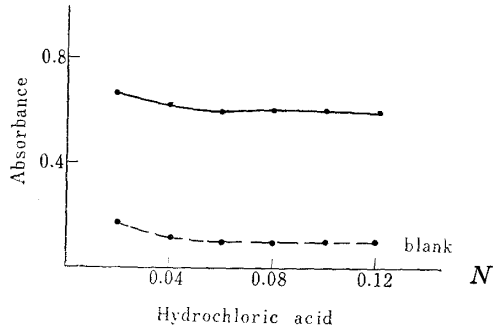

Fig. 3. Effect of Concentration of Hydrochloric Acid on Absorbance at 575 mμ

Kind of acid and its concentration : By keeping the concentration of PNPH at 0.1 mg. per ml. and varying the concentration of hydrochloric acid, the diagram shown in Fig. 3 was obtained, which indicated that the maximum absorbance were obtained at the acid concentration of 0.02*N* both for test and blank solution and that an acid concentration of 0.1*N* was suitable for practical purpose.

Effect of temperature : The range of temperature investigated was from 0° to the boiling point of methanolic solution. The development of the reaction at 60±1° is shown in Fig. 4, which indicated that the reading at any time after 70 minutes may be satisfactory. For practical purpose, the most convenient time and temperature to allow the reaction proceed were 90 minutes and 60° respectively.

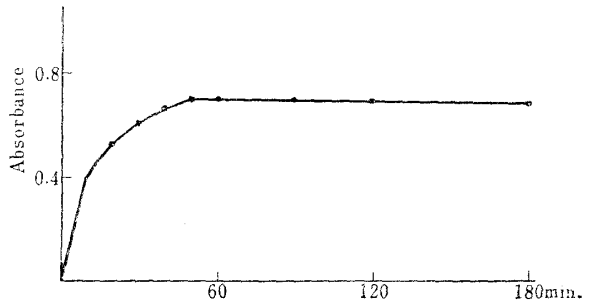

Fig. 4. The Relation between Time of the Reaction at 60±1° and Absorbance at 575 mμ from 0.10 mg. of Cholesterol

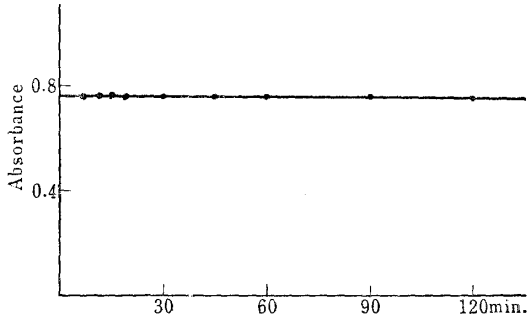

Fig. 5. Stability of the Colored Solution produced from 0.10 mg. of Cholesterol by the Standard Procedure

Effects of Alkali

The reaction mixture was yellow after heating. Addition of sufficient alkali shifted to a dark red color. The reagent blank gave an orange tint which faded slowly on standing. The fading of reagent blank could be accelerated without changing the relative value of sample by reheating the mixture after addition of alkali. It was, however, considered preferable to measure the color after standing 20 to 30 minutes at room temperature.

Since the absorption of carbon dioxide from the air tended to fade color more rapidly and caused precipitation, the reaction had to be carried out in stoppered tube.

The amount of alkali used proved to be critical. When 1.0 ml. of less than *N* of sodium hydroxide was used, the color tended to fade more rapidly.

The rate of fading was roughly inversely proportional to the concentration of alkali. On the other hand, a large quantity (4~5 ml.) of 2*N* sodium hydroxide proved to be critical for the undesirable precipitation of salt on standing. Several kinds of alkali

other than sodium hydroxide, such as tetraethylammonium hydroxide (TEAH) and alcoholic potassium hydroxide were tried, but found to be not satisfactory and inconvenient.

When 1.0 ml. of 10% TEAH was used, for example, color tended to fade rapidly than the same amount of 2*N* sodium hydroxide. Although this quantity of TEAH did not cause precipitation, absorbance of the solution was decreased. It was consequently found that the addition of 1.0 ml. of 2*N* sodium hydroxide was most convenient and satisfactory. The colored solution was stable for four hours under this condition (Fig. 5.).

Absorption Spectra

The colored solution produced from cholesterol by the standard procedure gave a strong absorption maximum at 575 mμ (Fig. 6.), which did not shift with time, the concentration of cholesterol and that of the other reagents, against reagent blank.

The absorption curve of reagent blank was measured in DMF, and was found to give small reading at 575 mμ (Fig. 6).

The spectrum of the colored solution of cholesterol carried out by the standard procedure and that of the colored solution given by cholest-4-en-3,6-dione with PNPH reagent in alkaline DMF were identical. Furthermore, the oxidation product of cholesterol under the standard procedure gave the same absorption maximum at 252 mμ as that of cholest-4-en-3,6-dione in ethanol. These experiments demonstrated the fact that the end product in this color reaction was *p*-nitrophenylhydrazine derivative of cholest-4-en-3,6-dione.

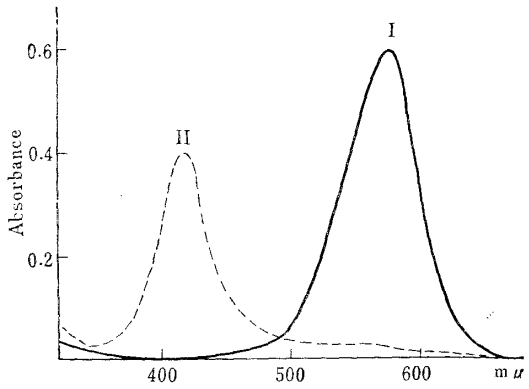

Fig. 6. Absorption Curves of the Colored Solution by the Standard Procedure

I : 0.10 mg. of cholesterol
II : Reagent blank

However, the spectrum of the colored solution given by cholest-4-en-3,6-dione and PNPH reagent under the standard procedure was different from those of its mono- and di-*p*-nitrophenylhydrazone in alkaline solution, which had absorption maximum at 612 and 540 mμ respectively.*[1] Work is in progress on the chemistry of the reaction between ketosteroids and *p*-nitrophenylhydrazine and of the behavior of these in alkaline solution and will be reported later.

Specificity

In order to study the specificity of this method for free cholesterol, several esterified cholesterols and ketosteroids were tested. The compounds to be tested were dissolved at concentration of 0.20 mg. in acetone and were tried under the present procedure exactly as described above. Esterified cholesterols tested were cholesteryl acetate, palmitate, stearate, oleate and linoleate. They were inactive for oxidation and gave no color.

Ketosteroids tested were cholest-4-en-3-one, epiandrosterone acetate, estorone, testosterone, progesterone and androsterone.

Δ^4-3-Ketosteroids such as cholest-4-en-3-one, progesterone and testosterone, which were stable under the oxidation in the standard procedure, reacted with PNPH reagent to form hydrazone, which had an absorption maximum at 540 mμ and interferred somewhat at 575 mμ. However, Δ^4-3-ketosteroids are in trivial amount in comparison with that of cholesterol in serum.*[4] Other ketosteroids having keto groups in other positions

*[4] The approximate range of progesterone level during pregnancy of healthy woman may be as low as 5.0 to 4.0 μg. per 100 ml. of serum.

such as epiandrosterone, estorone and androsterone did not react and developed no color under the same condition.

Acetone used as a solvent can produce hydrazone with PNPH reagent, showing absorption maximum at 520 mμ. It could, however, be removed by prolonged vacuum evaporation before the formation of hydrazone.

From the results presented, it may be concluded that the procedure is highly specific for free cholesterol.

Recovery Test on Serum

The method used for the preparation of cholesterol fraction from serum has been based upon a procedure slightly modified from those used by other investigators.[11,12]

Table I. Recoveries of Added Cholesterol on Determination of Free Cholesterol in Serum

Sample	Cholesterol added (mg./dl.)	Found (mg./dl.)	Recovery (%)	Sample	Cholesterol added (mg./dl.)	Found (mg./dl.)	Recovery (%)
1	0.0	49.0	—	2	25.0	84.1	99.4
	0.0	48.5	—		50.0	104.3	99.6
	25.0	76.0	108.0		50.0	103.8	99.5
	25.0	75.5	106.0		75.0	135.8	101.2
	50.0	96.0	94.0		75.0	134.4	99.2
	50.0	98.5	99.0			mean	99.2
2	0.0	59.4	—			σ	3.9
	25.0	84.1	99.4				

Table II. Recoveries of Added Cholesterol on Determination of Total Cholesterol in Serum

	Cholesterol added (mg./dl.)	Found (mg./dl.)	Recovery (%)	Sample	Cholesterol added (mg./dl.)	Found (mg./dl.)	Recovery (%)
3	0.0	135.0	—	4	50.0	178.0	98.0
	0.0	138.0	—		100.0	222.0	94.0
	50.0	188.0	102.0		100.0	226.0	98.0
	50.0	190.0	106.0		150.0	263.0	96.0
	100.0	238.0	100.0		150.0	273.0	96.0
	100.0	237.0	99.0		200.0	320.0	95.0
	150.0	296.0	106.0		200.0	316.0	92.0
4	0.0	128.0	—			mean	98.3
	0.0	138.0	—			σ	3.7

Table III. Comparison of the Present Method and Mann's Method for Total Cholesterol in Serum

Sample	Present method			Mann's method		
	N[a]	X[b]	σ[c]	N	X	σ
A	5	166.0	2.8	5	173.8	4.7
B	5	126.8	3.1	5	129.0	4.7
C	4	209.4	4.4	5	209.6	2.2
D	5	177.2	5.1	5	172.0	5.1
E	5	189.8	3.4	5	184.5	3.1

a) N: Number *b*) X: Mean level (mg./dl. serum)
c) σ: Standard deviation

An inspection of Table III indicated that the standard method for total cholesterol was in good agreement with the Mann's method both in terms of the equivalence of means and in terms of the precision. When different amounts of cholesterol were added to the residues from alcohol-acetone extract for free cholesterol and from non-saponifiable extract for total cholesterol, the recovery of the added material has been ranged from 94 to 105% (Tables I, II).

Conclusion

The method described here is based upon the selective oxidation of free cholesterol to cholest-4-en-3,6-dione with Kiliani's oxidizing reagent.

The amount of cholest-4-en-3,6-dione formed is determined colorimetrically with *p*-nitrophenylhydrazine reagent. The color produced by present method was stable for several hours and 0.05 to 0.20 mg. of cholesterol could be determined with accuracy (Fig. 7). It is seemed that this method might be enough sensitive to be scaled down.

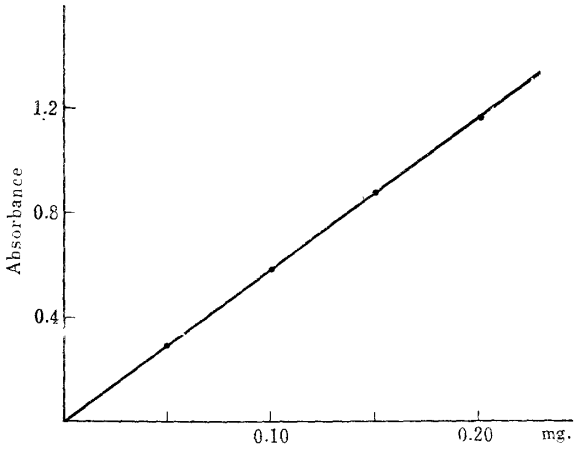

Fig. 7. Calibration Curve

Not only esters of saturated fatty acids, such as cholesteryl acetate, stearate and palmitate, but also those of unsaturated fatty acids, such as cholesteryl linoleate and oleate did not develop color. In view of this fact, it was suggested that by the present method free cholesterol could be determined even in the presence of esterified cholesterol. The application of the present procedure to the samples of serum for free and total cholesterol gave the results shown in Tables I, II and III.

The present method is not so rapid as the other methods. However, on the view of the complete batch analysis including the extraction of serum, it is not tedious and is more precise because of lacking the complicated digitonide procedure. Moreover, it is indicated that free cholesterol in serum could be determined independently in the presence of esterified cholesterol by this procedure.

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Summary

A new colorimetric determination of cholesterol was studied and the standard procedure was established. The principle was based on that cholesterol in acetone was oxidized to cholest-4-en-3,6-dione by the Kiliani's reagent, and its *p*-nitrophenylhydrazone produced a blue purple color in alkaline solution of dimethylformamide. This color had an absorption maximum at 575 mμ and was very stable. It has been found that free cholesterol could be determined specifically even in the presence of esterified cholesterols, which gave no color under the standard procedure. The method has been applied to the analysis of free and total cholesterol in serum. Known amounts of cholesterol added to extract from serum were measured using the calibration curve by the standard procedure and the recoveries were quite satisfactory.

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12) B. Zak, *et al.* : Amer. J. Clin. Path., **24**, 1307 (1954).