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# Quantitative Determination of Free Cholesterol and Cholesterol as Esters Without Digitonin.

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Cholesterol esters react more rapidly than free cholesterol with sulfuric acid and acetic anhydride to form the characteristic green color of the Liebermann-Burchard reaction. This difference, first observed for cholesterol palmitate[1] has been found to hold for the acetate and oleate ester as well. This difference in the reactivity of the free and combined cholesterol influences the results obtained by the usual colorimetric methods, and it is certain that in several of the widely used procedures true color equivalence is not attained.

By developing the Liebermann-Burchard reaction at 0-2°C., only the esters will develop color while the free cholesterol remains practically colorless, thus allowing the determination of cholesterol esters in the presence of free cholesterol. Then by completing the reaction at 38°C., total cholesterol can be determined in the same solution. This procedure, which obviates the use of digitonin, is carried out as follows:

Alcohol-ether extracts prepared according to Bloor,[2] containing the equivalent of 1 cc. of blood serum, are evaporated to dryness at a temperature of 60-70°C. The dry residue is extracted with anhydrous chloroform* which is filtered through cotton into a glass-stoppered 10 cc. cylinder. Two standard solutions, one containing cholesteryl oleate equivalent to 1.6 mg. cholesterol† in 10 cc. chloroform, the other cholesterol in the same amount and volume, are measured into similar cylinders. Both standard and unknown solutions are cooled in a refrigerator for 10 minutes. Acetic anhydride and sulfuric acid are mixed in the proportion of 1 cc. of acetic anhydride to 0.025 cc. of concentrated sulfuric acid, then cooled to 0-2°C. One cc. of the cold mixture is added to the cooled standard and unknown solutions. After mixing these are placed in an ice bath

[1] Reinhold, J. G., *J. Biol. Chem.*, 1934, **105**, lxxi.

[2] Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry, Vol. 2, Williams and Wilkins, Baltimore, 1932.

* Commercial chloroform should be washed, dried over anhydrous potassium carbonate and distilled. The distillate is dehydrated with phosphorus pentoxide and again distilled. The same chloroform should be used in standard and unknown solutions.

† 2.7 mg. cholesteryl oleate contain 1.6 mg. cholesterol.

at a temperature of 0-2°C. After 40-50 minutes, the unknown solutions and both standard solutions are compared in a colorimeter with an artificial standard consisting of an aqueous 0.0025% solution of naphthyl green B. A red eye-piece filter like that used by Bloor is employed in making the readings. Color that appears under the conditions described originates with esterified cholesterol, although a little color is developed by free cholesterol.

The solutions next are warmed in a water bath at a temperature of approximately 38°C. for about 40 minutes, and then are allowed to stand at room temperature for 10 minutes. A slight precipitate that often appears in the unknown solutions settles out or can be removed by centrifugation. The clear solutions are compared colorimetrically with either the cholesterol or cholesteryl oleate standards. On warming, free cholesterol rapidly develops color so that esterified and free cholesterol yield equivalent concentrations of color at this time. From readings made after the solutions have been warmed, the total cholesterol may be calculated by the usual formula.

Ester cholesterol is calculated from the first readings. Since free cholesterol, if present, adds slightly to the color developed by esterified cholesterol in this stage of the reaction, a correction is applied. It is calculated on the assumption that cholesterol of either type develops a basal intensity of color equivalent to the amount formed by free cholesterol, while esterified cholesterol produces an additional quantity. The latter is proportional to the concentration of ester, so that

$$\text{Ester cholesterol in milligrams} = \frac{B-C_2}{O-C_1} \cdot S$$

where B = reading of naphthyl green solution against the unknown,

O = reading of naphthyl green solution against cholesteryl oleate standard,

S = concentration of cholesterol in cholesteryl oleate standard,

$C_1$ = reading of naphthyl green against standard cholesterol solution,

$$C_2 = \frac{\text{total cholesterol in unknown solution}}{\text{cholesterol in standard solution}} \cdot C_1.$$

If more than 2 unknown solutions are to be read, it is advisable to prepare 2 sets of standards, one pair being read before and the other after reading the unknown solutions against the artificial standard. The time of each reading is recorded and the readings of the cholesteryl oleate and free cholesterol standards corresponding to the time at which each unknown was read can be obtained by interpolation.

Coprostenol (allocholesterol), which reacts under these conditions like esterified cholesterol rather than free cholesterol, apparently is not present in unbound form or exists in serum in concentrations too low to interfere with the application of the differential reaction.

Known mixtures of cholesteryl oleate and cholesterol have been analyzed correctly by the procedure described. Cholesteryl oleate added to alcohol-ether extracts of serum can be recovered with reasonable accuracy. Several comparisons of determinations of cholesterol and cholesterol esters by the new method and by a gravimetric digitonin procedure[3] indicate that the results agree closely.

## 7787 C

## Diuresis of Hyperthyroidism.

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During the course of investigations on diabetic animals it was observed that thyroid administration or injection of anterior pituitary extracts did not produce diuresis in pancreatectomized dogs. In a previous report[1] it was shown that an extract from the anterior pituitary, which caused marked diuresis failed to produce such an effect in thyroidectomized animals. The results indicated that the induced hyperthyroidism was responsible for the polyuria. This work was confirmed by Biasotti.[2] It was also observed[3] that if the pituitary and pancreas were previously removed, thyroid administration failed to cause the usual diuresis.

Hyperthyroidism was induced by feeding desiccated thyroid (Armour's), in doses of 1 gm. per kilo body weight per day or by injecting the anterior lobe extract previously described[1] for 7-10 days. In some cases both methods were used in the same animal, considerable time elapsing between each experiment. Twelve dogs were used, in which the following operations were performed:

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[3] Ewert, B., *Biochem. Z.*, 1933, **263**, 149.

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[1] Barnes, B. O., Regan, J. F., and Bueno, J. G., *Am. J. Physiol.*, 1933, **105**, 559.

[2] Biasotti, A., *Rev. Soc. Argentina Biol.*, 1933, **9**, 499.

[3] Barnes, B. O., *Am. J. Physiol.*, 1934, **109**, 5.