small sampling and time errors exists. The rotating-bottle method is quite obviously the least precise. This is not surprising, since the apparatus has to be switched off while the bottles are removed and the samples are taken.

SUMMARY AND CONCLUSIONS

A new nuclear *in vitro* dissolution-rate measuring apparatus has been developed and preliminarily evaluated for use in measuring the dissolution release of labeled materials from solid dosage forms as a precise means of studying the effects of modifying the different variables of dosage form on the *in vitro* dissolution of model systems. The new apparatus employed Geiger–Müller detection of a γ emitter and continuous recording of the events. ^{59}Fe was the tracer incorporated in a standard tablet formulation containing ferric chloride, calcium sulfate, and magnesium stearate. The dissolution-rate constant measured in the new nuclear apparatus was compared with the dissolution-rate constants measured in the U.S.P. disintegration apparatus and the rotating bottle. The tablets did not disintegrate, but released the ferric chloride solute by a leaching mechanism according to the model, $Q = k't^{1/2}$.

The conclusions that can be drawn are as follows.

1. The nuclear method of dissolution-rate measurement is the most convenient of the three methods because (*a*) the presence of an operator was not required after the tablet was introduced; (*b*) a permanent record of the dissolution of the ^{59}Fe, representative of the ferric chloride dissolution, was made; (*c*) a chemical analysis with its inherent difficulties in sampling, measuring, etc., was not needed.

2. The nuclear method was more precise. The variation-rate constants of the U.S.P. and the rotating-bottle methods were 2.7 and 4.1 times greater than the variation-rate constant of the nuclear method.

3. Of the three *in vitro* methods studied, the U.S.P. method was the most conservative in dissolution-rate measurement (rate constant = 0.856). The nuclear method was less conservative (rate constant = 1.56), and the rotating bottle was the least conservative (rate constant = 2.17). This is related to the degree of agitation and solvent exposure of the tablet in the apparatus.

4. The data conform to the general equation $Q = k't^{1/2}$, previously deduced on theoretical grounds by Higuchi.

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Fluorometric Method for Determination of Cholesterol in Microliter Quantities of Blood

By FREDERICK TISHLER and J. N. BATHISH

A fluorometric method for the determination of total cholesterol is described which is based on the Tschugaeff reaction. The procedure is applicable to the analysis of as little as 2 µl. of blood, which may be obtained, for example, from the finger. In order to determine the selectivity of this reaction, a number of related steroids have been studied.

There has been a need for a rapid method for the determination of cholesterol on the micro level using macro techniques. Albers and Lowry (1) have described a fluorometric method for as little as 0.1 mcg. of cholesterol in animal tissue utilizing the Liebermann-Burchard reaction; however, the final volume to be read is less than 0.25 ml. McDougal and Farmer (2), modifying the procedures of Albers and Lowry, have developed a fluorometric procedure for the determination of total cholesterol in blood from the tip of a rat's tail. Again, however, the method of extraction and development of fluorescence involve the use of micro techniques.

The Tschugaeff reaction (3) has been shown to be more sensitive than the Liebermann-Burchard reaction and has been applied to the colorimetric determination of total cholesterol in blood by several investigators (4–6). A fluorometric method has now been developed based on the Tschugaeff reaction that is many times more sensitive than the previously described procedures and that utilizes macro techniques on a micro sample.

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Optimum conditions for the zinc chloride–acetyl chloride induced fluorescence have been determined, and a number of related steroids have been studied in order to determine the selectivity of this reaction.

EXPERIMENTAL

Apparatus.—All measurements were made on an Aminco-Bowman spectrophotofluorometer with a quartz cell having a 1-cm. light path using cell-slit arrangement No. 3, minus cell slits 3 and 4, as described in the Aminco-Bowman spectrophotofluorometer instruction manual (7). The excitation and fluorescence spectra were obtained on a Moseley autograph XY recorder. Fluorescence was developed in a constant-temperature bath maintained at 65 ± 0.2°.

Reagents.—All steroids used in the study were either of reference standard quality as determined by phase solubility (8) when possible or chromatographically pure as determined by thin-layer chromatography. The zinc chloride–acetyl chloride reagent was prepared in the manner previously described (5). Dissolve 40 Gm. of anhydrous zinc chloride in 153 ml. of glacial acetic acid by heating the suspension at 80° with occasional stirring for 2–3 hr. Cool to room temperature, and filter through a sintered glass filter. Store in a glass-stoppered dark bottle.

Procedure for the Determination of Total Cholesterol in Blood.—Pipet 10 µl. of blood into 20 ml. of an absolute alcohol–ether (8:2) mixture contained in a 40-ml. centrifuge tube. Stopper and shake vigorously for 2 min. Allow the mixture to stand 5 min. and then centrifuge. Pipet 1.0 ml. of the clear alcohol–ether solution into a 25-ml. volumetric flask and evaporate to dryness on a steam bath.

Add 5 ml. of chloroform to the volumetric flask and mix. To two additional 25-ml. volumetric flasks add 5 ml. of chloroform (blank) and 5 ml. of a cholesterol standard (1 mcg. of cholesterol/5 ml. of chloroform). To all the flasks add 1 ml. of zinc chloride reagent, 10 ml. of acetyl chloride, shake, stopper, and place in a 65° water bath for exactly 20 min. After 20 min., cool the flasks to room temperature in an ice-water bath and dilute to volume with chloroform. Using an activation of 525 mµ and a fluorescence of 575 mµ (both uncorrected), set the instrument to give a reading of 70–80 with the standard. Then read the sample and the blank without changing any controls. Calculate the concentration of cholesterol in the sample taken as follows:

$$\frac{A_0 - A_b}{A_s - A_b} \times 20 = \text{mcg./10 } \mu\text{l.}$$

where A_0 = fluorescent intensity of the sample
A_s = fluorescent intensity of the standard
A_b = fluorescent intensity of the blank

DISCUSSION

The excitation and fluorescence spectra for cholesterol appear in Fig. 1. In order to determine the optimum conditions for the formation of the fluorogen, the fluorescence of the final solution as a function of time and milliliters of zinc chloride and acetyl chloride was investigated. From Figs. 2 and 3 it can be seen that at 65°, a maximum fluorescence is obtained when 1 ml. of zinc chloride and 10 ml. of acetyl chloride are used, and the solution is heated for 20 min. Temperatures below 65° required a longer heating time, while temperatures above 65° caused evaporation difficulties. A plot of fluorescent intensity *versus* concentration was found to be linear up to 5 mcg./25 ml. of final solution (Fig. 4).

Adopting the method of Sackett (9) for the extraction of cholesterol from blood, five 10-µl. samples of

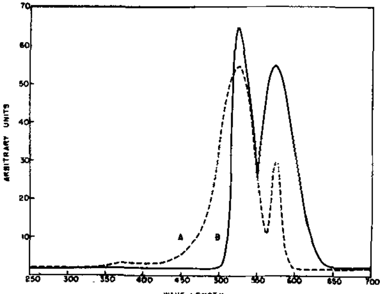

Fig. 1.—Excitation and fluorescence spectra of cholesterol. Key: A, excitation scan, fluorescence held at 575 mµ; B, fluorescent scan, excitation held at 525 mµ.

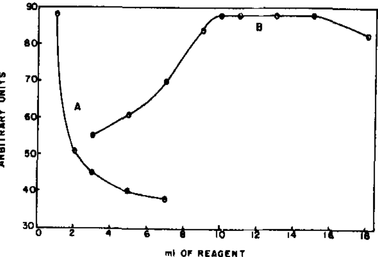

Fig. 2.—Plot of fluorescences of final solution against milliliters of reagent. Key: A, milliliters of zinc chloride reagent varied, acetyl chloride held constant at 10 ml.; B, milliliters of acetyl chloride varied, zinc chloride held constant at 1 ml.

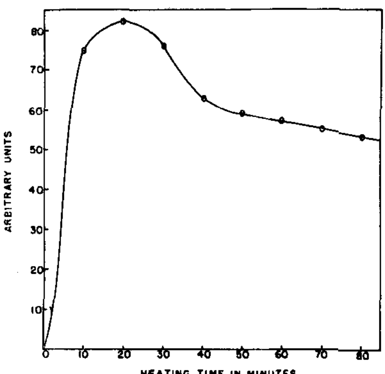

Fig. 3.—Plot of fluorescences of final solution against heating time of reaction.

blood were analyzed after the addition of cholesterol. The results obtained are summarized in Table I.

Tables I and II indicate the reproducibility of the procedure when a 10 and a 2-μl. sample are analyzed for cholesterol. Although a 1-μl. sample has not been analyzed, no difficulties would be anticipated;

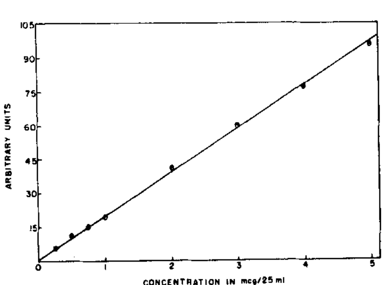

Fig. 4.—Plot of fluorescences of final solution against concentration of cholesterol.

Table I.—Recovery of Cholesterol from Blood in mcg./μl.

Sample	Present[b]	Added	Found	Difference[a]	% Recovery[a]
1	1.39	1.0	2.41	1.05	105
2	1.34	1.0	2.35	0.99	99
3	1.34	1.0	2.33	0.97	97
4	1.37	1.0	2.43	1.07	107
5	1.37	1.0	2.41	1.05	105
Av.	1.36 ± 0.02				

[a] Differences and per cent recovery are based on the average 1.36 mcg. [b] The results obtained from the analysis of 5 aliquots of the same blood sample.

Table II.—Reproducibility of Analysis on a 2-μl. Sample of Blood

Sample	Found, mcg./μl.	Deviation, mcg.
1	1.26	−0.05
2	1.34	+0.03
3	1.29	−0.02
4	1.37	+0.06
5	1.31	0.00
Av.	1.31 ± 0.04	

Table III.—Fluorescence of Cholesterol and Its Esters on an Equimolar Basis

Compd.	Relative Readings
Cholesterol	80
Cholesteryl acetate	77
Cholesteryl benzoate	76
Cholesteryl chloride	78
Cholesteryl palmitate	82
Cholesteryl stearate	75

Table IV.—Fluorescence of Cholesterol and Some Related Compounds

Compd.	Structure	Relative Fluorescent Intensity
Cholesterol		80
Lanosterol		Nil
Stigmasterol		33
β-Cholestanol		Nil
Coprostanol		8
Cholestane		Nil
5-Cholestene		8

TABLE IV.—(*continued*)

Compd.	Structure	Relative Fluorescent Intensity
Cholic acid	HO, H, H, H, COOH, HO, OH, H	Nil
7-Dehydro-cholesterol	HO	13
5-Cholestene-3-one	O	Nil
Estrone	O, HO	Nil
β-Estradiol	OH, HO	Nil
Desoxycorti-costerone acetate	$CH_2OOC—CH_3$, C=O, O	Nil

however, a greater standard deviation would be expected because of pipeting errors.

It has been noticed by many researchers (10–12) that when the Liebermann-Burchard reaction is carried out in chloroform, the color intensities obtained for cholesterol esters were greater than that for pure cholesterol when compared on an equimolar basis. Results for total cholesterol were, therefore, incorrect unless a factor was applied or a prior saponification was used. In the fluorometric procedure of Albers and Lowry (1), which utilized the Liebermann-Burchard reaction, fluorescent intensities were the same on an equimolar basis only after 1-hr. reaction time and when trichloroethane replaced chloroform. Table III shows the results obtained when cholesterol was compared with its esters on an equimolar basis. Since the results indicate that after 20-min. reaction time both cholesterol and its esters have similar fluorescent intensities when compared on an equimolar basis, a presaponification step and a factor are not necessary.

A number of related steroids were studied in order to determine the selectivity of the reaction and the effect of various groups on the molecule (Table IV). Based on the compounds studied, it appears that the reaction is selective for those steroids that possess both a hydroxyl group (or esters) at the 3 position and a double bond at the C_5—C_6 position. Kato (13) has recently developed a new colorimetric reaction for cholesterol using anhydrous aluminum chloride and anisaldehyde and has shown that cholesteryl chloride is an intermediate product formed during the reaction. Since cholesteryl chloride also showed fluorescence, it is also highly possible that this compound is formed as an intermediate. Further studies are now being carried out to determine the mechanism of zinc chloride–acetyl chloride induced fluorescence.

SUMMARY

A simplified fluorometric method is described for the determination of cholesterol in blood.

The method is at least 100 times more sensitive than the published fluorometric procedures that employ the Liebermann-Burchard reaction.

A number of related steroids have been studied in order to determine the selectivity of the reaction.

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