Improved Quantitation of Cephalin-Cholesterol Flocculation Test

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A rapid turbidimetric method for the determination of cephalin-cholesterol flocculation is described. Greater specificity and photometric sensitivity have been achieved along with adequate precision. The test is standardized with the conventional barium sulfate turbidity standards used for other laboratory procedures. Definition of results in turbidity units is proposed, and photometric standardization of the cephalin-cholesterol emulsion described. The stability of the emulsion is elucidated. Comparison is made with a previously described quantitative method.

A recent report (1) contains a comprehensive and authoritative article on the cephalin-cholesterol flocculation test. It fails, however, to include a discussion of the heat-accelerated test of Bunch (2). A number of modifications of the original flocculation test of Hanger (3) appear in the literature. Several workers have made serial dilutions of the serum with saline (4-7) to determine the point at which flocculation occurs. Others have analyzed the flocculated material for its cholesterol content (8, 9). Still others have photometrically measured the residual turbidity of the supernatant fluid (2, 10, 11). The latter approach affords the greatest facility and, therefore, is best suited to the needs of a clinical laboratory. It was this approach that we pursued further.

Although some workers still feel that a visual estimate of flocculation suffices, it is our feeling that such a view is inconsistent with a particular function of a clinical biochemistry laboratory, which is to supply data from tests which are sufficiently sensitive to enable the unequivocal resolution of clinically marginal or so-called “border-line”

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cases. It is in this area that the clinician most depends upon the laboratory findings and it is in this very area that a semiquantitated test may fail. It is true that variations in the sensitivity of purchased antigen is still a problem, even though a diminished one. Sufficient ripening of cephalin, as suggested by Mateer et al. (12), has probably been the reason for the reduced number of false positive reactions obtained in recent years with commercial antigens, as well as for the great reduction in photosensitivity. Furthermore, this problem of antigen variation has been resolutely met by enlightened laboratory workers through the medium of control serums. On the other hand, the problem of providing a quantitation which meets the criteria of good photometric sensitivity and minimum interference has remained only partially resolved.

Our purpose in this work was to modify the heat-accelerated technic of Bunch (2) in such a way as to insure relative freedom from interferences such as are found in serum pigments (bilirubin, hemoglobin, etc.) and yet provide a reasonably rapid procedure which requires only micro quantities of sample. Further, it was felt that the problem of a stable, reproducible working emulsion, incorporating saline, should be resolved in order to minimize day-by-day variations and interlaboratory differences. Finally, a suitable standardizing technic, coupled with a routine procedure affording adequate photometric sensitivity and integrated with means of more rapid flocculation, already reported (2), was considered a sine qua non for satisfactory quantitation of cephalin-cholesterol flocculation.

Reagent Preparation

Cephalin-cholesterol saline emulsion (stock) Mark a 250-ml beaker at 108 ml. by placing that amount of water in it and marking the lower part of the meniscus with a glass-marking pencil. Measure 128 ml. of distilled water into the beaker and heat on an electric hot plate, turned to medium heat, until the temperature of the water is 65°-70°. Add slowly, with stirring, 4 ml. of stock cephalin-cholesterol antigen solution (an ether solution of cephalin and cholesterol described by Knowlton (1)). Continue heating on the hot plate until the solution boils, stirring constantly. Then turn the hot plate dial to low heat and let the solution simmer until the volume reaches the 108-ml mark on the beaker. During the heating all coarse granular lumps are dispersed to a stable, milky, translucent emulsion and all traces of ether are removed. Cover the beaker with a watch glass and place in the
dark until the emulsion is cooled to room temperature. If the volume is below the 108-ml. mark, add distilled water to that mark and mix. Add 12 ml. Merthiolate solution (1 gm./L. sodium ethylmercurithiosalicylate—dye-free) and mix. This stock emulsion retains its activity unchanged for 2–4 weeks if kept stoppered and stored in the refrigerator (approx. 4°C).

**Working cephalin-cholesterol emulsion** In a 500-ml., graduated, glass-stoppered mixing cylinder place 400 ml. of an aqueous solution containing 0.9% (w/v) of sodium chloride and 0.1 gm./L. of Merthiolate. Add the well-mixed stock cephalin-cholesterol emulsion in approximately 20-ml. increments, with thorough mixing between additions, until a total volume of 500 ml. is achieved. Stopper the cylinder and mix thoroughly by vigorous inversions. If any curdled material is present, centrifuge at least 10 min. at an RCF of 800–1000. (Removal of curdled material prevents false positive reactions, as suggested by Knowlton (1)). Store in the refrigerator. This cephalin-cholesterol saline emulsion, preserved with Merthiolate, is stable for 3 months.

**Photometric standardization of cephalin-cholesterol saline emulsion** Place at least 3 ml. of the well-mixed cephalin-cholesterol saline emulsion in a 19-mm. round cuvet. Read in a Coleman Junior spectrophotometer, Model 6D* at 500 m\(\mu\) against a blank of distilled water adjusted to 100% transmittance. The reading should correspond to no less than 17.0 and no more than 18.0 cephalin-cholesterol turbidity units, as defined below. If outside this range, the emulsion must be remade. (In order to permit readings on as little as 3 ml. of solution in the 19-mm. round cuvets, cut a slice of rubber from a solid rubber stopper of sufficient thickness to elevate 3 ml. of solution, contained in the cuvet, into the optical path when the rubber slice is placed at the bottom of the cuvet adapter. This is used for all subsequent photometric work.)

**Procedure**

**Preparation of Calibration Curve**

The calibration procedure employed here, using barium sulfate standards, is the same as that used for the thymol turbidity test. This standardization technic was first proposed by Shank and Hoagland (13). It should be pointed out that, although the readings in the actual test are made at 500 m\(\mu\), the standardization is still made at 650 m\(\mu\).

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*Coleman Instruments, Inc., Maywood, Ill.*
This is done purely for convenience, to permit the use of the same calibration curve for all the quantitated liver function turbidity tests. For this test we propose the use of the cephalin-cholesterol turbidity unit, which is equal to one Shank-Hoagland unit of thymol turbidity. The curve should be checked at regular intervals. A typical calibration curve is shown in Fig. 1. A nomogram, such as that suggested by Schols (14), might also be constructed for everyday use.

**Assay**

Pipet 5.0 ml. of cephalin-cholesterol saline emulsion into each of two conical centrifuge tubes (12 ml.). Mark one tube "sample" and the other "blank." Place both tubes in a covered 37° water bath for 2–3 min. Then add to the sample tube 0.2 ml. of serum. Stopper, mix gently, and replace in the covered 37° bath. Exactly 3 hr. after adding the serum, remove both tubes from the bath and centrifuge for 5 min. at an RCF of 800–1000. Pipet 3.0 ml. of the supernatant fluid from each tube into properly labeled 19-mm. round cuvets. To the blank cuvet add 0.12 ml. of serum and mix gently by lateral shaking. (If the serum is visibly free from icterus, hemolysis, and turbidity, 0.12 ml. of 0.9% (w/v) sodium chloride may be substituted for the serum.) Readings are made at 500 mμ. Converse to the usual procedure, the instrument is adjusted to 100% transmittance with the sample, and the blank is read against it. The turbidities in the supernatant fluids remain

![Typical calibration curve obtained on Coleman Junior spectrophotometer, Model 6D, using reagent blank, and 19-mm. round cuvets.](image-url)
optically constant up to 25 min. This technic permits direct readings in terms of increasing turbidity with advancing liver pathology instead of increasing clearing as obtained in the methods of Bunch (2) and Kibrick (11). The cephalin-cholesterol turbidity units are read from the calibration curve.

**Experimental**

**Comparison of Methods**

In order to validate this modification, the present method was compared with that of Kibrick (11), results being converted to our cephalin-cholesterol turbidity units for direct comparison. The results of this comparison, shown in the scattergram in Fig. 2, further substantiate the claim of Bunch (2) that incubation of the reaction

![Fig. 2. Comparison of results of present method (3-hr. incubation at 37°) with those of Kibrick method (11) (24-hr. incubation at room temperature.)]
mixture for 3 hr. at $37^\circ$ results in approximately the same degree of flocculation as that observed with 24-hr. incubation at room temperature (approx. $25^\circ$).

On a larger series, involving 20 serums analyzed in duplicate and reported in our cephalin-cholesterol turbidity units, the standard deviation, calculated by the formula (15):

$$S.D. = \sqrt{\frac{\sum (x_1 - x_2)^2}{N - 1}}$$

was $\pm 0.35$ for the Kibrick method and $\pm 0.30$ for the present method.

A further comparison was made of the present method with the procedure of Hanger (3) in which a visual estimation of the flocculation is used. The results are shown in Table 1. This comparison is presented to enable the reader to evaluate the old procedure in the light of this new methodologic presentation.

Stability of Cephalin-Cholesterol Saline Emulsion

Microbial contamination and the possibility of sodium chloride breaking the emulsion are both problems inherent in the stability of the cephalin-cholesterol saline emulsion. Two batches of Merthiolate-preserved emulsion, stored in clear glass bottles in the refrigerator (approx. $4^\circ$) and used at weekly intervals, proved stable for 5 and 6 months, respectively, at which time mold developed. Without Merthiolate, a batch of the emulsion, stored and used in the same fashion as the Merthiolate-preserved batches, exhibited mold growth in about 2 months. It is suggested that Merthiolate be retained as a preservative and the emulsion be discarded after 3 months when in daily use. It

<table>
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<th>Test No.</th>
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<th>Present method</th>
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should be emphasized that in no instance did Merthiolate preservation spuriously raise the values; however, it is equally true that, according to Knowlton (16), Merthiolate does not prevent falsely high results.

Spectral Characteristics of the Reaction Mixture

Absorption spectra were determined on a serum with an abnormally high cephalin-cholesterol turbidity reactivity and on a normal serum (Fig. 3). No clearly defined absorption maximum was observed. The wave length of 500 m\(\mu\) was chosen for the routine test on the basis of affording the most practical approach to greater sensitivity in the determination. Although the effects of serum pigments are greater at 500 m\(\mu\) than at 650 m\(\mu\), the "true" serum blank advocated in this modification adequately nullifies these effects. The lack of such a blank, even when readings are made at 650 m\(\mu\), is one of the pitfalls of the former quantitative approaches. Serum pigments are found to interfere somewhat at 650 m\(\mu\) also. At wave lengths shorter than 500 m\(\mu\), it becomes impossible to adjust the sample to 100% transmittance when used as a blank, as outlined in the method. Thus, wave lengths shorter than 500 m\(\mu\) would require reading both the blank and sample against distilled water and subtracting the resulting absorbancies.

Attempt to Further Accelerate the Reaction with Heat

Use of the other temperature, 56°, at which water baths are available in clinical laboratories resulted in a further acceleration of the
reaction. Because of the nature of the flocculated material, it was found impossible to correlate the results with incubation for 3 hr. at 37° or for 24 hr. at room temperature (approx. 25°) regardless of what time of incubation was used at 56°. Temperatures between 37° and 56° were not evaluated.

Normal Values

Twenty fasting serum samples were obtained from hospital personnel having no signs of illness. Both sexes were about equally represented in this group and age ranged from 18 to 56 years. The cephalin-cholesterol flocculation determinations were performed on these serums within 1 hr. after clotting at room temperature (approx. 25°).

The 95% limits for cephalin-cholesterol flocculation were 0.3–5.0 cephalin-cholesterol turbidity units. No statistically significant difference between levels for males and females was found.

Discussion

It has been shown that the objectives of a precisely quantitated cephalin-cholesterol flocculation test can be achieved in such a manner that the method is rapid, accurate, sensitive, and requires only micro quantities of sample. It has been further demonstrated that a sufficiently stable cephalin-cholesterol emulsion, incorporating saline, is possible. The authors recommend the adoption of the cephalin-cholesterol turbidity unit, as defined in this article, to permit one basic standardization technic for all turbidimetric liver function tests.

References