A Comparison of Some Methods of Cholesterol Measurement

Betty E. Hawthorne

Five referee sera were analyzed for total cholesterol at several laboratories by up to 10 different methods or modifications. Results by any method varied less within a single laboratory than among laboratories, although the ranges of replicate analyses reported differed considerably. Those methods including procedures for saponification with potassium hydroxide and some isolation of cholesterol from other chromogens by digitonin precipitation or re-extraction into petroleum ether, in general, yielded results somewhat lower than those obtained by methods which did not include these procedures.

A large number of methods are currently in use in clinical and research laboratories for the quantitative measurement of cholesterol in serum. The increasing frequency of cholesterol analyses emphasizes the importance not only of the precision and reproducibility of individual methods but of variations that occur among different laboratories using the same method and among laboratories using different methods. Cholesterol methodology was critically reviewed.
by Zak and Ressler in 1955 (1) and by Cook and Rattray in 1958 (2). Comprehensive studies of sources of errors in various procedures and comparisons of results of measurement by different methods continue to be published (3–5). Although almost every paper on cholesterol methods reports comparisons among two or more methods within a single laboratory or between two laboratories, comparisons of analytical results on test sera from several laboratories routinely using different methods are limited (6).

In planning the Western Regional Research Project W-44, "The role of diet and certain environmental factors in cholesterol metabolism," procedures were established for periodic comparisons of analyses of referee serum samples by the nine participating laboratories: the Experiment Stations of Arizona, California, Colorado, Hawaii, Idaho, Montana, Oregon, Utah, and Washington. Later, seven laboratories were added: six from the northeastern region—the Experiment Stations of Connecticut, Maine, Maryland, New Hampshire, New York, and Rhode Island—and one laboratory from the Human Nutrition Research Division of the U. S. Department of Agriculture. This paper reports results for total cholesterol in five different referee sera analyzed at 8–16 laboratories by a total of 6–10 different methods or modifications (Table 1).

Table 1. Number of Laboratories Participating and Methods Used in Analyzing Five Referee Sera

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<th>No. of laboratories</th>
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<tr>
<td>High-lipemic</td>
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Procedure

In 1957 a supply of vials from a single lot of commercial freeze-dried serum* was obtained and distributed to the participating lab-

*Clinical Chemistry Control Serum (freeze-dried), Hyland Laboratories, Los Angeles, Calif.
oratories in the western region. Distribution to the other laboratories was made early in 1959 after verifying that no change had occurred in the lyophilized samples during the period of refrigerated storage. These samples were reconstituted and analyzed during 1957, 1958, and/or 1959 by a total of 16 laboratories. Vials of another lot of the same brand of control serum were distributed in 1958 and analyzed by eight of the same laboratories. Each participant was asked to reconstitute the dried sera according to the directions provided: "Add exactly 5.0 ml. of distilled water. Replace the stopper and agitate gently until the solution is complete. Treat the control serum in the same manner as an unknown serum." Samples were analyzed by procedures routinely in use in each laboratory, the number of replications being determined by their policy. Data were reported as mean values, with the number of aliquot analyses represented by the mean and the range of values indicated.

As a continuation of the study, frozen sera of significantly lower and higher cholesterol concentrations were distributed in 1960 and analyzed by 13 of the same laboratories. It was not possible at the time to obtain these variations in commercial control sera in the limited quantities desired, so test human sera of low and high cholesterol concentrations were collected and prepared in one laboratory.*

The samples were prepared by pooling a number of analyzed sera to obtain approximately 25 ml. of each of three samples. The first sample consisted largely of sera from children in whom the cholesterol concentrations were low. The second sample was pooled from a number of hospital patients who were hypothyroid, diabetic, or hypertensive and whose cholesterol concentrations were high. Because one patient's serum was extremely lipemic as well as hypercholesterolemic (over 600 mg. cholesterol per 100 ml.), a third sample was prepared. Pooling with other sera reduced the cholesterol concentration and the lipemia, but the consistency and color of the serum remained very different from the other two. For identification, these samples were labeled "low," "high," and "high-lipemic," respectively.

Aliquots (exactly 1 ml.) of each pooled serum were transferred to 1-ml. volumetric flasks which were stoppered and sealed with protective tape.† The samples were frozen, packed in Dry Ice in styrene containers† and shipped by air to the participating laboratories. Participants reported that all samples arrived in the frozen state.

*Oregon State University.
†NASCO, Inc., Port Atkinson, Wis.
The following directions were provided for handling of the frozen sera after arrival: "1. Transfer to freezer for storage until time for analysis. 2. At time of analysis, remove from freezer and place in a 37°C water bath for 30 min. Mix thoroughly by inverting tubes slowly and repeatedly; avoid development of foam. 3. Allow serum to attain room temperature before measuring aliquots." Participants again analyzed samples by their own routine procedures and reported results as mean values, range of values, and the number of aliquot analyses.

Eleven different methods or modifications were used by the participating laboratories. Seven of the methods depended for final measurement on the Liebermann-Burchard, or a modified Liebermann-Burchard, color reaction, but employed various extraction and isolation procedures: Sperry and Webb (7), in which extraction from 0.1-ml. samples is by acetone-ethanol with isolation of the cholesterol effected by digitonin precipitation after saponification by potassium hydroxide; Galloway et al. (8), a micromodification of the Sperry and Webb method requiring only 0.04 ml. of serum; Abell et al. (9), in which 0.5 ml. of serum is treated with alcoholic potassium hydroxide for simultaneous deproteinization and saponification, and cholesterol is separated from other lipids by extraction into petroleum ether; Clayton et al. (10), an adaptation to samples of 0.075 ml. of the methods of Kibrick et al. (11) and Abell and associates (9), which involves hydrolysis of the cholesterol esters by benzyltrimethylammonium hydroxide simultaneously with the evaporation of an alcohol-ether extract; Pearson et al. (12), a direct procedure in which 0.1 ml. of serum is treated with glacial acetic acid, p-toluenesulfonic acid solution, and acetic anhydride with no prior isolation; Adamson (13), a microadaptation of the method of Pearson and co-workers which reduces the serum required to 0.005 ml. and adds a preliminary deproteinization and extraction with acetone-ethanol; and Carr and Drekter (14), another procedure similar to that of Pearson and associates but in which acetic anhydride is used for the simultaneous precipitation of protein and extraction of cholesterol from 0.2-ml. samples.

One method was fluorometric—that of Albers and Lowry (15) as modified by Koval (16), in which the fluorescence of a modified Liebermann-Burchard reaction is measured. In this method lipids from serum samples containing 2–100 μg. of cholesterol are precipitated with trichloroacetic acid and extracted by alcoholic potassium acetate and absolute alcohol, esters are saponified with potassium
hydroxide, cholesterol is extracted by petroleum ether, and an acetic
anhydride-trichloroethanesulfuric acid color reagent is added to the
dried residue.

Three of the methods used made the final measurement with the
ferric chloride color reagent introduced by Zlatkis et al. (17): the
method of Koval (16), in which the direct procedure of Zlatkis and
associates is modified by preliminary precipitation, extraction, and
saponification of samples containing 0.2–10 mg. of cholesterol (pro-
cedures identical to the steps in his modification of the method of
Albers and Lowry); the method of Rosenthal and Jud (18), which
employs the use of an iron solution prepared originally in phos-
phoric rather than acetic acid on acetone-ethanol extracts from 0.05
ml. of serum; and a modification of the method of Rosenthal and Jud
by Standal (19), in which the amount of serum is reduced to 0.005
ml. and total cholesterol is separated for measurement by digitonin
precipitation after potassium hydroxide saponification.

Results and Discussion

The total-cholesterol values reported for the two lyophilized sera
are shown in Table 2, and for the three frozen sera, in Table 3.

The most extensive comparisons among methods, among labora-
tories, and among analyses within a single laboratory can be made
from the data for lyophilized Serum 1 (Table 2). Mean values re-
ported for this sample ranged from 148 to 208 mg. of total cholesterol
per 100 ml. of reconstituted serum. Although the range of mean
values (148–188 mg./100 ml.) reported from the laboratories using
the methods of Sperry and Webb (7), Galloway et al. (8), Abell et al.
(9), and the Koval (16) modifications of Albers and Lowry and Zlat-
kis et al. was lower than the range of mean values (186–208 mg./100
ml.) reported from those laboratories using the methods of Pearson
et al. (12), Adamson (13), Carr and Drekter (14), and Rosenthal
and Jud (18), it is difficult to generalize concerning the effect of
method. Values determined by the same method but in different labora-
tories sometimes varied considerably. Differences between the
lowest and highest mean values for methods used by two or more
laboratories were 15–35 mg./100 ml. Further, the procedures of origi-

nal extraction and/or type of final measurement among those meth-
ods yielding the higher or the lower range of values are not con-
sistent. However, there are two procedures common to the methods
yielding the lower values that are not included in the other methods:
(1) saponification with potassium hydroxide, and (2) further isola-
Table 2. **Total Cholesterol Concentrations (mg. Cholesterol per 100 ml. Serum)**  
Reported for Two Lyophilized Control Sera

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*Clinical Chemistry Control Serum, Lot No. 369E1, Hyland Laboratories, Los Angeles. (Value found: 195 mg./100 ml.; Acceptable range: 185-205; Methods used: Bloor, and Pearson et al.)

†Clinical Chemistry Control Serum, Lot No. 369F11, Hyland Laboratories, Los Angeles. (Value found: 205 mg./100 ml.; Acceptable range: 195-215; Methods used: Bloor, and Pearson et al.)

‡Figures in parentheses indicate number of aliquots represented by range and mean.

§Analyst for this year differs from that of other year(s).
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*Figures in parentheses indicate number of aliquots represented by range and mean.
†Analyst differs from one who contributed data from same laboratory for Table 2.
tion of the cholesterol either by digitonin precipitation (7, 8) or re-
re-extraction into high boiling-point petroleum ether (9, 16). The
importance of the preparation of a nonsaponifiable extract for an ac-
ccurate determination of cholesterol by a variety of colorimetric re-
actions has been emphasized (5). Many current reports stress that,
if the isolation of cholesterol is incomplete, certain chromogens—e.g.,
tryptophan (20–22), bilirubin (20, 21), vitamin A (23), and polyun-
saturated fatty acids (24)—interfere with the color reaction.

Data from laboratories using more than one method—i.e., Labora-
tories F, M, and P—do not add much clarity to the comparisons be-
cause of the particular methods used. From Laboratory P similar
low values of 177 and 168 mg./100 ml. were reported using Koval’s
modifications of the methods of both Albers and Lowry and Zlatkis
et al. A value reported from Laboratory M by the method of Clayton
et al. was lower than that by the method of Carr and Drekter—175
and 188 mg./100 ml., respectively, but the only other value de-
determined using the method of Clayton and associates was considerab-
ly higher—199 mg./100 ml. However, in 1957 mean values of 184 and
208 mg./100 ml. were reported from Laboratory F by the methods of
Abell et al. and Pearson et al., respectively.

Although there were gross differences in values reported among
laboratories using the same method, values reported by a single lab-

oratory from year to year were quite consistent. Among the seven
laboratories in which Serum 1 was analyzed in two or three separate
years, differences ranged from 3 to 8 mg./100 ml., even when there
was a change of analyst, as in Laboratory C. Variations in the use of
a method within a single laboratory would appear to be less than
variations in the use of a method among different laboratories. From
the range of values reported for aliquots, the consistency of replica-
tion of aliquot analyses also would appear to be more characteristic
of a laboratory using a method than of the method itself.

Lyophilized Serum 2 was found to contain more cholesterol than
Serum 1 by all eight laboratories that analyzed both samples (Table
2), the range of increase reported being 15–25 mg./100 ml. These in-
crements are somewhat higher than the 10-mg. difference between the
values reported by the distributor of the two control sera. The values
reported by the distributor for Serum 1 and Serum 2 (Table 2), using
the methods of Bloor and Pearson et al., were 195 and 205 mg./100
ml., respectively. The range of values reported by the use of differ-
ent methods was smaller for Serum 2 than for Serum 1, although
comparisons are limited by data being available for only six methods.

The data reported for the “low” and “high” frozen sera (Table
3) present a pattern quite similar to that of the lyophilized sera. The range of mean values reported from laboratories using the methods of Sperry and Webb (7), Galloway et al. (8), Abell et al. (9), and the Koval (16) modification of Albers and Lowry was lower than that reported from the laboratories using the methods of Adamson et al. (13), Carr and Drekter (14), Rosenthal and Jud (18), and the Standal modification of Rosenthal and Jud (19). The ranges were 109-128 and 131-154 mg. cholesterol per 100 ml. serum for the "low," and 313-351 and 343-417 mg./100 ml. for the "high." It is significant to note that, although Laboratory D used for this series a modification of the method of Rosenthal and Jud which included saponification and isolation with digitonin, the values determined were in the higher range. Gross differences again occurred among laboratories using the same method. Variations among replicate analyses within a single laboratory were in several cases proportionately greater than were those of the lyophilized sera. Adequate mixing of thawed, 1-ml., frozen serum samples would appear to require more care than uniform mixing of 5 ml. of reconstituted lyophilized sera.

The problems encountered in adequate mixing and sampling of highly lipemic sera are emphatically demonstrated by the data summarized for the "high-lipemic" Serum in Table 3. Mean values reported from the 13 laboratories ranged from 350 to 563 mg./100 ml., and aliquot ranges were high. Problems of inadequate sampling from a lipemic serum appear to be accentuated by those micromethods using samples of 0.005-0.05 ml.—i.e., the methods of Adamson (13), Galloway et al. (8), Rosenthal and Jud (18), and Standal (19).

Wide variations in results of measurements of serum cholesterol have been attributed to the unavailability of an acceptable universal standard as well as to the use of diverse methods (25). In 1957, following the analysis of Serum 1 by the collaborators in the several laboratories of the western region, a purified cholesterol sample* was distributed to each participant as a referee standard. Appropriate dilutions were made and the density of color produced by the Liebermann-Burchard reaction was read at the 625 or 635 m\(\mu\) wave length routinely used. Among the five laboratories, C,E,F,G and H, using the Sperry and Webb or Galloway et al. methods, absorbance readings of 0.1 mg./1.0 ml. dilutions of the referee standard ranged from .141 and .146. Absorbance readings of identical concentrations of standards routinely used in each laboratory varied from those of the referee standard from +.001 to -.0045. Differences in results deter-

*Prepared and distributed by the University of Arizona.
mired on referee Serum 1 among these laboratories did not appear to be due to differences in the standards used for comparison.

The methods compared in this study involve only a small fraction of those currently in use for serum cholesterol measurements. The need for a widespread survey to evaluate current procedures in clinical and research laboratories was recognized by the U. S. Public Health Service when it initiated the Cooperative Cholesterol Standardization Program in 1960. More recently, the Standards Committee of the College of American Pathologists has undertaken a study “to characterize a best cholesterol standard for universal use and to recommend an analytical procedure which may serve as a consistent point of reference” (25). It is reassuring that concerted efforts are being directed toward evaluation and improvement of cholesterol methods.*

References

*Since the submission of this manuscript, Tonks has published a paper on the accuracy and precision of clinical chemistry determinations in 170 Canadian laboratories (26). Mean cholesterol values for one referee serum determined by different methods varied in the same direction as those reported for this study, but the range of values was greater.