Cholesterol Methodologies: A Review

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Introduction

The introduction of enzymes as reagents into the methodologies of cholesterol determination (1–6) has drastically altered the limited strong-acid approaches taken by most analysts of the past 90 years (7–19). There has been a considerably heightened interest in determining the compound because of new diagnostic approaches such as hyperlipoprotein phenotyping (13) with the implied relationship of high cholesterol values as an underlying cause of cardiovascular disease (14, 15), and abnormal cholesterol values in several other disease states (16, 17).

From a historical point of view, modern cholesterol determinations had their beginnings in the late nineteenth century when Salkowski described a color reaction (18) for this analyte, which was isolated from gallstones about a century earlier (19). Three studies then gave impetus to what might be described as a great analytical chemistry movement for the determination of cholesterol, and these were the development of a practicable color reaction (20), its modification (21), and the discovery that digitonin precipitates cholesterol quantitatively and relatively easily (22). The latter was accomplished by Windaus, even though as late as 1928, in his Nobel Prize address, he described the cholesterol structure incorrectly, showing an ethyl group at carbon 10 (23, 24). When it was discovered that oxidation with selenium resulted in chrysene (25), and on the basis of x-ray measurements on the mycosterol, ergosterol, it was then believed that the ring nucleus of cholesterol was perhydrochrysene. Later, several investigators (26–28) concluded that the correct nucleus was cyclopentanoperhydrophenanthrene, a structure that seemed in harmony with data derived from earlier experiments (24).

The combination of a color reaction for quantification and digitonide precipitation for purification led to the first colorimetric procedure for serum (29), which was based on the gravimetric procedure of Windaus (22). By that time he had introduced saponification, a step that showed that some idea of the presence of free cholesterol and the nature of ester cholesterol in blood was already known to these early analysts (22, 30). It is interesting that the first acceptable reference procedure for the determination of cholesterol was this gravimetric method of Windaus (29). It was later used by Schoenheimer and Sperry (10) to prove the accuracy of their own elegant procedure, which then became the reference method used to prove the Abell et al. procedure (7), a significant chain of events. Both have now been accepted as reference procedures, which indicates that present day accuracy is easier to accomplish but perhaps no better than that obtained by the gravimetric means of the past.

Between the colorimetric, fluorometric, and electrochemical enzyme-reagent techniques of the present—some manual, some mechanized—and the beginning of cholesterol methodologies of the late nineteenth century, all of which were manual, many suggestions were set forth for procedures to determine this interesting and important compound (31–41). Literally hundreds of papers, many little more than miniscule modifications of others, have been proposed. Oddly, early studies showed the necessity for purification of the analyte before resorting to an endpoint reaction. But as time went on and mechanization of methodology appeared, the direct reactions became more popular because mechanical devices were limited in the sorts and numbers of reaction steps they could perform. It was then suggested that a reference procedure such as the Schoenheimer–Sperry and a direct Liebermann–Burchard procedure produced results that were indistinguishable from one another, a complete turnabout from the past (42). More will be said of this later under interferences and standardization.

Before attempting to discuss the various methods available for the determination of cholesterol, it might be useful to classify them in order that one may peruse and choose according to the application and need to which the measurement is to be applied. Such a chemical categorization could simplify the quandary most users feel when faced with variety and a number of choices. Because of the advent of newer methodologies involving completely enzymatic systems (43, 62) as well as the procedures leading to them (1–6), it appears that some revisions in classification may be necessary that

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would include the newer approaches, even though it is apparent that the new systems should fit into the older classification (40).

Several methodological classifications have previously been proposed (36, 38–40). Among these, one was described in which methods were grouped according to the procedures of handling (40) rather than by the color reaction used as the endpoint device at the equilibrium of the last step in the procedural outline (39). This seemed and still seems quite suitable as a classification plan if it were modified somewhat to include enzyme reagents and other kinds of endpoint reactions other than spectrophotometric ones. It demonstrates in brief fashion the techniques involved, and enables the selector to make a decision that is based on the limitations of the procedure selected for the kind of data required in terms of the accuracy and precision that is adequate for the needs of the user. One should also consider the medium in which the determination was to be done. It would be of critical importance, for example, if the medium of interest contained insoluble particulate matter, which might preclude the use of a simple, direct reaction that is better suited to a simpler sample that presents no such solubility problems. However, it might be possible in the future to avoid problems engendered by particulate matter in the endpoint reaction, for example an electrochemical one rather than an optical one, could react with an analyte generated in that procedure.

Classification of Cholesterol Methods

I. Direct Reactions for the Determination of Cholesterol

A. No separation or partial separation of cholesterol is carried out. The reagents are mixed with sample and the endpoint is determined by spectrophotometry (9, 12, 63–83), electrochemical measurement of a product of reaction (49, 56), or any other kind of endpoint device deemed suitable to that purpose. A direct reaction would undoubtedly have to be considered to be one in which the reagents are added to the sample and in which no fraction of the sample such as the proteins are removed before a measurable reaction is allowed to take place; that is, there is no phase separation in direct reactions. The Wybenga procedure with an Fe(III) reaction (63), the procedures of Pearson et al. (9) or Huang (64), each based on the Liebermann–Burchard reaction, and the Allain procedure (5) in which a coupled enzymatic system is used are examples of direct reactions. The Parekh–Jung modification of an Fe(III) procedure is improperly described as a direct reaction (84–88). They themselves stated that the reagent precipitates out the proteins of serum and extracts the cholesterol from its binding sites (88). Like the similar Zak (89), Henley (90), Chiamori and Henry (91), or Bowman and Wolf procedures (92, 93) that are the basis on which the Parekh–Jung procedure is founded, it would have to be considered an extraction procedure. Any claims to a direct reaction for a material such as serum (85) or adrenal tissue (84) should be viewed as an error. The belief is also incorrect that a certain kind of organic solvent or mixture is required to extract cholesterol from serum, as perhaps a liquid–liquid system. Any solvent that can precipitate proteins and at the same time solubilize cholesterol from its binding site is an extraction solvent.

B. Blanking

1. Only a reagent blank is used (5, 68, 64, 94–101). The assumption is made that no irrelevant absorption due to color or turbidity could be present in the sample, perhaps because of high dilution, and therefore no serum blank is required, or it is assumed that the serum blank effect is small enough to be ignored, or that a fast kinetic rate measurement obviates the blank.

2. Reagent blank and a serum blank are both subtracted. A serum blank of several kinds has been suggested. A change in the solvent matrix is made such that cholesterol cannot react, so the background color of the serum itself is subtracted (40, 66, 69), or the reagents are changed so that cholesterol does not react but the interfering compound (or compounds) can react, allowing a blank correction for a dynamic interference along with any residual irrelevant absorption caused by other static colors or turbidities in the serum itself (75).

C. Advantages and (or) limitations. The primary advantage of a direct procedure lies in its simplicity. If a procedure yields reasonable values so easily, it is readily amenable to manual and simplified automated systems. In the latter methodologies, automated liquid–solid or liquid–liquid extractions are rather uncommon, although each has been reported (102, 103). Obviously such systems can be subject to problems involving static and (or) dynamic errors of either an absolute or relative type (104). In the case of the latter, the situation can be improved by use of the principle of internal standardization (105), a process that will be discussed later.

II. Partial Purification of Cholesterol with Organic Solvents

A. Partial isolation can be achieved by liquid–solid or liquid–liquid extractions (106–120). In the case of liquid–solid extractions, the extracting fluid is soluble in water and serves to remove the cholesterol from their protein-binding sites into the liquid phase, where the measurement can be made either directly (121) or on a later solubilized residue of an evaporated extract (122). Alternatively, the serum is dried into one of several solid materials, from which it can be eluted into a separating liquid (123–125). Biphasic liquid–liquid extractions with (7) or without (8) saponification of cholesterol esters before the extraction have been used. In some procedures, saponification is required because the extracting fluid will not completely separate both free and esterified cholesterol (7). In the automated mode, there has been at least one report in an enzymatic procedure (47) of dialysis of product, peroxide, where the latter could be measured in a diffusate, which seemed free of interferences. Any potential competition
of bilirubin with the color reaction was avoided by a preliminary treatment of the sample with alkali to oxidize it when total cholesterol was determined, because peroxide was generated on the same side of the membrane where the bilirubin was present. However, the reaction of bilirubin with peroxide is minimized if no peroxidase is present to catalyze it.

B. Blanking. Irrelevant absorbing compounds of the static type (i.e., absorption does not change during the reaction) can be isolated from cholesterol (126) by the extraction process. In addition, known dynamic interferences can also be separated (126). Therefore, procedures involving extraction systems do not usually describe the use of serum blanks, and rely primarily on reagent blanks.

C. Advantages and limitations. Partial purification and elimination of most interferences should result in a more nearly pure end-point determination and more nearly accurate values. However, the more steps that there are in the reaction process, the more likely the possibility that errors can occur. In addition, the potential for automation decreases as the complexity of the process increases. Because the determination of cholesterol is frequently requested, it intensifies the problem of handling many samples on a daily basis.

III. Complete Isolation of Cholesterol

A. Free cholesterol has been isolated, after extraction and saponification, by precipitation with one of a variety of precipitating agents (30, 114, 127–133) and this purified derivative has then been subjected to endpoint analyses (10, 30, 117, 118). The precipitation has sometimes been hastened with the aid of a gathering step for which either aluminum hydroxide (128) or aluminum chloride (127) is used.

B. Cholesterol or its esters, or both, have been separated by means of different chromatographic processes after a preliminary partial purification by extraction into an organic solvent. These have included gas chromatography (133–141), liquid column chromatography (142, 143), and paper chromatography or thin-layer chromatography (144–149).

C. Advantages and limitations. The advantage of purification of an analyte before it is determined is that it can then serve as a reference procedure for the more common procedures that are used routinely. The disadvantages of complexities of processes and equipment along with the requirement of greater skills for the analyst are outweighed by the need for reference procedures (150). In the case of purification by precipitation with digitonin, some compounds that are structurally similar to cholesterol and present in measurable amounts appear to be present in serum (137, 141), but the extent of their interference is not clearly defined as yet. In addition, if they are not esterified, they would contaminate free cholesterol disproportionately as compared to total cholesterol. An advantage of the digitonide, however, is that it provides an additional analytical grouping for measurement because of the carbohydrate moiety on digitonin (151–156). Another advantage of isolation and purification by digitonide formation is that this treatment would exclude potential interfering sterols whose structures preclude reaction with digitonin.

IV. Miscellaneous Procedures

A. Cholesterol and its esters have been extracted from the protein zones known to contain them after electrophoresis of serum proteins was carried out (157–160).

B. Lipoproteins containing cholesterol have been selectively precipitated out with dextran sulfate before colorimetric determination by either the Liebermann–Burchard or Fe(III) reactions (161, 162).

C. A selective partition procedure has been described for isolating free cholesterol (163).

D. Any procedure devised in the future that does not fit into I, II, or III would undoubtedly fit here in IV.

V. Screening, Definitive and Reference Procedures

No established definitive procedure for the determination of serum cholesterol has been described as yet. However, at least three procedures have proven worthy as (at least unofficial) reference methods (7, 10, 22), while all of the rest have been applied to the routine circumstances. The techniques that have provided most of the diagnostic data in recent years might best be termed the screening procedures (9, 42, 63, 64), whose function it is to find abnormal values quickly, although perhaps not providing nearly the accuracy of the reference procedures when the latter are carefully carried out (40). The advent of such simple screening procedures as a part of automated multiphasic instrumentation (42), or high-speed discrete sampling analyzers (5) where they can be dedicated for screening in hyperlipidemia, has generated an era in which all can easily obtain the advantage of relatively inexpensive cholesterol assay. The introduction of enzymes as reagents into the more recent methods for the determination of serum cholesterol appears to serve the ideal: a screening procedure that is as accurate as the reference procedures for most samples (40). Because the enzymatic procedures are very simple in terms of handling steps as compared to the considerably more complicated reference procedures (7, 10) and because the reagents are easier to handle, the precision of routine determinations is superior to what it was in the recent past, and may very well be better than that of the reference procedures unless the latter are carried out with an extreme care not usually practiced in the routine area. We appear to be approaching that analytical circumstance where some techniques for the determination of cholesterol are simple, accurate, and rapid, making them ideal for automation and still offering the promise of narrower "gray" areas for perhaps better diagnostic definition. It seems obvious even at this early period in the development of the enzymatic methods, that a cholesterol oxidase approach to a substitute analyte for cholesterol, namely hydrogen peroxide, when coupled to a preliminary purification process as in the Abell procedure (7) should lead to a more nearly ideal reference procedure than those in which the strong-acid endpoint reactions
are used, with all of their known attendant problems. However, in considering reference procedures, one must
determine how a reference method is established by a
contemporary group of scientists. In recent years one
has seen the introduction of the concept of a Reference
Method for calcium (164), alleged to be the first and
only Reference Method in all of clinical chemistry. From
it a reference procedure was established and later con-
firmed as acceptable (165). It behoves us to question
whether other reference procedures, such as those de-
scribed for the determination of cholesterol, have been
established by similar rigorous investigations or simply
accepted by common usage. This question has been
raised—and it should be answered—as to who estab-
lishes reference procedures (166).

Other Analytical Considerations

Interferences

This is one of the most important areas of any ana-
lytical procedure. Innovators of analytical procedures
cannot always predict what the future of any analytical
system will hold when testing it in a heterogeneous
sample subject to wide-swinging changes in concentra-
tion of its constituents owing to abnormal physiological
states and often characterized by the presence of com-
pounds (drugs) perhaps ingested by an individual or
administered to a monitored individual in an attempt
to return a patient back to the normal state. Even a
clean-looking biological sample such as normal serum
contains many compounds that are not always sus-
ppected as interferences because they do not become
troublesome until they are present in rather high con-
centrations in the tested fluid. Samples that are jaun-
diced, turbid, lipemic, or hemolyzed may present an
important problem, especially if a direct determination
is to be made. The most important of these problem
samples is caused by jaundice, because bilirubin is a
reactive compound, differing in the way it acts in the
several endpoint systems that are in common use in
present cholesterol methodologies. In the Lieber-
mann–Burchard and iron reactions, if bilirubin is not
removed, it reacts to form stable biliverdin, which gives
a positive absolute interference. The magnitude of that
interference is greater for the Liebermann–Burchard
reaction because bilirubin forms more measurable
absorbance by conversion to biliverdin than does chole-
sterol in generating a similarly shaped spectral absorb-
ance curve in the region of the measurement wave-
length. This easy conversion of bilirubin to biliverdin
exemplifies an oxidative action of the Liebermann–
Burchard reagent. In methods in which enzymes are
used as reagents, authors of three similar systems de-
scribed how bilirubin results in a small positive error
(43), a small negative error (5), or no error at all (167).
If one considers that the effect of interference could
be determined by testing bilirubin in albumin without the
possibility of peroxide/peroxidase side reactions, the
correct interpretation would be for a positive error. If

cholesterol and bilirubin were present simultaneously,
then the error could be negative. However, it is difficult
to rationalize no error at all when a very high bilirubin
concentration is present and a competitive substrate
reaction should take place to destroy a portion of the
peroxide generated. In other words, bilirubin can in-
terfere with enzymatic procedures for the determination
of cholesterol positively in the static mode and nega-
tively in the dynamic mode for colorimetric endpoints.
It would seem that it could only interfere negatively in
fluorescent methods and possibly negatively in elec-
trochemical measurements. Investigators of fluorescent
or electrochemical methods may believe that there
should be no error because bilirubin does not fluoresce
under these conditions since it requires an acid medium
and albumin (168) or that it does not react with a se-
lective-ion electrode. The latter technique should suffer
minimally from bilirubin because no peroxidase is
present. However, the potential reaction of bilirubin
with peroxide may be critical to all endpoint measure-
ments. The more peroxide side-reacts under the con-
ditions specified for a particular endpoint measurement,
the lower will be the final result and the greater the
deviation from the true indirectly obtained value for
cholesterol. Enzymes used as chemical reagents for
producing peroxide, where the latter becomes the de-
rived or substitute analyte for cholesterol, have some
selective specificity in generating peroxide, but from
that point in the sequence of reactions the enzymes are
no longer involved. Since the peroxide is capable of a
side reaction with a competitive substrate such as bili-
rubin in preference to those of the anticipated endpoint
reaction, if aminoantipyrene–phenol is the example,
then bilirubin can interfere by lowering the concen-
tration of final compound, hydrogen peroxide, which
is supposed to reflect cholesterol content. In addition,
the measurement wavelength becomes important for
the final result because bilirubin absorbs somewhat at
the endpoint peak maximum of 500 nm, and any re-
sidual bilirubin will act compensatorily for the substi-
tute colored complex that was not formed (177). If the
wavelength is shifted toward the infrared to get away
from bilirubin absorbance, as has been proposed (167),
then it would seem reasonable to assume that the
compensating irrelevant absorption of bilirubin is
eliminated and the final apparent concentration of
cholesterol should be lower.

Interferences in Fe(III) reactions can cause either
absolute or relative errors, which depend in part on how
the sample is treated before the colorimetric step. If one
embarked on isolation processes such as those used in
the procedure of Abell et al. (7) or Schoenheimer–
Sperry (10), and then substituted an Fe(III) reaction
for the Liebermann–Burchard reaction, the results
should be no different than those of the Liebermann–
Burchard reaction on the finally treated residue, unless
rather high concentrations of reactive compounds,
perhaps other Δ5-sterols, were extracted along with
cholesterol during the isolation. With the Fe(III) re-
tion, the final color obtained would be more stable and
considerably more sensitive. It is for the direct mode or for the partly isolated system that some interferences have been reported. Bromide causes relative errors of enhancement (169), although it is not often encountered and may be easily removed (91, 126, 169). Its enhancing effect is more suitably obviated by the method of internal standardization (105) rather than by isolation from the interference. Thiouracil is now known to be unnecessary for the now-defunct test for butanol-extractable iodine, azide is not a highly regarded preservative anymore because of its potential explosive qualities, while the amount of nitrate required for notable inhibition, presumably that contaminating the sulfuric acid, is much greater than the tolerable amount in that reagent (174). Bilirubin is quickly oxidized to biliverdin by any of the Fe(III) reactions, but the extent of its interference is less than that of most other colorimetric procedures, including the Liebermann–Burchard reaction and the Hantzsch and Trinder reactions of the enzymatic procedures (176, 177).

Many substances were previously reported as interferences in the Liebermann–Burchard reaction for cholesterol (39, 40, 178–182). The actual chemical documentation is fragmentary, mostly stemming from the early days involving visual bicolorimetric measurements. The compound that causes the most such trouble is bilirubin (39, 40). The reported extent of this interference has ranged from questionble (70), 3 mg/mg (42), and 10–12 mg/mg (183) to erratically high and variable (66, 76). However, it can easily be shown that, in the most common modification of the Liebermann–Burchard procedure for cholesterol (64), the reaction with bilirubin is linear when allowed to go to completion, the final color is stable (far more stable than that for cholesterol itself), the color formed is linearly related to concentration of bilirubin, the reaction is independent of the presence of cholesterol, and the product formed absorbs more strongly than does that of cholesterol. Several of these points are also identical to those found with all Fe(III) reactions for cholesterol, in all of which bilirubin is easily converted to biliverdin. However, if the procedure for cholesterol based on the Liebermann–Burchard reaction is carried out at low temperature as suggested (68), which would slow up the bilirubin oxidation, and if the wavelength of measurement is 540 nm as also suggested (68), then the bilirubin interference owing to unstable intermediates formed in its oxidative conversion might be too formidable to overcome by blanking.

Interferences in which enzyme reagents are used for the subsequent colorimetric determination of cholesterol through its oxidation to form peroxide have been evaluated to some extent (5, 6), but it is perhaps really the peroxide itself that needs the most study. If one considers the entire analytical scheme, peroxide seems the most vulnerable to side reactions, and the problems associated are quite similar to those with other oxidase systems in which peroxide reflects the concentration of the analyte. If peroxide is also the compound actually being measured in the fluorometric procedures (51), it would appear that it should result in a similar problem, if a side reaction could compete with the fluorescence reaction for the measured compound. Compounds already tested as interferants both for cholesterol (3, 5) and in other oxidase procedures for other analytes (184–186) include steroids, bilirubin, hemoglobin, drugs, uric acid, urea, creatinine, glucose, bromide, ascorbic acid, glutathione, L-dopa, and epinephrine. Bilirubin has already been shown to be a competing substrate for peroxide, and a mechanism in proof was described (176). Cholesterol was allowed to react to completion to deplete the peroxide formed before bilirubin in a solution of albumin was added and the resulting spectrum scanned. This combined spectrum was then compared to exactly the same amount of bilirubin plus cholesterol reacted simultaneously, where the bilirubin had an opportunity to compete for the peroxide with the intended color reagent. The latter combined spectrum subtracted from the first combined spectrum should have resulted in a straight line of 0.0 A along the absorbance axis what if no bilirubin interference occurred, but instead the second spectrum was considerably lower than the first spectrum, proving bilirubin interaction. In addition, when a spectrum for pure cholesterol representing exactly the same concentration of cholesterol was subtracted from the simultaneous reaction spectrum, then the remaining absorption did not account for all the bilirubin. Conversely, when the spectrum for a pure bilirubin solution was subtracted from the simultaneous reaction spectrum, then something less than the true cholesterol spectrum resulted (177).

The enzymatic procedure in which the oxygen consumed in the cholesterol oxidase reaction with cholesterol is measured electrochemically and where the peroxide is blocked (by the addition of azide) from any reaction with contaminating catalase in the glucose oxidase to form oxygen is less likely to be interfered with by bilirubin (56). The pH at which the oxidase action takes place and the short time of reaction, including the hydrolase action to free the esterified forms, allows more specificity than when the peroxide is coupled sequentially to catalase or peroxidase reactions as final spectrophotometric endpoint reactions.

To get around any problems engendered by the peroxide-peroxidase reaction with interferences, several alternatives present themselves for the enzymatic procedures in which peroxide is normally the measured compound. Bilirubin can be destroyed by preliminary alkaline action, which also hydrolyzes the esters before cholesterol oxidase action (47); peroxide can be dialyzed before it reacts with peroxidase and color reagents, a process in which the reaction between bilirubin and peroxide is minimized by the absence of the enzyme to catalyze it (45); or cholesterol can be removed from the serum in which the bilirubin is present before peroxide is generated (4). In addition, the reacting compound, oxygen, of the cholesterol oxidase step in polarographic systems can be measured instead of measuring reaction products of enzyme action while the peroxide generated is protected from catalase action by the addition of so-
dium azide (56). In the event that cholestenone rather than peroxide is measured, it is a strong ultraviolet absorber (240 nm) or its dinitrophenylhydrazone can be measured in the near-visible range (1, 4).

Other Sterols

From time to time reports have appeared in which sterols other than cholesterol are said to be present in serum (137, 141, 187). In procedures in which cross reactions are possible, a positive interference results, whereas if a separatory step is incorporated into the procedure before the detection and determination step, then a more nearly true value is obtained, as is the case with extraction followed by gas chromatography–mass spectrometry for identification and quantification (138). Procedures in which cholesterol, either free or total, is isolated by precipitation with saponins (10) should be more free of interference by other reactive sterols, for only those sterols of the alleged contaminants with the correct spatial configuration should precipitate. In addition, digitonides of these interfering sterols may be unreactive in a color reaction or have much lower molar absorptivities than does cholesterol. This area of the other reactive sterols for the several colorimetric procedures needs more clarifying investigation than we have seen, because results which are the basis of our present suppositions are based on quite poor analytical techniques (187).

Blanking

The simplest approach to the determination of any analyte of serum would be its direct measurement without treatment except by means of the measuring device itself. For the moment that is not an available alternative for cholesterol measurement. Therefore, an alternative way to determine cholesterol in serum would be to react the analyte of the sample directly with reagent, a reaction that ideally would involve only the desired constituent, with all other components of the sample inert or at least subliminal to the chosen measuring device. Primarily, such treatment of sample would result in a reaction product that most suitably would be monitored spectrophotometrically. More recently, electrochemical detectors have been suggested with which suitable products of enzymatic reaction action could be adequately determined (49, 56). In several procedures, blanking—either of reagent, sample, or both—has been described. Blanking remains one of the question mark areas of cholesterol methodology. One sees proposals for reagent blanks and serum blanks for direct reactions in which the serum blanks may be static (9, 188) or dynamic (86, 75, 76), and in at least one case (189) a blank in which it was claimed that the medium was inhibitory to the reaction of cholesterol, allowing only the interferences to react in that particular matrix. The static approach can only correct for the background color of serum, and since this must involve a change in solvent character that obviates the reaction of cholesterol, it must be assumed that there are no changes in the irrelevant absorption measured at the peak maximum in spite of the matrix differences. Obviously, this would fail with either the Liebermann–Burchard or Fe(III) reactions if the interference was reactive; for example, bilirubin would be easily and quickly oxidized to biliverdin. The biliverdin spectrum has a minimum where the Fe(III) reaction has a maximum, but its peak absorbance is at about the same wavelength as that for the Liebermann–Burchard reaction for cholesterol. Therefore, there is a near 10-fold difference between the two reactions in the extent of its interference and interference is greater with the Liebermann–Burchard reaction—in some procedures (76) the latter amounts to about 6:1 on a weight basis and therefore 9:1 on an equivalent basis.

The previous discussion on blanking was primarily concerned with interferences yielding absolute errors, but it can easily be shown that in Fe(III) reactions relative errors can occur that are corrected for by neither static nor dynamic blanking. It then becomes necessary either to remove the interference or separate the analyte from the interfering matrix, a task which few would relish. Blanking serves no purpose in this case because the interferant itself does not react with the reagents, but interacts only when the analyte is present (105). An alternative proposal would be to put standard into the sample, allow it to come under the inhibiting or enhancing influence of the sample matrix, and then calculate from the additive absorbances of the analyte and its standard when both are subjected to the same influences (105).

Another novel form of blanking in direct reactions was by bichromatic measurement wherein it was assumed that bilirubin would constitute the only interference (82). Two wavelengths were chosen, the measurement one at the peak for cholesterol and the corrective one toward the red end of the spectrum at which the absorbance reading for biliverdin was the same as at the peak, thus nulling out the effect of the bilirubin conversion.

Dynamic blanking by means of a change in reagent matrix to create an analytical circumstance in which cholesterol is not reactive but bilirubin or perhaps other interferences are reactive (189) has been shown to be inaccurate for at least one such version of an Liebermann–Burchard reaction (190) because the green spectrum formed with the Liebermann–Burchard reaction for bilirubin is displaced bathochromically in the blanking version, causing an underestimation of the interference effect at the measurement wavelength. Although described as a reaction in which the cholesterol reaction with the Liebermann–Burchard reagent is inhibited, it is really not an Liebermann–Burchard reagent when a compound such as dimethylformamide is substituted for a large portion of the Liebermann–Burchard reagent. Other organic compounds used in place of dimethylformamide were shown to have a similar spectral effect (190).

In studying interference effects for blanking, it is perhaps important to see if the interference reacts with the reagent or is only interactive when both reagent and
analyte are present with the interfering compound. For example, in the case of the Liebermann–Burchard and Fe(III) reactions with bilirubin, the reaction takes place independently from the reaction with cholesterol and the error is additive and absolute. In the case of the enzyme reagents sequencing to the peroxide/peroxidase reaction with the Trinder reagent or the catalase/methanol/peroxide of the Hantzsch reaction, there is no reaction with bilirubin without cholesterol because no peroxide has formed. But when cholesterol is present to generate peroxide by the cholesterol oxidase reaction, then bilirubin in the presence of peroxide can and does act as a competing substrate by destroying peroxide and causing a lowered Trinder or Hantzsch reaction (176).

However, if one looks at the spectrum of bilirubin one can see that the infrared side of the curve gives a substantial reading at 500 nm, the peak of the Trinder reaction, while the ultraviolet side gives an even more substantial one at 410 nm, the measurement wavelength of the Hantzsch reaction. Therefore, the user faces a dilemma in blanking for bilirubin in either reaction. If a serum blank in the Trinder reaction is subtracted where part of the bilirubin has been converted by the action of peroxide, the full concentration of bilirubin absorbance will be subtracted, whereas somewhat less than that concentration is actually residual in the final measurement, an overcorrective effect (176). Similarly, in the Hantzsch reaction, bilirubin in the sample will be partly or completely converted and the Hantzsch reaction yield is low, whereas a serum blank will contain all of the bilirubin, again resulting in oversubtraction of blank absorbance (176). Different versions of what appear to be identical reaction systems may show somewhat smaller or larger interferences, depending on reagent makeup, especially with enzyme reagents, because, for example, reaction rates in the sequence of reactions can be quite different, allowing more time for the bilirubin and peroxide to react. In any sequence of reactions in which conditions are favorable for peroxide/bilirubin reaction or peroxide-competing substrate reaction, the endpoint reaction will underestimate the cholesterol because the substitute analyte for cholesterol, having itself been converted by the competing substrate, is no longer available for the necessary endpoint reaction, and the overall effect is a decreased apparent cholesterol concentration. It would appear that this should be true if peroxide is measured fluorometrically, colorimetrically, or sequenced to yield a coenzyme-coupled ultraviolet measurement (5, 44, 51, 191). This blanking problem would not be encountered if the cholestene none could be determined, a difficult measurement in a direct reaction, or if the oxygen used in the oxidation of cholesterol to cholestene is measured while the peroxide generated is hindered or removed by side reactions, since it would not be the endpoint device in this kind of a procedure (56). If a colorimetric measurement is made based on a peroxide/peroxidase coupled reaction, and any bilirubin remains as an interfering color to contribute absorbance at the measurement wavelengths, then that reading will serve as a compensating error unless a full serum blank is used to aggravate the measurement.

What Should Constitute a Sample

Although there may be secondary objectives for cholesterol determinations, the usual sample in which it is to be measured is blood. If it seems reasonable to assume that the most useful portion of that sample is serum, it might still be worthwhile to recapitulate a bit on why this should be so. To begin with, one must consider how cholesterol is distributed in blood, in what forms it exists, where those forms are found, and in what concentrations they could be measured. One often reads statements claiming that cholesterol esters are the predominant form in plasma whereas free cholesterol predominates in the erythrocytes (187). However, there is no cholesterol inside the erythrocyte; it is all in the membrane, and apparently none of it is esterified—that was an artifact of previous analytical error (192). The cholesterol in the membrane is dynamic and has been described as existing in equilibrium with the serum pool of free cholesterol (192). There is also the buffy coat, which contains cholesterol and which varies in volume in certain diseases. Because the erythrocytes also vary in volume and thus act as a diluent of unknown proportions, analysis of whole-blood suffers from serious deficiencies that are alleviated by the use of serum. The use of plasma rather than serum also can be the source of certain problems owing to the anticoagulants, which could interfere with some reactions by interaction or by water transfer between compartments of blood (40).

Standardization

The process of standardization of cholesterol for the different kinds of methodologies has not been a simple one in several colorimetric procedures. In fact, it is one of the most complex and confusing aspects of cholesterol determinations. Because the cholesterol in serum is present mostly in the esterified form and the esters have been suspected of reacting differently than does free cholesterol in some procedural versions, then the problem of the form measured must be considered in those cases (10, 37, 40, 194). Different approaches have been taken to circumvent the problem. These have included saponification to convert all esters to the free form (10, 115), mixing ester and free cholesterol in a calibration standard to approximate the normal value (109), use of a factor to correct the ester value to the free cholesterol value (112), or changing the solvent character of the medium to make free and ester cholesterol react equivalently (9, 188).

Simple, direct colorimetric procedures of the acid type, such as those involving the still dominant Liebermann–Burchard reaction, have not been simple to standardize, especially if one wished to adapt them to automation, and even more so in the automated multiphasic systems where detergent-solubilized or derivatized cholesterol would not be helpful because a serum control was required for calibrating the other serum constituents measured in the multidetermination process.
package offered. Originally, when the Huang procedure (64), which had the advantage of a more stable reagent than those devised for earlier methods (7, 10), was compared in an automated version (42) to the Scho-heimer–Sperry method (10) by using nonicteric serum standards as calibrators whose values had been determined by their own reference extraction procedure (42), it was proven that the results were reasonably similar, indicating that the direct automated method was very accurate indeed. However, after the Framingham studies were reported (195), the values of the serum standard for cholesterol for multiphasic systems were determined by the Abell et al. procedure (7, 196), a second reference procedure, and these values were then assigned to the lock-in controls. This value represented only some 85% of the color generated, which meant that an arbitrary subtraction of about 15% of the absorbance of the standard would then be applied to all patients’ samples. This standardization treatment leads to several interesting points to consider. Fifteen percent subtracted from any samples, could only roughly approximate the background of irrelevant absorption for all of them because pathological samples, unlike controls, are apt to be quite different one from the other in background absorbance characteristics. The major interferant in direct Liebermann–Burchard reactions, bilirubin, gives an interference equivalent to about 50–60 mg of serum cholesterol per liter for each 10 mg of serum bilirubin per liter (177). Therefore, 200 mg of bilirubin per liter should cause an absolute error of at least 1000 mg of serum cholesterol per liter. But that too would be corrected partly by the standardization step in which an absolute percentage is subtracted from all samples. As a consequence, one must use a suggested (197) corrective equation (bilirubin − 10 mg/liter) × 4 = cholesterol to be subtracted, in mg/liter. For 200 mg of bilirubin per liter, one would then subtract 760 mg/liter rather than 1000 mg/liter, and the error would appear smaller. To compound the confusion, investigators who developed new procedures and compared their values to those of the automated procedures might find that results of the compared procedures did not appear to be very far apart (5, 48) even though they were actually much further apart than they appeared to be because of the serum correction factor. Uncorrected jaundiced specimens, if compared, would obviously be worse if every 100 mg of bilirubin per liter appeared to be about 500 to 600 mg of cholesterol per liter in the automated procedures. This deliberate devaluation of a standard value in an effort to make results conform to those believed to be more nearly correct is confusing. It may be questionable, in fact, to assign a true value to a standard in which interfering and variable background colors, either static or dynamic in nature, are arbitrarily subtracted from that standard. There is an implied assumption here that all sera, normal or pathological, must have the same characteristics as the control with respect to irrelevant absorbances.

Assuming that the value for control serum can be established by the Abell et al. procedure, perhaps its true cholesterol value, it was also rather surprising to find values for a single multiphasic control serum stated to be 2100 mg/liter for the Liebermann–Burchard direct reaction and 1450 mg/liter for the enzymatic procedure, a difference of about 30%. If the Liebermann–Burchard reaction uses a value derived from the Abell et al. procedure and the enzymatic procedure gives the same values as the Abell et al. procedure (Technicon Lot No. B5J810) it is difficult to understand how the two stated values could be 30% apart.

**Reaction Mechanisms**

Undoubtedly, a main reason why the enzymatic procedures for cholesterol are more easily accepted than other techniques is that they are the best understood in terms of their reaction-sequence mechanism than are the methods that are based on either the Fe(III) or Liebermann–Burchard reactions. In the enzymatic procedures, every step in the entire sequence not only is clearly understood but the products of each sequence, including that of the quantifying equilibrium reaction, have been identified (1–6). Relatively few studies describe the mechanisms for the Liebermann–Burchard reactions (198–204) or the Fe(III) reactions (200, 205, 206). This is partly because it is difficult to define products that are relatively unstable and that defy easy isolation for characterization. It is also not clearly understood whether those products that represent the endpoint reactions of Liebermann–Burchard and Fe(III) procedures can exist in stable form when isolated from the milieu in which they are generated. In the Liebermann–Burchard reaction, if one waits until final color stability is achieved, the peak maximum of the final product spectrum is at 430 nm. From this finding, one might infer that the green product most frequently measured, which peaks at 625 nm, is an unstable intermediate whose structure might be postulated from that of the compound which it theoretically precedes as a final product.

Some attempts have been made to characterize the structures of the final products of the Liebermann–Burchard (201, 204) and Fe(III) reactions (200). The product of an L-B reaction has been isolated and reported (204). A recent quite elegant study on both mechanisms has been described, in which it was determined that the product of the Fe(III) reaction that absorbs maximally at 563 nm is a cholestetraenyl cation and the Liebermann–Burchard product which exhibits a peak maximum at 620 nm is a pentaenyl cation (200). In addition these investigators believe that both reactions involve similar oxidative mechanisms, which result in a series of cholestapolyenes. They also present evidence that the two colored species are “the corresponding enylic carbonium ions of the respective conjugated polyenes” (200).

**Phases of Procedure**

Even though the tendency of routine laboratories is toward direct determination of cholesterol as classified in IA above, mostly avoiding a serum blank, there are
still many reports in which the isolation procedures of the other analytical classifications are described. Indeed, two of the reference procedures (7, 10) include alkaline saponification and extraction while one of them (10) also uses digitonin for more nearly complete purification before color development. It is unlikely that all investigators will rely on direct systems or that every sample will necessarily be a uniform aqueous liquid such as serum, or even that sera are uniform in composition, including lipid concentrations and in vivo or in vitro interferences. Thus it would seem worthwhile first to discuss these preliminary phases of the analysis before discussing the means of final determination, the end-points.

Extraction. The primary reason for extraction is the partial purification of cholesterol before it is reacted in the extract (70) or in a residue of the extract (7). In other procedures this might be the first step in a multi-step purification scheme. For example, extraction might be followed by isolation as a precipitated derivative (10, 114) or by thin-layer chromatography (207, 208), column chromatography (209), or gas chromatography (210).

Saponification. Hydrolysis of the cholesterol esters to convert them to the free form has been resorted to for several reasons. It would be necessary to saponify the esters before extraction if the solvent of choice was not capable of quantitatively extracting esterified cholesterol (7). Moreover, free cholesterol and its esters may react at different rates or result in unequal molar absorptivities in the Liebermann–Burchard reaction (10, 194). If precipitation by digitonin (10) or tomatine (114, 211) is required for a purification step, or if the turbidity (or nephelometry) of the precipitate is to be what is finally measured (212, 213), or if ultraviolet measurement is the determinative device (214), then it might be necessary to saponify the esters of cholesterol.

Endpoint Devices
Most cholesterol procedures result in visible colors which have useful quantitative properties in that they obey Beer’s law over a wide analytical range. In the modern era, two reactions (12, 20, 21) and the many modifications of modifications of them have been a part of the profuse offerings during a relatively short time. Reviews describing the strengths and shortcomings of both color reactions are available (36–40). Other spectrophotometric procedures have been also suggested (11, 31, 215–218) along with physicochemical measurements (153, 219–221) that are resourcefully devised but have yet to achieve popularity. Of the latter, the electrometric approach involving enzymes as reagents appears quite promising and it will certainly be an available system to be offered in a compact instrument for measurement of peroxide generated by cholesterol oxidase in sequence with hydrolysis by cholesterol esterase. It will be unnecessary to add catalase, peroxidase, and color reagents coupled to those enzymes because the procedure can end with either the determination of the peroxide itself or the rate of oxygen uptake in the cholesterol oxidase step.

Liebermann–Burchard reaction. The Liebermann–Burchard reaction has the longest history of common use of all of the endpoint devices now available (20, 21). Reagent modifications have been suggested which alter reaction rates or color intensity achieved, by changing the ratio of acetic anhydride to sulfuric acid or the organic solvent in which the reaction takes place (109, 211, 222). In recent years, the stability of the reagent has been increased from hours (10) to weeks (64) by a change in reagent makeup. The inclusion of a high concentration of Na2SO4 or p-toluenesulfonic acid (9) resulted in reagents with longer shelf lives. The stabilities of the several similar reagents proposed popularized them as one-step direct procedures for use with mechanized instrumentations (42). However, because interferences were difficult to overcome with this type of method (66, 76, 82) and because of the confidence engendered by the advent of enzymatic approaches, use of this reaction is beginning to decline and it will soon probably disappear entirely. At present, its single advantage over enzymes is cost per test.

Iron reactions. Modifications of the original Fe(III) reagents (12), all of which resulted in more sensitive color reactions than the Liebermann–Burchard reactions and with color that was far more stable, enhanced the use of this reaction. Its sensitivity made it useful at very low concentration of cholesterol extracted from small amounts of tissue (84, 223) or with micro amounts of sample (68), and its fluorescent properties made it amenable to the determination of cholesterol in cerebrospinal fluid (224). Although, it is reported to be markedly affected by bilirubin, the error resulting from this interference is small when compared to this error with the Liebermann–Burchard reaction (76) and in fact is smaller than the error from bilirubin in some cholesterol oxidase procedures (176). However, the strong acids used, the viscosity of the final mix, reported errors from interferences—some valid (169, 170, 174) some not (225, 226)—and the advent and immediate acceptance of the enzymatic techniques undoubtedly will lead to disuse of this reaction in routine laboratories. Like the Liebermann–Burchard reaction, its major advantage is low materials cost, which could keep acid systems in use for screening procedures.

Enzyme reagents. When cholesterol reacts with cholesterol oxidase to generate a cholestenone and peroxide it provides several potential reaction possibilities, most of which have been exploited to some extent. The original procedure was quite clumsy and time consuming (227) and involved measurement of the disappearance of cholesterol by use of the Liebermann–Burchard reaction. Alternatively, the cholestenone generated by the action of a cholesterol dehydrogenase from a Mycobacterium was measured at 240 nm, or the 2,4-dinitrophenyldrazone of this ketone was measured at 390 nm (1, 4). Later, Flegg (4) also measured cholestenone in the ultraviolet at the wavelength of the peak maximum or alternatively, the 2,4-
dinitrophenylhydrazone was formed and measured. It was in this paper that the author suggested other ways of measurement if the reaction by the oxidase proved to be a peroxide generator. Once it was determined that peroxide did form, the way was open to treat the procedure in a manner similar to other analytes such as glucose, uric acid, and galactose, whose oxidase enzymes also generate peroxide during the reaction with their analytes. Thus colorimetry, fluorometry, and electrochemical detection became available for cholesterol, already having been established for the determination of the peroxide generated in other procedures. The primary thrust from the inception of available procedures has been to modify from one endpoint device to another, to subject those procedures to mechanized handling, and to study the interferences, many of which had already been investigated in the other techniques involving peroxide generating analytes.

Miscellaneous other endpoint reactions. There has been a great deal of interest in method development with respect to cholesterol and this has resulted in reactions for and modifications of numerous endpoint devices for the final step of cholesterol procedures. Aside from the Fe(III) and Liebermann–Burchard reactions, which some believe to be variations of the same reaction (200, 201), there has been a continuous stream beginning with gravimetric determination of the digi- tonide (30) and terminating for the moment in the enzymatic reagents that have already resulted in a myriad of variations for determination. In between, one can find the Tschugaeff–Bernouli reaction (11, 31, 228), turbidi- metric or nephelometric measurements (212, 213), carbohydrate determinations on the pentasaccharide chain of saponin derivatives (151, 229), metal reactions other than iron (126, 230, 231), an erythrocyte hemolysis technique (232), titrmetry (233), gasometry (234), oscillographic polarography (229), colorimetry by a differential thermal effect (125, 235), mass spectrometry or flame ionization following gas chromatography (136, 138), unesterified cholesterol by hemolysis inhibition by licensomycin (236), radioactivity (153), oxidation with perchloric acid-acetyl chloride in ethylene dichloride (217), ultraviolet spectrophotometry (214), reaction with o-pthalaldehyde (216, 237, 238), and oxidative charring (152). This list is by no means complete (39, 40) and other miscellaneous techniques for cholesterol exist. However, their use in hospital or research laboratories is negligible compared to Liebermann–Burchard, Fe(III), and enzymatic procedures.

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