ministration deserves the confidence and support of all physicians in coping with a difficult task.

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RELIABILITY OF BILIRUBIN DETERMINATIONS IN ICTERUS OF THE NEWBORN INFANT

In view of the increasing necessity for close collaboration between the clinical laboratory and the pediatrician who is faced with multiplying problems in management of diseases of the newborn, it may not be amiss for a clinical chemist to point out some of the limitations to the assistance he is called upon to render in chemical evaluation of jaundice.

Granting that symptomatic battles against increasing levels of bilirubin in the serum constitute the clinical objective in these cases, there appears to be a growing sensitivity of the pediatrician in his reaction to smaller and smaller changes between consecutive bilirubin assays. Although bilirubin determinations are perhaps the most notoriously unreliable of any in clinical chemistry, in situations where the pediatrician has a fair degree of confidence in the laboratory reports, it is my impression that he often goes overboard in demanding precision, riding the infant’s bilirubin “curve” as he would a broncho—every small buck is to be combatted with spurs, a hard bit and a platelet pack; each small subsidence is a victory. One can appreciate the squeeze such situations put upon him and his dependence upon any objective evidence he can find to help him. Although it is granted that clinical evaluations other than chemical results always contribute to his decisions, he can grossly mislead himself in a too-close reliance on the reported levels of bilirubin in the serum—often in the face of any reservations he may have about their reliability.

Two chief factors contribute to variance in the observed values when one is following the concentration of bilirubin closely: 1) In attempting to quantify the bilirubin in any given specimen, there is an irreducible range of variation in laboratory results themselves, even with the best laboratory support in the world; and 2) in a sick infant, who may actually be in bilirubin equilibrium for some hours, there may be appreciable variations in the absolute level of bilirubin from time to time.

ABSOLUTE ("TRUE") VALUES

It is probably safe to say that no laboratory in the country is prepared to state categorically what the true concentration of bilirubin in any given serum might be. The various forms in which bilirubin exists in serum give grounds for skepticism as to the means for standard comparison by either spectrometric or diazotization procedures.

Bilirubin standards are notoriously unreliable; until recently, the universal use of organic solvents in preparing them precluded any valid comparison with natural forms of bilirubin in serum, and free (reagent bottle) bilirubin is so insoluble that a wide variety of calibrations can be obtained by ostensibly identical methods of preparation. For example, in the classic method of preparation, chloroform stock-solutions are
prepared; these last perhaps a day or two before their apparent concentrations begin to decrease perceptibly, although in some laboratories they may be used for weeks. From these stock solutions the bilirubin must be teased into alcoholic dilutions for standardization. From methanol, particularly, the bilirubin begins to precipitate on the walls of the container in a matter of minutes, so that a factor obtained within 5 minutes will give lower (presumably more accurate) calculated values than one obtained 15 minutes later. The color values differ in methanol and ethanol media, and neither are identical with those in serum. As may be imagined, these facts have been the source of more difficulty and chemical profanity than any other single laboratory gremlin.

The availability of bilirubin standards in the form of "synthetic serums" (fairly easily prepared in the laboratory and somewhat more stable) has been of great value in increasing confidence in standardization of results with serum, since protein-binding has a significant effect on the results with both diazotization and spectrophotometric procedures, as shown by White and Duncan (Canad. J. M. Sc., 30:522, 1952). Thus the estimates may be considerably closer to actual concentrations than formerly, as exemplified by an experience in the Wilmington area, where results obtained in estimation of bilirubin in the various hospitals varied so widely from each other as to render chaotic any clinical interpretation of reports from more than one hospital. Even though methodology was quite similar and Malloy-Evelyn standards were prepared from the same commercial bilirubin, discrepancies of as much as 50% were observed in identical test specimens, until a uniform method of preparation of bilirubin standard in buffered albumin was adopted; whereupon the various results came within acceptable range of each other, and no particular complaints on this score have been heard from clinicians since.

REPRODUCIBILITY

The van den Bergh reaction cannot possibly be considered specific for bilirubin—indeed, it is rather surprising that it can be used at all in estimating the extremely low normal concentrations of that substance in serum—and its colored products (several forms of "azobilirubin") are not much more highly colored than the pigment itself. It is quite fortuitous that bilirubin is the major component in the chromogen fraction and that diazotization seems to proceed fairly consistently in methanolic solution with respect to protein-bound forms of free (indirect) and conjugated (direct) pigment, or at any rate so we at present assume. Still it is hardly a chemist's dream of an ideal quantitative reaction:

1) The extent of interference from heme-derived pigments and other unknown serum constituents of possibly variable concentration is as yet unelucidated, but hemolysis can and does depress the results in the van den Bergh reaction.

2) The "standard" Malloy-Evelyn reaction must be a delicate balance between release and determination of a colored product, on the one hand, and serum protein turbidities, on the other.

3) As alternatives to methanol, the use of other protein-releasing agents has not been uniformly successful, although turbidities may be overcome in this manner.

4) In any diazo reaction the determinable range requires fairly large multiplier factors in the calculation, and small variances in optical density (introduced by turbidity, cuvet blanks or photometer insensitivity) exert a large effect upon the results.

5) While one must ordinarily depend upon precalibration of the method used, diazo reactions do not produce consistent color densities from day to day.

6) With increasing demands for micro determinations on capillary samples of blood, the well known variance of these specimens from truly representative blood samples often introduces additional error.
With these sources of variation potentially present during any determination one must under the best of conditions accept a considerable variability in the results. For levels of bilirubin associated with jaundice, the author is at present quite satisfied with the laboratory staff if he can feel we are in the main hitting within 5% of an arbitrary "true" value (based upon our standards), with the realization that in any single determination this error might double.

CROSS-CHECKING

How can one determine the variation or precision of one's results in such a case? Shipping identical frozen specimens to the laboratories of one's friends in order to obtain a consensus may help, although the results may be disheartening, and while this practice may give some approach to the average values measured in one laboratory, it does not constitute any control over day-to-day variation. The only answer at present lies in checking results by the van den Bergh reaction with some form of spectroscopic determination.

Abelson and Boggs (Pediatrics, 17:452, 461, 1956) opened up this field for the pediatrician several years ago by measuring bilirubin spectrophotometrically and correcting for interference from hemoglobin; refinements in the technic have been introduced so that one may obtain a reference value for the bilirubin content of a serum, based upon presumably unvarying physical properties of the pigment, which may be used to check one's chemical results. We have been using a procedure based upon that of Abelson and Boggs for several years with quite satisfactory results, since it constitutes a cross-check of the degree of confidence with which our chemical results are to be trusted.

STABILITY OF LEVELS OF BILIRUBIN IN SERUM

Without greater technical precision than is currently available, it is difficult to estimate the extent of variations in the bilirubin in the serum from hour to hour. In an infant in bilirubin equilibrium (that is, with pigment entering the blood stream at the same rate it is being removed by the liver and tissues) the circulating pigment is temporarily locked in the plasma compartment by combination with albumin; any abrupt change in fluid status, hydration or dehydration, will obviously affect its concentration while not affecting its total quantity. How wide are these fluid shifts in the infant, even under closely-supervised management of hydration? In view of the exaggerated ratio of plasma water to body water in the newborn infant and its lability to fluid demand and supply, these shifts can at the very least be as great as the laboratory variance and have an equal chance of being added to them. Thus the evaluation of a "significant change" in levels in the serum must take into account the possibility that even under the best conditions of laboratory reliability and fluid management, it may require a shift between two consecutive estimations (say, 4 hours apart) of bilirubin concentration of the order of 15% to be significant of alterations in the pigment load. Repeated determinations can be helpful in showing consistent trends one way or another—if they are not read too closely.

SIGNIFICANCE

What significance, then, can be attached to any one bilirubin estimation? This of course depends upon the individual laboratory and upon the pressure to which it has been subjected in handling pediatric chemical determinations. Thanks to a rather large investment of time and effort and continuing study to the problem, our own results may in most cases be more consistent and possibly closer to "true" values than all of the preceding leads one to believe, but from some experience in following levels of bilirubin in the serum of infants for days at a time I am only too aware of the impossibility of pinning down the sources of the variations that do turn up if one checks and analyzes one's results often enough.
Our own modest gains have been self-defeating: our pediatricians have now given up their 3 or 4 mg/100 ml grains-of-salt with which (perhaps with good reason) they used to interpret our results, and are now reading significance in changes below 1 mg/100 ml. Although they are most reasonable and will readily admit that they do not rely on our results to this extent (pity the chemist who has to indoctrinate a clinical staff in the unreliability of his reports), they still betray evidences of influence by the increasing or decreasing written figure—or decimal. To consecutive reports of, say, 18.5 and 17.9 mg/100 ml, a not unusual response is: "Ah, coming down!"—when, since one could have been obtained on venous, and the other on capillary specimens of blood, the results could be guaranteed only to within 1 or 2 mg/100 ml each way. While they accept values of 1.7 and 1.9 mg/100 ml as being excellent checks, it is still difficult to get the point across that one cannot do better on a corresponding serum dilution for values 10 times these, and that under the best of conditions (within 5% accuracy) a reported value of 17 mg/100 ml has only a good statistical chance of being significantly different from one of 19 mg/100 ml.

The laboratory has undoubtedly boxed itself in by reporting concentrations to the first decimal, and at higher concentrations it might more properly issue reports such as: "12 to 13 mg/100 ml," or "18 ± 1 mg/100 ml." Perhaps if it reported results in milligrams of bilirubin per milliliter of serum, this particular difficulty would solve itself.

There are other indices in certain phases of the jaundice problem that may be of help. In problems possibly involving a defect in conjugation or elimination, an occasional quantitative determination of direct-reacting bilirubin is indispensable. In hyperbilirubinemia of hemolytic origin, estimation of the heme-pigment or hemoglobin level itself is of value in predicting the pigment load that will soon be presented as bilirubin. Owing to the possibility of mechanical hemolysis, this is rather risky—repeatedly high levels of hemoglobin are an unfavorable, and at least one low level of hemoglobin is a favorable prognostic sign in these cases. But the over-riding problem will for some time continue to be the interpretation of the level of bilirubin itself.

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Appendix

Because some of the methodology cited is scattered or not readily available in the literature, several recent developments which we have found most useful have been appended for evaluation in the reader's own laboratory.

White's Bilirubin Standard

The best bilirubin standard for spectrometric and diazo methods is one made in aqueous protein solutions. A satisfactory standard may be made up in a clear, analyzed pooled serum; a more convenient one was suggested by Dr. David White. A "synthetic serum" is made by diluting 10 ml of 30% human serum albumin (Cutter Laboratories' buffered preparation) to 100 ml with M/15 phosphate buffer at pH 7.4. The desired quantity (20.0 mg) of bilirubin (Hoffman-LaRoche) is weighed out to 0.1 mg and dissolved by rubbing up in 3 to 4 ml of 0.01N NaOH. When completely dissolved this is washed quantitatively into a 100-ml volumetric flask with the "serum." Small aliquots of this standard are tubed, stoppered and frozen; the refrigerated standard is stable for a day or two, the frozen standard keeps about a month before its color reactions show appreciable change. For calibration curves, appropriate dilutions of the thawed standard are made in the buffered albumin "serum."

Spectrophotometric Determinations of Elevated Levels of Bilirubin

Bilirubin shows a broad absorbance with the maximum in the neighborhood of 457 m$. This reading may be interfered with by the intense Soret band of hemoglobin at about 412 m$ (45 m$ lower than the bilirubin peak) which spreads over much of the bilirubin range. At 457 m$, the absorbance of hemoglobin is one-
ninth that at its own 412 mÅ peak, and this interference may be subtracted from the bilirubin reading at 457 mÅ. White and associates (Clin. Chem., 4:211, 1958) correct for the hemoglobin at another wave length, but since it is the Soret band that does the interfering, we have preferred following the original Abelson-Boggs suggestion, although the corrections and calculations have been modified to give much better correlation with diazo values—for the most part within 5% for sera from jaundiced individuals.

A 1:51 dilution of clear, cell-free serum in 0.9% saline solution is read in a good spectrophotometer at a fairly sharp nominal bandwidth; the Model B Beckman spectrophotometer is perhaps most convenient, but less discriminating instruments cannot be used. Readings of optical density (O.D.) through 1.0-cm rectangular cuvets are made at 412 mÅ and at 457 mÅ—the exact wavelengths of the peaks for hemoglobin and bilirubin are determined for one’s own instrument. Total bilirubin concentration is given by the following calculation:

\[
\text{Total bilirubin (mg/100 ml)} = \left[ \frac{\text{O.D.}_{457} - \text{O.D.}_{412}}{9} \right] \times \text{Factor}
\]

Besides the correction for hemoglobin in the second term, a correction for the bilirubin reading at 412 mÅ (about 80% of the 457 mÅ maximum) is also incorporated into the factor, which is most simply obtained by using the equation backwards with known bilirubin standards; the value of the factor with our instruments and technic is 74. The procedure is simple and easily adaptable to micro determinations: 50 µl of serum are diluted in 2.5 ml of saline solution, and glass beads or plastic inserts are used in the cuvets to bring the solution into the light beam. Hemoglobin may be estimated similarly from the same two readings, if the saline dilution has been completely oxygenated by gentle mixing with oxygen:

\[
\text{Hemoglobin (mg/100 ml)} = \left[ \text{O.D.}_{412} - (0.8 \times \text{O.D.}_{457}) \right] 	imes 700
\]

Micro van den Bergh Determinations of Elevated Levels of Bilirubin (above 5 mg/100 ml)

Aside from the indispensability of a protein-stabilized bilirubin standard, accurate results with diazo procedures depend upon: 1) increasing the concentration of reagent to improve linearity and speed up the reaction; 2) liberating or dissolving the unconjugated bilirubin from its associated albumin in order to complete its reaction; and 3) preventing turbidities with alcoholic diluents. Complete reaction of all of the protein-bound bilirubin cannot be effected by the ordinary caffeine or urea and benzoate mixtures, and methanol is ordinarily employed. The protein turbidities encountered with final concentrations of 50% methanol can be practically eliminated by the use of final concentrations as low as 40% with slight decrease in color values.

Many filter photometers may be modified for the use of small aliquots of sample. For those in which 4 ml can safely be read through a light path of around 1 cm, the following method is recommended, although the technic may be scaled down for 10-mm micro cuvets. To 2.0 ml of fresh 2% diazo reagent (a mixture of 1 volume of 0.5% NaNO₂ with 20 volumes of 2% sulfanilic acid in 0.2N HCl) is added 0.10 ml of serum and, with mixing, 2.0 ml of 90% methanol. After exactly 10 minutes at room temperature, the optical density is read at a wavelength near 570 mÅ (560 to 580 mÅ filters are satisfactory). A serum blank run similarly in 2.0 ml of 1% sulfanilic acid plus 2.0 ml of methanol is subtracted. A “direct” reaction may be run by adding 0.1 ml of serum to 2.0 ml of diazo reagent plus 2.0 ml of water. Calibration with suitable dilutions of White’s standard gives the factor by which corrected serum readings are multiplied, and the results are linear to about 25 mg/100 ml. For the Coleman instrument with 16-mm cuvets, the volumes of diazo reagent and methanol are increased to 3.0 ml.
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