Extraction of Unconjugated Bilirubin from Serum

To the Editor:

Royer and Ko (1) reported a system for extraction of triglycerides from serum, based on the heptane-isopropanol method of Dole (2). Royer and Ko substituted nonane for heptane and altered the proportions. The nonane and isopropanol divide into two phases, with the nonane forming the upper phase, when dilute sulfuric acid is added. In working with their method, we observed that when extractions were performed on sera containing bilirubin in abnormally high concentrations, bilirubin was present both in upper and lower phases, while that from the sera of normal individuals was predominantly in the upper phase. These observations led to the supposition that a mixture of nonane and isopropanol might also serve as a solvent fractionation method for bilirubin.

If the volume of acid is increased three times over that suggested by Royer and Ko, more bilirubin is extracted into the upper layer because more nonane is separated from the alcohol–water mixture. In the case of normal sera the bilirubin is then consistently all present in the upper (nonane) phase. As a further experiment, extraction of the bilirubin from the serum of a patient in sickle cell crisis into the nonane–isopropanol mixture, followed by phasing with the three volumes of dilute sulfuric acid, showed virtually all of the bilirubin to be in the nonane phase.

The upper layer of the two phases consists of nonane and the lower layer of isopropanol, water, and acid. If the concentration of the acid is increased (e.g., to 1 or 1.5 mol/liter), the bilirubin in the lower phase, assumed to be conjugated bilirubin, undergoes rapid oxidation. That in the upper phase shows no indication of oxidation, even though the serum bilirubin may have been partially oxidized to biliverdin before extraction. This is presumably due to two factors: (a) the absence of acid in the upper phase and (b) the greater resistance of unconjugated bilirubin to oxidation (3).

Extraction of the bilirubin (all of it in the unconjugated form) from a commercial control containing 20 mg/dl in a 6 g/dl solution of human albumin (DADE Bilirubin Control) indicates that the solubility of unconjugated bilirubin in nonane is only about 5–7 mg/dl, as determined by making dilutions of the control with the albumin solution.

All testing was done with the 2:3.5 proportions of nonane–isopropanol described by Royer and Ko, but the results indicate that changing the proportions may make it possible to extract a greater concentration of unconjugated bilirubin into the nonane phase while still retaining enough isopropanol in the lower (water–alcohol–acid) phase to accommodate the conjugated bilirubin in large concentrations.

At this point various proportions have not been tested, nor has an attempt been made to develop a method for determination of bilirubin concentration. It would seem logical to assume that an oxidation method would be preferred over that of acid diazotization. However, for purposes of determining the predominant fraction, or the approximate relative proportions of the two fractions, visual observation is adequate.

References

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On Iodine Calibration Curves

To the Editor:

A minor controversy has recently arisen over the method of preparation of calibration curves in iodine determinations (1–3). Although the matter has received attention in clinical chemical circles, some doubts still exist as to the function to be plotted (4), and an explanation of the reasons for making the choice seems to be desirable.

The reaction involved is the iodide-catalyzed reaction between cerium(IV) and arsenic(III) in sulfuric acid solution:

$$\text{As(III)} + 2 \text{Ce(IV)} \rightarrow \text{As(V)} + 2 \text{Ce(III)} \quad (1)$$

In the absence of iodide the reaction proceeds extremely slowly (5). Traces of iodine increase the speed of the reaction enormously, and the rate has been found to be directly proportional, or nearly so, to the iodine concentration, provided that other conditions, such as the ratio of [As(III)] to [Ce(IV)] (brackets indicate concentration) and the temperature used in clinical determinations, are ideal and constant (5, 6). In other words, if the rate constant for reaction 1 without catalyst is $k$, the rate constant of the reaction when the catalyst is added at a concentration of iodide $[I^-]$ would be $k_{1-1}$ in the rate expression:

$$-d[\text{Ce(IV)}]/dt = k_{1-1}[\text{Ce(IV)}] = (n[I^-] + k)[\text{Ce(IV)}] \quad (2)$$

where $n$ is a proportionality constant that can be determined experimentally for a given set of conditions, and is about $10^{-1}$ s$^{-1}$ at [Ce(IV)] = $7.5 \times 10^{-4}$ mol/liter and [As(III)] = $1.62 \times 10^{-3}$ mol/liter in 0.5 molar sulfuric acid medium, for example (6). Since the noncatalyzed reaction is many orders of magnitude slower than the catalyzed reaction (5), $k$ in Equation 2 is negligible and, from Equation 2,

$$k_{1-1} = n[I^-] \quad (3)$$
It should here be pointed out that the rate expression in Equation 2 implies that the catalyzed reaction is a pseudo-first-order reaction in Ce(IV). The reaction is a complex one, but it is practically first-order in Ce(IV) under most experimental conditions studied (6, 7), although it is not a real first-order reaction as has sometimes been claimed (6). It should further be pointed out that expression 3 can be derived from Rodriguez and Pardue's (6) rate expression by approximation if [As(III)] \gg [Ce(IV)] and that [As(III)] can be considered constant in the reaction.

In actual chemical analysis for iodine we determined the value \(-\frac{d[Ce(IV)]}{dt} = k_{1-1}[Ce(IV)]\) is convenient for constructing a concentration-time curve and measure the slopes at different [I\(^-\)] used. If a colorimetric method is used to measure [Ce(IV)], then the absorbance, A, should be used, because the absorbance at 410 nm as a function of [Ce(IV)] obeys Beer's law when sera are being analyzed (8).

The method of integration is more convenient than the differential method, and it is adopted in most clinical laboratories for determining iodine.

In the method of integration, the expression

\[
\frac{d[Ce(IV)]}{[Ce(IV)]} = k_{1-1}dt
\]

is integrated as follows:

\[
\int_{[Ce(IV)]}^{[Ce(IV)]} \frac{d[Ce(IV)]}{[Ce(IV)]} = \int_{t_i}^{t_f} k_{1-1}dt
\]

\[
ln[Ce(IV)]_f - ln[Ce(IV)]_i = k_{1-1}(t_f - t_i)
\]

In a given measurement [Ce(IV)]\(_i\) and (t\(_f\) - t\(_i\)) are constant. Let a = ln[Ce(IV)]\(_i\) and b = (t\(_f\) - t\(_i\)). For a given set of experimental conditions, we then have

\[
a = a - b[n(I^-)]m
\]

Since the concentrations [Ce(IV)]\(_i\) are proportional to the A's in the measurements, we can combine expressions 3 and 5 to give

\[
ln A = (a - bn[I^-])m
\]

or

\[
log A = a' - b'[I^-]
\]

where m = ln A/[ln[Ce(IV)]\(_b\), a' = ma'/2.303 and b' = mbn/2.303. It is then obvious that a plot of log A (or ln A) against [I\(^-\)] should give a straight line (7) having a slope of -b'.

In practice, almost all spectrometers are built to measure either % T or A. Because it can be shown (Figure 1) that in the range of 20% T to 60% T, where most clinical measurements are done, % T (or to a less extent A) is a good linear approximation of log A; % T's or A's therefore have been used in most established clinical determinations of iodine. It is assumed that these approximations introduce no error of medical significance. Results when % T is used are reportedly little different from those when log A (or ln A) is used (4). In such work, when both % T and A values are used in calibration and are compared for the same samples, the results differ about (or less than) 1 \(\mu g/100\) ml in samples with an iodide concentration ranging from 2 to 7 \(\mu g/100\) ml (3).

To verify that A's provide values of significantly improved accuracy in clinical iodine determination, we measured iodine in a series of aqueous solutions of potassium iodate and serum PBI in commercial controls with a Technicon PBI AutoAnalyzer, by Technicon's N-56 methodology. The potassium iodate was "Baker Analyzed" reagent (J. T. Baker Chemical Co., Phillipsburg, N. J. 08865). The controls were "IODO-TROL" (assayed value 3.0 \(\mu g\) of I\(^-\) per 100 ml) and "IODO-TROLL" (5.1 \(\mu g\) of I\(^-\) per 100 ml) supplied by Dade Division, American Hospital Supply Corp., Miami, Fla. 33152. Linearity is better when log A values are used, especially so when data outside the 20% T to 60% T range are included (Figure 2). The relation between % T, A, and log A follows the theoretically predicted pattern. The controls measured have values as shown in Table 1. All of the results are satisfactory owing to the fact that measurements were restricted to the optimum range of 20% T to 60% T. Outside this range, the approximations involved in the use of % T or A are unjustified.

We thank Dr. Alan D. Westland of University of Ottawa and Dr. Cooper H. Langford of Carleton University for reading the manuscript and making suggestions on the publication of this letter.

Table 1. Values for Iodine Concentration (\(\mu g/100\) ml), Expressed in Various Ways

<table>
<thead>
<tr>
<th>Standard</th>
<th>Functions Values</th>
<th>A ((\mu g/100) ml)</th>
<th>%T</th>
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<tr>
<td>IODO-TROL.L</td>
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<td>A</td>
<td>%T</td>
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<tr>
<td></td>
<td>A</td>
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<td>5.0</td>
</tr>
</tbody>
</table>

References

2. Clinical Newsletter 2, 3 (1972), BIO-RAD Laboratories, Richmond, Calif.

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