CLIN. CHEM. 26/1, 22-25 (1980)

Mechanism of Interference by Hemoglobin in the Determination of Total Bilirubin. I. Method of Malloy–Evelyn

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Oxyhemoglobin is the species of hemoglobin in erythrocyte hemolysates that inhibits the diazo reaction. Ferric hemoglobin derivatives and species with relatively low molecular mass do not interfere. Conversion of oxyhemoglobin to acid hematin under assay reaction conditions is associated with rapid destruction of bilirubin, which accounts for the diazo reaction error. The most probable mechanism for this destruction of bilirubin is an oxidative reaction involving H_2O_2, formed in the oxidation of hemoglobin, and acid hematin acting as a pseudoperoxidase. We could find no evidence for other mechanisms of interference such as spectral error or azobilirubin destruction. Addition of potassium iodide, 4.0 mmol/L final concentration in the reaction mixture, eliminates interference from hemoglobin added to give concentrations as great as 10 g/L. It also eliminated the effects of hemolysis in the method of Ertingshausen et al. (*Clin. Chem.* **19**:1366, 1973), in which ethylene glycol is used as the accelerator.

Additional Keyphrases: *variation, source of · methemealbumin*

That hemolysis interferes in the Malloy–Evelyn assay (*1*) of bilirubin is well documented (*2–4*), but the mechanism of this interference is poorly understood. Michaëlsson (*2*) made a detailed study of the effect of hemolysis on the Malloy–Evelyn assay. The error was attributed mainly to "destruction" of bilirubin in the dilutions of serum with methanol before diazo reagent was added. A small portion of the error was caused by increased sample blanks and azobilirubin destruction.

Conclusive evidence is lacking that hemoglobin alone causes the interference with assay of bilirubin in hemolyzed serum. Glutathione reportedly inhibits the diazo reaction (*5*), indicating the possibility of other interfering species.

Several reports show that spectral error does not account for the interference caused by hemolysis (*6, 7*). Brody et al. (*6*) have recently suggested that the interference is chemical in nature.

Engel (*8*) proposed that bilirubin is oxidized by oxyhemoglobin when the diazo reaction is conducted at low pH. Saturation of whole-blood samples with CO plus the addition of ascorbic acid before diazotization eliminated interference from hemoglobin. More recently, Ichida and Nobuoka (*9*) found that converting oxyhemoglobin to carboxyhemoglobin greatly reduced the error caused by hemolysis in the Malloy–Evelyn assay.

Ferric heme derivatives are known to form from oxyhemoglobin during the diazo reaction (*6*). However, Kapitulnik et al. (*10*) found no interference by methemoglobin or methemealbumin in the assay of amniotic fluid bilirubin by the Malloy–Evelyn method.

Here we present data that identify oxyhemoglobin alone as the interfering species in hemolysates and support a mechanism to account for interference by oxyhemoglobin.

Materials and Methods

Instrumentation

Absorbances and spectral absorbance scans in the Malloy-Evelyn assay were obtained with a Model 240 spectrophotometer with wavelength scanner (Gilford Instruments, Oberlin, OH 44074).

The method of Ertingshausen et al. (*11*) was performed with a Rotochem IIA/36 centrifugal analyzer (American Instrument Co., Silver Spring, MD 20910).

Reagents

Sulfanilic acid, 1.0 g/L in 0.18 mol/L HCl.

Sodium nitrite, 5.0 g/L. Prepare a fresh solution weekly.

Methanol, absolute.

Working diazo reagent. Mix 10 mL of sulfanilic acid reagent with 0.3 mL of sodium nitrite. Use within 1 h.

HCl, 0.18 mol/L.

Total bilirubin reagent kit. Reagents for the method of Ertingshausen et al. (*11*) were purchased from Union Carbide, Rye, NY 10580. Each vial of stabilized diazo sulfanilic acid is reconstituted with 9.5 mL of the ethylene glycol/HCl diluent provided.

Bilirubin samples. Dade bilirubin control (Dade Reagents, Miami, FL 33152) was used throughout. The bilirubin concentration was 206 mg/L in human serum albumin, 55 g/L. Dilutions for standard curves were made with human serum albumin, 55 g/L, from Dade.

Other materials. Human methemoglobin, bovine hemin chloride, and reduced glutathione were from Sigma Chemical Co., St. Louis, MO 63178. Oxyhemoglobin was prepared by reducing a stock solution of methemoglobin with sodium dithionite, followed by dialysis against distilled water and equilibration with air. Hemolysate was prepared by washing erythrocytes from heparinized blood three times with saline, 85 g/L, and adding to the packed cells an equal volume of distilled water. These materials were used to prepare stock solutions, which were added in known amounts to aliquots of bilirubin samples and to human serum albumin, 55 g/L. Hemoglobin concentration in stock solutions was assayed with a Coulter Model S (Coulter Instruments, Hialeah, FL 33010).

Low M_r fraction of erythrocyte hemolysate. One volume of freshly prepared hemolysate was dialyzed against three volumes of distilled water for 3 h. The dialysate was used to

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Presented by B.C.S. in partial fulfillment of requirements for the M.S. degree, Department of Medical Technology, SUNY at Buffalo.

An account of experiments related to the method of Jendrassik-Grof is described in the accompanying article in this issue.

Received June 26, 1979; accepted Sept. 13, 1979.

check for interference from components of low relative molecular mass.

Procedures

Malloy–Evelyn assay. The assay was run by mixing 50 μL of sample, 950 μL of H_2O, 200 μL of diazo reagent, and 1.2 mL of methanol. After 30 min, absorbances were read at 540 nm. Sulfanilic acid solution replaced working diazo reagent in the sample blank tubes. The effect of preincubation in neutral methanol was studied by adding diazo reagent at various times after the addition of methanol. Similarly, the effect of preincubation in acidic methanol was studied by adding 200 μL of 0.18 mol/L HCl in place of diazo reagent, then methanol; then, after various periods of time, 200 μL of diazo reagent was added. Under these conditions acidity was doubled and final methanol concentration decreased by 4% over the usual concentration in the final reaction mix. In both these experiments the diazo yield was compared with 0 and 10 g/L of added hemolysate.

Spectral absorbance scans. Spectral absorbance scans from 440–640 nm of samples in neutral or acidic methanol were read against a reagent blank. The change in bilirubin concentration was considered a function of the slope of the plot at 490 nm, estimated by the difference in absorbance at 485 and 495 nm (*12*). Formation of methemoglobin in methanol dilutions was monitored by changes in the ratio of absorbance at 576 and 540 nm, $A_{576/540}$. In 500 mL/L methanol the ratio for oxyhemoglobin was 1.04, which decreased to 0.698 upon complete conversion to methemoglobin.

Measurement of bilirubin by difference spectroscopy. Bilirubin was measured directly by reading the absorbance of bilirubin at 455 nm (A_{455}) against a reference from which bilirubin was omitted (sample with hemolysate added to human serum albumin). The stability of bilirubin in neutral and acidic methanol was determined by recording the A_{455} with time. These data were compared with the diazo yield after preincubation under the same conditions.

Method of Ertingshausen et al. (11). Using the Rotofill, we loaded transfer discs with 50 μL of sample and 100 μL of distilled water to the inner well, and 500 μL of reagent to the middle well. A_{550} was read after 5 min. Ethylene glycol/HCl diluent replaced working reagent in sample blank runs. KI, 50 mmol/L, was used instead of distilled water as the sample diluent to study the effect of a reducing agent on hemolysis interference.

Results and Discussion

Interfering Species

The decrease in test absorbance in the Malloy–Evelyn assay was linearly related to concentration of added oxyhemoglobin up to 10 g/L (Figure 1). Regression lines relating test absorbance to hemoglobin concentration did not differ significantly, $p > 0.05$, whether hemolysate or the oxyhemoglobin preparation was added. Added methemoglobin, to 10 g/L, or methemealbumin, to 11 g/L, expressed as hemoglobin equivalent, did not interfere with the assay, confirming observations made by Kapitulnik et al. (*10*). Reduced glutathione, up to 100 mg/L, did not interfere. Dialysate from hemolyzed erythrocytes, when used in place of distilled water to make the initial serum dilution, did not interfere. All these experiments demonstrate that oxyhemoglobin is the interfering species in hemolysates.

Nature of Hemoglobin Interference

Addition of hemolysate significantly, $p < 0.05$, depressed the slope of the standard curve, but the intercepts were not significantly different from 0 ($p > 0.05$, Figure 2). The absorbances of oxyhemoglobin in the test and blank, measured with samples of hemolysate alone in human serum albumin, were identical. These data indicate that interference by oxyhemoglobin is chemical rather than spectral in nature, in agreement with the recent report of Brody et al. (*6*).

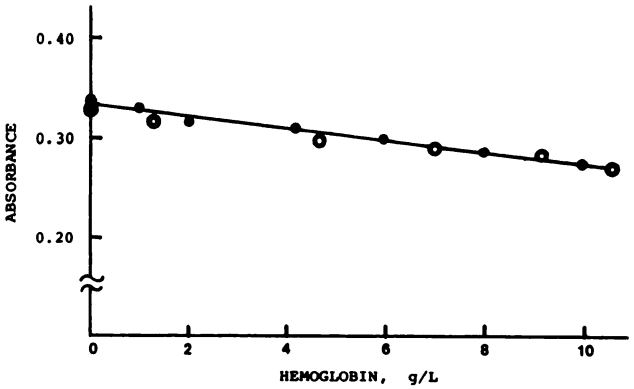

Fig. 1. The effect of hemolysate (●) and oxyhemoglobin (O) on the Malloy-Evelyn assay

Bilirubin concentration was 187 mg/L. Absorbance data were obtained at 540 nm

Repeated absorbance readings 30 to 90 min after initiation of the reaction were stable with added hemoglobin up to 10 g/L. This differs from the findings of Michaëlsson (2) that azobilirubin fading and increasing sample blanks contribute to the error caused by hemolysis.

Effect of Hemolysate on Bilirubin Stability

In neutral methanol, bilirubin decreased over time when hemolysate was added (Figure 3). Spectral absorbance scans, as illustrated in Figure 4, demonstrated the progressive formation of methemoglobin, and the destruction of bilirubin was highly correlated, $r = 0.97$, with methemoglobin formation. Because ascorbic acid stabilized bilirubin in the presence of hemoglobin, an oxidation reaction may be responsible for the observed loss of bilirubin. Diazo yields for the same samples preincubated for various times in methanol before adding diazo reagent showed a parallel decrease, suggesting that the destruction of bilirubin accounts for the diazo reaction error caused by hemolysis.

A very rapid loss of bilirubin occurred in acidified methanol with added hemolysate; this was also prevented by the addition of ascorbic acid (Figure 5). Spectral absorbance scans, as illustrated in Figure 4, demonstrated that acid hematin

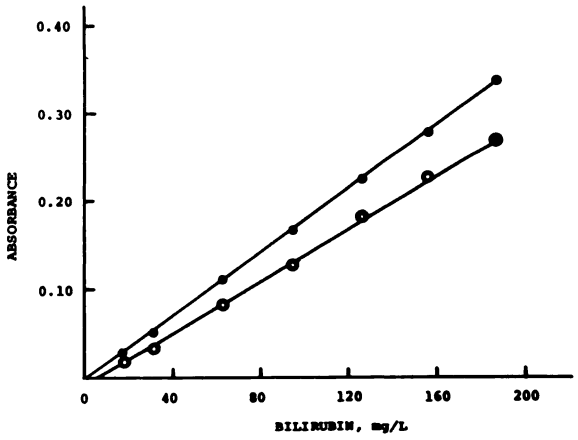

Fig. 2. The effect of added hemolysate on standard curves in the Malloy-Evelyn assay

No hemolysate, ●; 10 g/L hemolysate added, O. Absorbance data were obtained at 540 nm

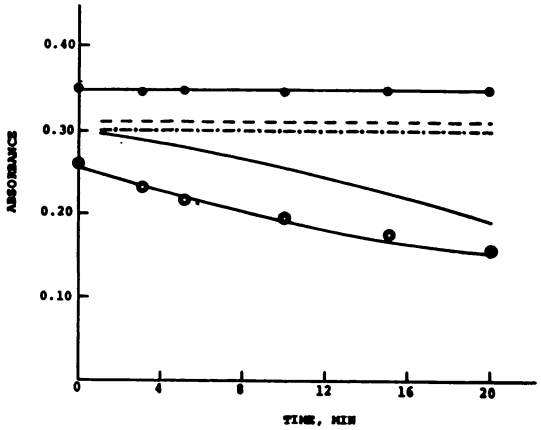

Fig. 3. The effect of hemolysate on the stability of bilirubin in neutral methanol

Diazo reaction initiated after preincubation in neutral methanol for the times indicated: ●—●, no hemolysate; O—O, with added hemolysate, 10 g of hemoglobin per liter. A_{455} for bilirubin in neutral methanol was recorded against a reference from which bilirubin was omitted: - - -, no hemolysate; ——, with added hemolysate, 10 g of hemoglobin per liter; — · — · —, with added hemolysate, 10 g of hemoglobin per liter and ascorbic acid, 10 mmol per liter.

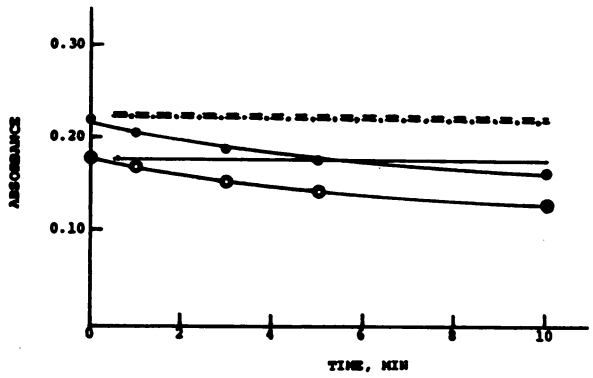

Fig. 5. The effect of hemolysate on the stability of bilirubin in acidic methanol

Diazo reaction was initiated after preincubation in acidic methanol for the times indicated. A_{455} for bilirubin in acidic methanol was recorded against a reference from which bilirubin was omitted. Symbols as in Fig. 3

formed within 30 s and that oxidation of bilirubin also occurred within this time. Without added hemolysate, the diazo yield decreased as the preincubation time increased, probably because the increased acidity in this experiment inhibited the diazo reaction. The error caused by hemoglobin in the diazo reaction was constant and independent of preincubation time, suggesting that the interference occurs rapidly and early in the diazo reaction.

The destruction of bilirubin in acidic methanol was linearly related to hemoglobin concentration (Figure 6). The slope of the line relating A_{455} to the hemoglobin concentration did not differ significantly ($p > 0.05$) from the slope relating diazo reaction absorbance to hemoglobin concentration. These data directly demonstrate that bilirubin destruction is responsible for the diazo reaction error caused by hemolysis. The extremely rapid loss of bilirubin in acidic methanol accounts for the apparent independence of hemolysis interference on the time of preincubation in acidic methanol.

Effect of Reducing Agents on Hemolysis Interference

Ferrous sulfate, 4.0 mmol/L final concentration, partially stabilized the diazo reaction, whereas ascorbic acid completely stabilized bilirubin (Figure 6). KI, 4.0 mmol/L final concentration, not only stabilized bilirubin but also prevented the diazo reaction error in as much as 10 g of added hemoglobin

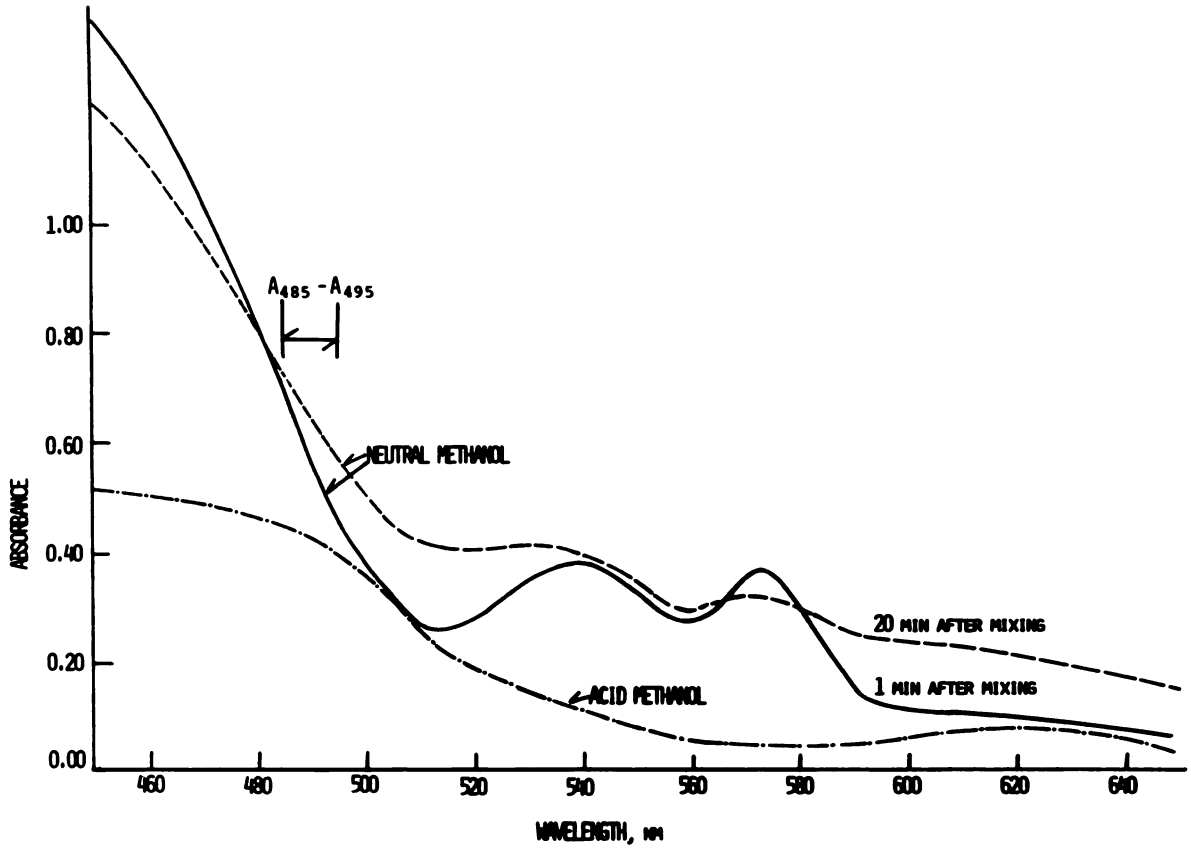

Fig. 4. Spectral absorbance scans of a bilirubin sample with added hemolysate

Bilirubin, 186 mg/L. Hemoglobin, 8 g/L. Scans were taken in neutral or acidified methanol at various times after mixing

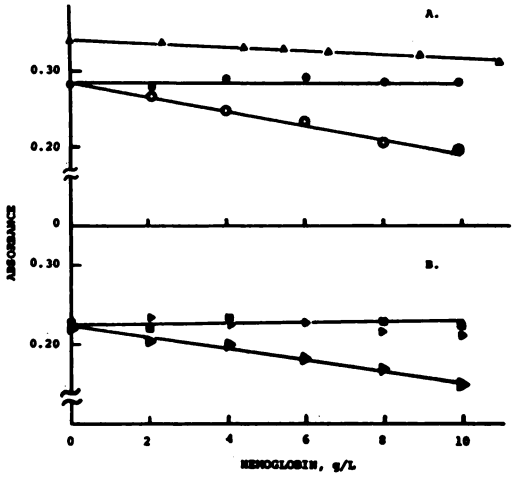

Fig. 6. Comparison of diazo-reaction absorbance and bilirubin absorbance in acidified methanol with added hemolysate and various reducing agents

A. Diazo reaction: ▲—▲, $FeSO_4$, 4 mmol/L, added; ●—●, KI, 4 mmol/L, added; O—O, no reducing agent added. *B.* A_{455} read after 1 min: ▶—▶, KI, 4 mmol/L, added; ■—■, ascorbic acid, 10 mmol/L, added; ▷—▷ no reducing agent added

per liter. This further indicates that destruction of bilirubin accounts for the interference caused by hemolysis, because preventing bilirubin destruction also eliminates diazo reaction error.

The effect of hemolysis on the method of Ertingshausen et al. (*11*) was studied briefly. Addition of hemolysate, up to 8 g/L, to a sample containing 160 mg of bilirubin per liter, caused a decrease in absorbance that was proportional to hemolysate concentration. The regression line relating test absorbance, A, to hemoglobin concentration was $A = 0.7401 - 0.0331$[Hemoglobin, g/L]. KI, 7.7 mmol/L final concentration, completely eliminated this interference.

Proposed Mechanism

Conversion of oxyhemoglobin to ferric derivatives may proceed with the formation of H_2O_2 under denaturing conditions (*13*). The formation of an O–O bridge between two heme groups, a reaction that is sterically hindered in the native conformation (*14*), leads to the formation of H_2O_2 and ferric heme.

In an additional experiment, direct addition of hydrogen peroxide to bilirubin samples in aqueous methanol did not result in bilirubin oxidation. However, the subsequent addition of methemoglobin to the reaction mixture caused very rapid oxidation of bilirubin, although methemoglobin alone did not oxidize bilirubin. Bilirubin is known to be oxidized by hemoglobin and H_2O_2 (*15*). This mechanism could explain the observed dependence of the rate of bilirubin destruction on the rate of ferric heme formation.

KI, which is known to reduce H_2O_2 (*16*), prevents the diazo reaction inhibition caused by hemoglobin. Spectral absorbance scans revealed the formation of iodine when hemoglobin was present in the Malloy–Evelyn assay, which directly confirms the antioxidant effect of iodide ion. KI did not rapidly reduce the working diazo reagent, as ascorbic acid does, and appears useful in preventing interference by hemoglobin in the methods of Malloy–Evelyn and Ertingshausen et al. (*11*).

The proposed pseudoperoxidase oxidation of bilirubin is very similar to the original proposal made by Engel in 1939 (*8*), who stated that oxyhemoglobin causes the oxidation of bilirubin. The proposed mechanism of oxidation is consistent with the observation that hemoglobin catalyzes the oxidation of bilirubin with H_2O_2 but not O_2 as the oxidant (*15*).

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