Elimination of the "Chromogen Oxidase" Activity of Bilirubin Oxidase Added to Obviate Bilirubin Interference in Hydrogen Peroxide/Peroxidase Detecting Systems

G. A. Maguire

The use of bilirubin oxidase to remove interference by bilirubin in hydrogen peroxide/peroxidase detecting systems is hampered by its inherent "chromogen oxidase" activity (its ability to oxidize the chromogens used in the systems). This unwanted activity is >99% inhibited by 0.5 mmol/L cyanide, 97% inhibited by 20 mmol/L azide. At these same concentrations, they inhibit bilirubin oxidase activity by 95% and 73%, respectively. Sequential addition of reagents allows the use of bilirubin oxidase without interference by the chromogen oxidase activity.

Many assay methods are based on the detection of hydrogen peroxide by the Trinder detection system (1), peroxidase (POD; EC 1.11.1.7) catalyzing the reaction of H2O2 with 4-aminophenazone and phenol (or a phenol derivative) to form a highly colored quinone-imine dye. Unfortunately, other substances capable of being oxidized by POD/H2O2 (e.g., bilirubin) may be present and interfere, especially in methods for detection of substances present in relatively low concentrations, such as creatinine. Addition of ferrocyanide to the reagent diminishes (2) but does not eliminate (3) the interference, whereas use of bilirubin oxidase (BOX; EC 1.3.3.5) eliminates the interference (3).

A major drawback of BOX is that it will itself catalyze the oxidation of the chromogen system, acting as a "chromogen oxidase" in the absence of POD/H2O2 and producing a large reagent-blank absorbance. In the course of developing a colorimetric assay for serum 5'-nucleotidase (manuscript in preparation) based on the production of H2O2, I found that bilirubin interferes substantially. Before I could use BOX to eliminate this interference, it was necessary to find reaction conditions under which the chromogen oxidase activity of BOX was minimal. Experiments that achieved this objective are described.

Materials and Methods

Materials. BOX was purchased from Amano Pharmaceutical Co., 2-7, 1-chome, Nishiki, Naku-ku, Nagoya 460, Japan. All other enzymes were from Sigma (London) Chemical Co., Ltd., Poole, Dorset, U.K. All chemicals were "Analar" grade, from BDH Chemicals Ltd., Poole, Dorset, U.K. Tri bromohydroxybenzoic acid was synthesized as described by Trinder and Webster (4).

Instruments. For all experiments I used either of two centrifugal analyzers—a Multistat III (Instrumentation Laboratory (UK) Ltd., Warrington, U.K.) with a red filter fitted to the heating lamps or a Cobas Bio (Roche Products Ltd., Welwyn Garden City, Herts., U.K.).

Bilirubin solution. Bilirubin was dissolved in 1 mmol/L sodium hydroxide to give a concentration of 1000 mmol/L and bovine serum albumin was then added to give a concentration of 30 g/L.

5'-Nucleotidase assay reagent. This reagent contained, per litre, 2 mmol of sodium phosphate, 50 mmol of Tris HCl (pH 8.0), 2.7 mmol of tribromohydroxybenzoic acid, 0.4 mmol of 4-aminophenazone, 2 mmol of MgSO4, 20 mmol of beta-glycerophosphate, 1 mmol of adenosine monophosphate, 20 mmol of sodium azide, 50 U of xanthine oxidase (EC 1.1.3.22), 20 U of nucleoside phosphorylase (EC 2.4.2.1), 200 U of adenosine deaminase (EC 3.5.4.4), 1000 U of POD, and 6.4 mg (19.2 kU) of superoxide dismutase (EC 1.15.1.1).

BOX reagent. This reagent contained, per litre, 0.4 g (0.60 kU) of BOX and 10 mmol of sodium dodecyl sulfate.

5'-Nucleotidase assay. Serum (50 μL) was mixed with 50 μL of BOX reagent and incubated at room temperature for 5 min, then 10 μL of this (plus 30 μL of water) was mixed with 160 μL of assay reagent and incubated at 37 °C. Absorbance was measured at 520 nm 120 s after mixing and at 10-s intervals for 210 s (in the Cobas Bio) or at 20-s intervals for 220 s (in the Multistat).

Results and Discussion

Bilirubin interferes in H2O2/POD detecting systems because it is oxidized by this system. When all the bilirubin has been so oxidized, the interference ceases. This leads to a delay in oxidation of the chromogen. Indeed, there is an initial decrease in absorbance as the bilirubin is oxidized. The duration of the delay in chromogen oxidation depends on the rate of H2O2 production and therefore, in the 5'-nucleotidase assay, will be inversely related to the activity of 5'-nucleotidase in the sample. This leads to differences in sensitivity to bilirubin inhibition at different 5'-nucleotidase activities. The inhibition caused by 500 μmol of bilirubin per litre was 99% when the sample nucleotidase activity was 30 U/L but only 50% when its activity was 40 U/L. In assays for analytes other than enzymes in which only the initial and final absorbance is measured, the interference ought not to be affected by the analyte concentration.

In investigating the use of BOX to eliminate the interference by bilirubin I observed significant loss of BOX activity after overnight storage of solutions of it at 4 to −20 °C. For maximum activity, only freshly prepared BOX solutions should be used. In subsequent studies the BOX solutions I used (0.1–10 g/L) were kept at 4 °C and used the same day they were prepared.

BOX reacts preferentially with unbound bilirubin. The detergent sodium dodecyl sulfate, by removing bilirubin from albumin, enhances the degradation of bilirubin catalyzed by BOX (Figure 1); in its absence 10-fold more BOX was required to achieve a similar rate of bilirubin degradation (Figure 1).

BOX also oxidized the chromogens 4-aminophenazone and tribromohydroxybenzoic acid, the rate of color formation being proportional to BOX concentrations. At a concentration of BOX of 20 mg/L, the concentration used in the 5'-
Ferro cyanide enhances the chromogen oxidase activity by several fold and therefore should not be included in the assay reagent. Fluoride partly inhibits the chromogen oxidase activity of BOX. However, the maximum inhibition was only 50% at a fluoride concentration of 10 mmol/L. Inhibition by azide or cyanide was more successful, 0.5 mmol of cyanide per litre sufficing to inhibit the chromogen oxidase activity of BOX by 99%, but also inhibiting the bilirubin-degrading activity by 95%. Azide, 20 mmol/L, inhibited the chromogen oxidase activity of BOX by 97% while inhibiting bilirubin degradation by 73%. Therefore, to use these inhibitors without affecting the degradation of bilirubin requires preincubation of the sample with BOX in the absence of inhibitors.

Cyanide interfered in the 5'-nucleotidase assay and therefore for this assay I used azide to inhibit BOX. Under the conditions described for the 5'-nucleotidase assay, interference from bilirubin in concentrations as great as 1000 μmol/L was eliminated, the sample-blank being equivalent to an activity of only 0.4 U/L. Optimal concentrations of BOX and inhibitors remain to be determined for other H₂O₂/POD based assays. Nevertheless, use of the inhibitors described here should facilitate the wider use of BOX for removal of bilirubin interference.

I thank Professor C. N. Hales, Dr. J. P. Luzio, and Dr. C. P. Price for their encouragement.

References