

## Chromogenic System for Measuring Hydrogen Peroxide: The Enzymatic Uric Acid Assay

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**Featured Article:** Fossati P, Prencipe L, Berti G. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. *Clin Chem* 1980;26:227–31.<sup>3</sup>

Our 1980 report in *Clinical Chemistry* described an improved chromogenic detection system that, coupled with the enzyme oxidation of uric acid, led to a direct method for assaying uric acid in biological fluids. The system assay was reliable, simple, rapid, and suitable for either manual or automated procedures. The work was developed in the context of the Sera-Pak line of clinical chemistry reagents at Miles Italiana SpA's Ames research and development laboratories. This line of reagents was marketed in 58 countries throughout the world. Before our investigation, chemical and enzymatic methods for uric acid assay had been described, but these assays had practical disadvantages: lack of direct assay in a small sample with a single reagent, need for a serum blank, long incubation times, and false negatives or positives due to interfering substances. The oxidation coupling reaction between phenol and 4-aminophenazone to yield red quinoneimine dye had long been known, and the reaction widely used in clinical chemistry since Trinder applied it to the enzymatic determination of glucose (1). We speculated that a similar approach might be suitable for measuring uric acid. However, difficulties were encountered, which included the low uric acid concentration in serum and incompatibility between the working pH of horseradish peroxidase and that of animal-originated uricase. Furthermore, the Emerson-Trinder chromogenic system had the major drawback that the oxidative coupling reaction was affected by bilirubin and reducing compounds. This drawback could decrease reaction color as concentrations of these substances increased (2). The interference by reducing compounds such as ascorbic acid primarily consists of either competition with the chromogen in the

peroxidase-catalyzed reaction of hydrogen peroxide or bleaching of the color being formed. Bilirubin interference is a significant obstacle to determining serum metabolites through the Trinder chromogenic system, and is thus a major drawback when hyperbilirubinemic samples are analyzed.

Collaborative studies between Ames Laboratories and the Maggiore Ca' Granda Hospital led us to find several solutions. First, use of a substituted phenol, the 3,5-dichloro-2-hydroxybenzenesulfonic acid by oxidative coupling with 4-aminophenazone yielded a quinoneimine dye with 4 times the molar absorptivity relative to the colorimetric systems then available. Second, use of a bacterial uricase from *Aspergillus flavus* avoided major loss of activity at the pH of maximum horseradish peroxidase activity. Third, use of ferrocyanide, through which peroxidase-peroxide oxidation becomes ferricyanide, in turn oxidizes the substituted phenol leading to the final quinoneimine dye, thus avoiding many potential interferences from substances such as bilirubin. Bilirubin's reaction mechanism is quite complex and, even today, not fully understood. The best approach to the problem found so far was that of Witte et al. (3), who ascribed bilirubin interference to one or more of the following factors: spectral effects, bilirubin acting as alternative peroxidase substrate, or bilirubin-destroying peroxidase reaction intermediates. The spectrum of dye formed in the reaction was kept from overlapping by use of a chromogenic system with 520 nm absorptivity. Chemical interference was avoided by the inclusion of ferrocyanide ions in the reagents (4). To prevent ascorbic acid from hindering the peroxide and its generated chromogen, we added ascorbate oxidase to the reagent. Ascorbate oxidase was therefore produced for the first time on an industrial scale. We grew green squash for this purpose and, after peeling and separation, we extracted, purified, and stabilized the enzyme.

In the 1980s automation was coming into widespread use. As a 1-step procedure, our method was designed to perform well on a broad variety of automatic instruments. Within a year of the report, the method saw application on more than 50 different laboratory instruments. Because of its advantages, our chromogenic method was adopted for measuring other important analytes, such as triglycerides and creatinine, which may generate hydrogen peroxide by reacting

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<sup>3</sup> This paper has been cited more than 300 times since publication.

with enzymes (5–6). The research reported gave rise to a method still widely used today, with minor ongoing changes to reagent composition.

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*design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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