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Formamidine Sulfinic Acid as a Biochemical Reducing Agent

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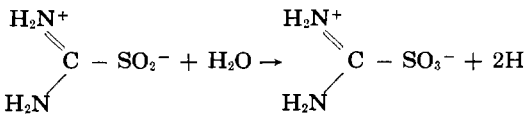
Formamidine sulfinic acid (thiourea dioxide) is described as a useful biochemical reducing agent for converting substrates such as ferredoxin, cytochrome *c*, methemoglobin, and the like, to their reduced forms. The reagent can be used in aqueous buffered systems (*p*H 4–8), and has the advantages of producing no by-products which can interfere with spectroscopic measurements. The chemical and spectroscopic properties of the compound are described.

Frequently, in biochemical reactions involving electron transport, a chemical reagent is required to convert an oxidized molecule to the reduced state at mild reaction conditions. The two widely used reagents for this purpose are sodium borohydride and sodium dithionite. These have certain disadvantages, however, which prevent their application in spectroscopic studies; sodium dithionite produces colloidal sulfur with its inherent light-scattering properties, and sodium borohydride evolves hydrogen gas which interferes with absorption measurements. In the course of experimental work with cytochrome *c* (Shashoua, 1964), we observed that formamidine sulfinic acid (thiourea dioxide) can readily reduce the oxidized ferric molecule to the reduced ferrous state at room temperature in aqueous solutions without the above-mentioned disadvantages. This feature and a number of other properties recommend the use of thiourea dioxide as a general biochemical reducing agent.

Properties of Formamidine Sulfinic Acid

Formamidine sulfinic acid is known as a useful reducing agent for both inorganic and organic compounds, such as tin and copper salts in ammoniacal solutions (Böeseken, 1936), and certain quinones and dyes (Gore, 1954). The mechanism of its reducing action is reputed (Gore, 1954) to involve an irreversible conversion to formamidine sulfonic acid in accordance with the reaction scheme:



Recent evidence (Golunyad and Bolovtova, 1962) has suggested that its reducing action at 70° involves further decomposition to give sulfur dioxide as a by-product. However, in using an aqueous medium and a maximum reaction temperature of 40°, no indication of such a problem was detected. A study of the redox potential of formamidine sulfinic acid as a function of concentration and *p*H gave a value of −1.5 volts for the *p*H range 4–8 at concentration levels of 4×10^{-4}–1×10^{-2} M.

One major advantage of formamidine sulfinic acid as a biochemical reducing agent is that both the reduced and oxidized (sulfonic acid) forms of the compound are water soluble and optically transparent above 300 mμ.

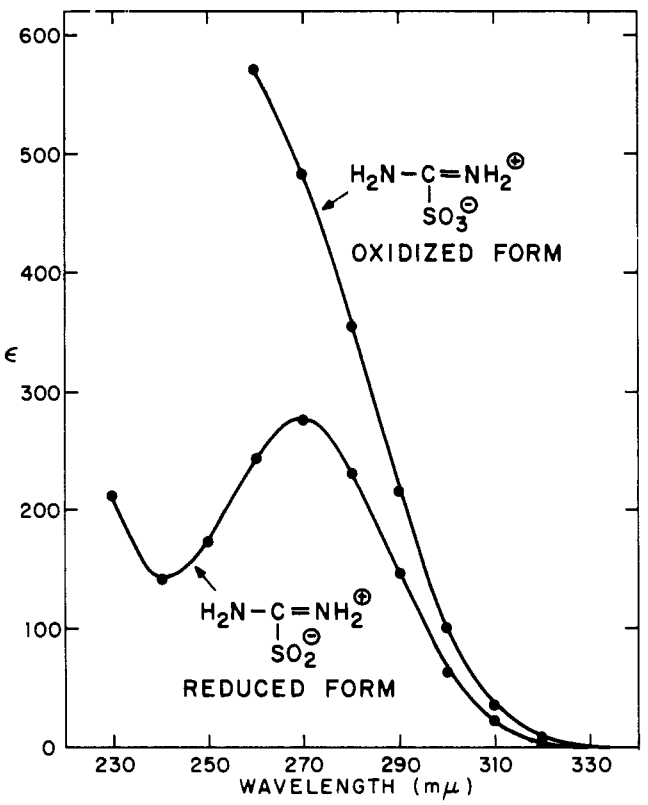


FIG. 1.—Absorption spectra of aqueous solution of formamidine sulfonic acid (oxidized form) and formamidine sulfinic acid (reduced form).

Figure 1 shows a plot of the absorption spectra of the reduced and oxidized forms. (The oxidized form, formamidine sulfonic acid, was prepared by reaction with excess hydrogen peroxide.) One disavdantage of the compound is perhaps its slow reaction rate of 4–16 hours in a typical experiment.

Experimental Results and Discussion

Oxidized cytochrome *c*, methemoglobin, ferredoxin, peroxidase, vitamin B_{12}, and ferritin are readily converted by formamidine sulfinic acid to their reduced forms. A typical reaction consists of treating a 0.02% solution of the oxidized substrate at *p*H 7 with about 50–200 molar equivalents of formamidine sulfinic acid

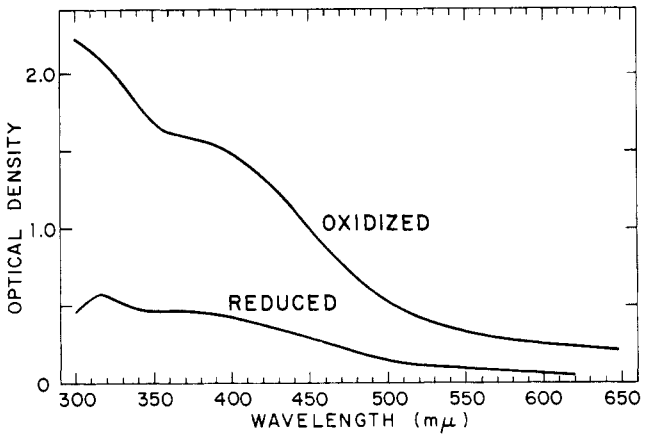


FIG. 2.—Absorption spectra of aqueous solution of oxidized and reduced ferredoxin, 0.027% concentration.

at room temperature for 4 hours. The concentration of the substrate is chosen so that the absorption spectra of the initial and final reduced products can be readily measured in a spectrophotometer (i.e., a 1-cm path length with an optical density of 1–2 at the absorption maximum).

With the exception of ferredoxin (obtained from Drs. E. Knight and D. Blomstrom, Central Research Department, Du Pont Co., Wilmington, Del.), the materials studied were all commercial-grade samples (Nutritional Biochemicals Corp., Cleveland, Ohio). The oxidized cytochrome *c* and methemoglobin were prepared from the reduced forms by oxidation with potassium ferricyanide and purified by rigorous dialysis. The formamidine sulfinic acid was obtained from Aldrich Chemical Co., Milwaukee, Wisc. In the case of methemoglobin, a steady stream of nitrogen was bubbled through the sample during the course of the reaction.

The results for ferredoxin are shown in Figure 2. Here, the reduction was carried out under nitrogen to prevent the rapid return to the starting material by oxidation with oxygen from the air. The course of the reduction was followed spectrophotometrically and was shown to come to equilibrium in about 4 hours. The reaction could be speeded up to 5 minutes by heating to 40° at *p*H 7. The reduction of peroxidase was also extremely sensitive to oxygen from the air; in fact, a small amount of peroxidase behaved as a good catalyst for converting formamidine sulfinic acid to the sulfonic acid form in the presence of air. In every case, the published literature spectral data were used to confirm the formation of the reduced product.

These experiments illustrate the application of formamidine sulfinic acid as a suitably mild reagent for converting certain oxidized biochemical substrates to their reduced forms; it is particularly useful where the reaction is to be studied by ultraviolet (above 300 mμ) or visible spectroscopy.

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The Activation of Antihemophilic Factor (Factor VIII) by Activated Christmas Factor (Activated Factor IX)*

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The reaction of activated Christmas factor with antihemophilic factor has been investigated. Evidence is presented indicating that activated Christmas factor is an enzyme which reacts with antihemophilic factor converting the latter to an activated product which accelerates clotting. The reaction requires the presence of calcium ions and phospholipid. For the latter requirement, a mixture containing equal amounts of phosphatidylcholine and phosphatidylserine is most effective. Diisopropylphosphofluoridate and soybean-trypsin inhibitor have no effect on the activation of antihemophilic factor, whereas heparin is a potent inhibitor. Preincubation of activated Christmas factor with thorium tetranitrate also inhibits the reaction. Evidence for the participation of phospholipid in a reaction following the activation of antihemophilic factor is also provided.

The requirement of antihemophilic factor (AHF[1] or factor VIII) for normal blood clotting in the intrinsic system is well established. In hemophilic plasma the formation of thrombin is slow (Addis, 1911), and prothrombin consumption is greatly impaired (Brinkhous, 1939). Antihemophilic factor participates in the clotting sequence after the interaction of Hageman factor (factor XII), plasma thromboplastin antecedent (PTA or factor XI), and Christmas factor (PTC or factor IX) (Ratnoff and Davie, 1962). The initial reactions are shown in equations (1), (2), and (3).

$$\text{Hageman f.} \xrightarrow{\text{surface}} \text{activated Hageman f.} \quad (1)$$

$$\text{PTA} \xrightarrow{\text{activated Hageman f.}} \text{activated PTA} \quad (2)$$

$$\text{Christmas f.} \xrightarrow[Ca^{2+}]{\text{activated PTA}} \text{activated Christmas f.} \quad (3)$$

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[1] Abbreviations used in this work: AHF, antihemophilic factor; PTA, plasma thromboplastin antecedent; PTC, Christmas factor, plasma thromboplastin component; DFP, diisopropylphosphofluoridate.