Effects of an Isolated Dehydrogenase Enzyme and Flavoprotein on the Reduction of Triphenyltetrazolium Chloride[1]

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The enzymatic reduction of triphenyltetrazolium chloride (TTC) to its red formazan has been used as a means of measuring dehydrogenase activities of biological systems (*1–6*). Bielig *et al.* (*7*), through an analysis of the pH dependence and temperature sensitivity of TTC reduction by bacterial systems, suggested the role of a flavoprotein, diaphorase, as the immediate site of the reduction. Kuhn (*8*) also has reported that TTC is reduced via the oxidation of reduced coenzyme by diaphorase. Mattson *et al.* (*9*) reported the reduction of TTC by a glucose dehydrogenase-coenzyme I system, but it was not mentioned whether flavin enzymes were involved.

We have investigated the effect of an isolated dehydrogenase on the reduction of TTC. Phosphoglyceraldehyde dehydrogenase was isolated and purified from baker's yeast as described by Meyerhof and Junowicz-Kocholaty (*10*). The activity measured was the reduction of coenzyme I (DPN) in the presence of phosphoglyceraldehyde and sodium arsenate in a borate buffer (0.3 *M* at pH 7.0). The reduction of DPN was determined by measuring the increase in absorption at 340 mμ in the Beckman spectrophotometer. When TTC (30 μg/ml) was added to this system, the rate of the reduction of DPN was not affected, nor was formazan produced, as shown by the absence of any absorption at 485 mμ (maximum peak). The millimolar extinction coefficient (485 mμ) of formazan in an acetone-water solvent at pH 6.1 was 12.4.

The oxidation of reduced DPN by diaphorase was next investigated. The diaphorase was prepared from baker's yeast by a modification of the method of Green and Dewan (*11*). When this was added to the above-mentioned system in which DPN had been reduced enzymatically, the reduced DPN was readily reoxidized as measured by decrease in absorption at 340 mμ. Under these conditions the addition of 30–250 μg TTC/ml had no effect on the reoxidation of the reduced DPN; nor was any formazan produced. However, after preliminary investigations using reduced DPN prepared by the method of Ohlmeyer (*12*), it became evident that the reduction of TTC could be accomplished by the diaphorase system under anaerobic conditions. Under aerobic conditions, however, the reduction occurred either not at all, or very poorly, over a narrow pH range. Fig. 1 shows the rate of formation of formazan at pH 7.0. Under these conditions, formazan is produced only under the anaerobic conditions obtained with conventional Thunberg tubes which had been evacuated, flushed with nitrogen, and reevacuated.

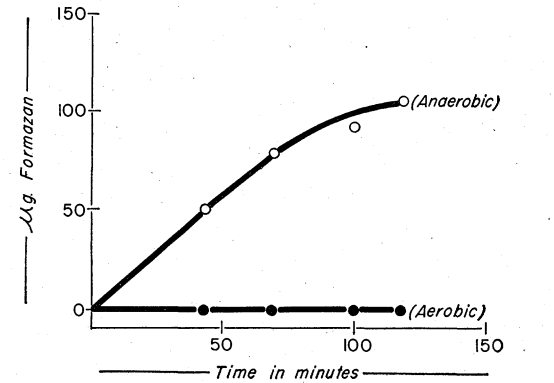

FIG. 1. Reduction of TTC by yeast diaphorase (pH 7.0). (Conditions same as in Table 1.)

It can be see from Table 1 that the reduction of TTC can occur aerobically in the narrow pH range of 7.5–8.3. The amount of formazan formed was still considerably less than was produced anaerobically under the same conditions. Under the optimal conditions (pH 8.3) of the aerobic reduction of TTC by this system, it could be demonstrated that for each mole of reduced DPN oxidized, one mole of formazan was produced (Table 2). At pH 7.0 the oxidation of the reduced DPN still occurred, though at a somewhat slower rate, and this was not affected by the presence of TTC; nor was the latter reduced.

TABLE 1

THE EFFECT OF PH ON THE REDUCTION OF TTC BY YEAST DIAPHORASE*

pH	Formazan formed after 120 min (μg/ml)	
	Anaerobic	Aerobic
5.7	0	0
6.9	150	0
7.5	350	20.5
8.0	500	50.0
8.3	650	58.0
9.2	0	0

* The system consisted of: 2.5 ml 0.5 *M* phosphate buffer (pH 6.9–8.3) or 0.2 *M* borate buffer (pH 9.2–10.0); 0.5 ml diaphorase (1/30 dilution); TTC (200 μg/ml); reduced DPN (200 μg/ml)—reduced by sodium hydrosulfite. Total system of 5 ml.

As applied to isolated systems, diaphorase has been shown to reduce TTC both aerobically and anaerobically, although the aerobic system was much less effective than the anaerobic. This may be due to (1) an elevation of the O/R potential to an unfavorable level, (2) a direct competition between oxygen and TTC for flavoprotein, or (3) the involvement of other mechanisms in the aerobic reduction of TTC. Whether

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TABLE 2

OXIDATION OF REDUCED DPN AND REDUCTION OF TTC BY YEAST DIAPHORASE*

Time (min)	pH 7.0			pH 8.3		
	TTC present			TTC present		
	DPN mμM	Formazan mμM		DPN mμM	Formazan mμM	
0	0	0	0	0	0	0
5	–	9	0	–	12	12
10	9	13	0	27	21	24
20	21	24	0	54	51	39
50	53	34	0	64	58	67

* System same as in Table 1.

other mechanisms, such as the cytochrome-cytochrome oxidase systems, are involved, either directly or indirectly, is at present under investigation.[3]

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[3] Since submission of this paper, the work was continued with an isolated bacterial (*E. coli*) DPN-H_2 oxidase and TTC, as well as neotetrazolium. The findings were similar and will be the subject of a future report.

The Use and Toxicity of Pontamine Sky Blue

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The need for a better method of visualizing the primary and secondary lymphatic echelons in resectional surgery in carcinoma has long been recognized. Any agent used for this purpose should have the following characteristics: (1) high water solubility, (2) high degree of contrast compared to the tissues into which it is injected, (3) high specificity for and low diffusibility from lymph and lymph nodes, (4) low toxicity, (5) color stability, particularly near pH 7.2, and (6) ability to penetrate into the surgical area within 15 min. Braithwaite (*1*) found that indigo carmine fulfilled most of these criteria, but unfortunately the rate of dye uptake and its penetration into the surgical area left much to be desired. A review of the work of McMasters and Parsons (*2*) and Hudack and McMasters (*3*) showed that Pontamine Sky Blue (sodium salt of dimethoxydiphenyl-diazo-bis-8-amino-1-naphthol-3, 6 disulfonic acid) fitted all these criteria. In a study involving 35 cases of gastric resection for carcinoma, Weinberg and Greaney (*4*) established the utility of the dye for this purpose.

Recently the dye has been employed in more than 50 cases of intrathoracic surgery to delineate the surgical anatomy of the endothoracic lymphatics. The patient is prepared for such surgery by the usual procedure, an endotracheal catheter introduced, the thoracic cage opened and 5 ml of a 4% solution of Pontamine Sky Blue injected into the lung parenchyma near the hilum. The dye spreads rapidly, outlining the lymphatics not only in the immediate area but also in the more remote areas, thus allowing more radical surgery to be performed. This has extended the scope and completeness of extirpation of the diseased and potentially diseased tissue in bronchiogenic carcinoma. During the course of this work, 8 patients showed a residual blue coloring of the subcutaneous tissues. This was of concern not only to us but to those investigators using the dye under our directions because nothing was known about the possible toxicity of the dye.

The intravenous toxicity of Pontamine Sky Blue was determined in mice, and the LD_{50} was calculated by the Litchfield-Wilcoxon (*5*) method (Table 1).

TABLE 1

LD_{50} OF PONTAMINE SKY BLUE IN MICE

No. animals	LD_{50} g/kg	Confidence limits (odds 19/20) g/kg	Slope	Confidence limits (odds 19/20)
45	2.26	2.154–2.371	1.02	0.93–1.11

The symptoms of toxicity observed were acute respiratory embarrassment and death by cardiac and respiratory failure. The respiratory phase can be readily counteracted by artificial respiration.

Thirty animals that survived doses between 1.5 and 2.5 g/kg of the dye were observed for 28 days. Slight discoloration of the skin was observed in all the animals, but no evidence of residual toxicity was observed at necropsy.

In the use of any dyestuff, there is the possibility of traces of inorganic and organic materials being carried through the process and ending up in the final product. Such materials can produce toxic symptoms unrelated to the over-all toxic symptoms produced by the dye itself. Spectrographic analysis of Pontamine Sky Blue revealed that there were minute traces of Mn, Pb, and Cr. However, these ions were in such small quantities that they could not possibly cause any of the toxic manifestations seen when the dye was injected into humans or animals.

The possibility of toxicity caused by aging of the