Technical Papers

The Reduction of Tetrazolium Salts by an Isolated Bacterial Flavoprotein[1]

Arnold F. Brodie[2] and Joseph S. Gots

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia

In a previous publication (*1*) the authors demonstrated that 2,3,5,triphenyltetrazolium chloride (TTC) could be enyzmatically reduced by the yeast flavoprotein, diaphorase, with the simultaneous oxidation of a molar equivalent of reduced DPN. Recently, Kun (*2*) presented evidence which clearly demonstrated that other flavin enzymes (i.e., amino acid oxidases) could also reduce this compound. He could not demonstrate the reduction of TTC with glycolytic enzymes unless mitochondrial fractions were added. Shelton and Schneider (*3*) were able to show that purified xanthine oxidase, and diphosphopyridine nucleotide-cytochrome *c* reductase, were able to reduce tetrazolium salts rapidly. The present investigation was undertaken with an isolated bacterial enzyme which could reoxidize reduced DPN concurrently with the reduction of TTC or neotetrazolium (NTC).

Mattsen *et al.* (*4*) reported the reduction of TTC by a glucose dehydrogenase–coenzyme I linked system, but it was not mentioned whether flavin enzymes were involved. In a later publication Jensen *et al.* (*5*) reported that the tetrazolium salts could be reduced by a series of DPN linked dehydrogenase enzymes, including lactic and phosphoglyceraldehyde dehydrogenases. They claimed that these systems did not contain flavoproteins or cytochrome oxidase, although no experimental data were presented to support this conclusion. In a previous publication (*1*) we investigated the ability of phosphoglyceraldehyde dehydrogenase to reduce TTC. Under the conditions of the experiment the formazan was not produced. We have continued the dehydrogenase study with crystalline lactic dehydrogenase isolated from rabbit muscle according to the method of Korkes *et al.* (*6*), alcohol dehydrogenase isolated from yeast according to the method of Racker (*7*), and glycerol dehydrogenase isolated from *Escherichia coli* by the method of Asnis and Brodie (unpublished). The reduction of DPN was followed spectrophotometrically at 340 mμ in the Beckman spectrophotometer (model DU), and in no instance did the tetrazolium salts interfere with this reduction. The formation of formazan and diformazan was followed at 485 mμ and at 530 mμ, respectively, with no evidence of formazan production by any of the dehydrogenases tested.

An enzyme was extracted from *E. coli* strain ECFS, which could reoxidize reduced DPN (*8*). The cells were disrupted by sonic vibration and fractionated with ammonium sulfate. The yellow enzyme showed a typical flavin spectrum when analyzed in the spectrophotometer. This oxidase had no other dehydrogenase activity and was inactive with TPN reduced with glucose-6-phosphate and its dehydrogenase.[3]

This enzyme behaved similarly to yeast diaphorase in that it could reduce TTC and NTC in the presence of reduced DPN. The pH optimum for this reduction with the bacterial enzyme was found to be 7.8. The reduction of neotetrazolium could now be reconstituted enzymatically with various fractions from one organism. In Fig. 1, DPN was reduced with the glycerol

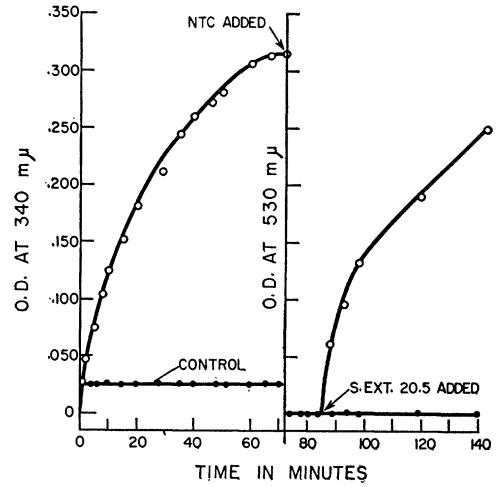

FIG. 1. The reduction of neotetrazolium by bacterial fractions. The system consisted of 500 μg DPN, 30 μM glycerol, 0.5 ml glycerol dehydrogenase, 1 ml *M*/10 pyrophosphate buffer pH 7.8, and distilled water to make a final volume of 3 ml. After the reduction of DPN had reached a plateau, 100 μg NTC was added and followed at 530 mμ for 15 min, whereupon 0.1 ml bacterial oxidase (S. Ext. 20.5) was added.

dehydrogenase fraction isolated from *E. coli* (ECFS). Upon the addition of NTC no reduction occurred during a 15-min period. When the oxidase fraction of the same organism was added, the reduction of NTC (and the simultaneous reoxidation of reduced DPN) occurred. Two moles of reduced DPN were found to be necessary for the reduction of one mole of NTC—i.e., 0.10 μM of reduced DPN were oxidized for 0.056 μM of neotetrazolium reduced.

To characterize the prosthetic group of the enzyme, the following method was employed. Manometric experiments were performed in which the oxygen uptake was followed with reactivated D-amino acid oxidase apoenzyme prepared from pig kidney.[4] Various con-

[1] This work has been aided by a grant from the Eaton Laboratories, Inc., Norwich, N. Y.

[2] Present address: Biochemical Research Laboratory, Massachusetts General Hospital, Boston.

[3] The reduced TPN was kindly supplied by Dwight B. McNair Scott, of Children's Hospital, Philadelphia.

[4] The D-amino acid oxidase apoenzyme and flavin adenine dinucleotide (95% of the total flavin present) were generously supplied by Leslie Hellerman, of the Johns Hopkins Medical School.

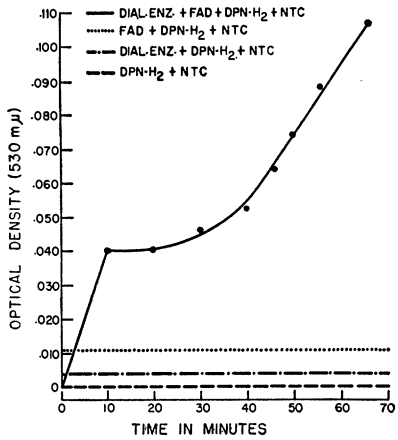

FIG. 2. Reactivation of bacterial oxidase after dialysis in dilute HCl. The system consisted of 0.5 ml bacterial enzyme, 24 μg FAD, 100 μg neotetrazolium, 1 ml *M*/10 pyrophosphate buffer pH 7.8, and distilled water; incubated for 10 min before the addition of 1000 μg reduced DPN to make a final volume of 3 ml. Control systems are indicated.

centrations of flavin adenine dinucleotide (FAD) were added in order to determine the rate of oxygen uptake as a function of the flavin concentration. The flavin content of the hydrolyzed bacterial oxidase was then determined in the same manner. It was capable of reactivating the D-amino acid oxidase apoenzyme. The nonhydrolyzed enzyme failed to reactivate the oxidase.

Although the data indicated the presence of FAD, it seemed essential to demonstrate the necessity of this coenzyme for enyzmatic activity. By dialyzing the bacterial enzyme for 24 hr in the presence of dilute HCl and potassium monobasic phosphate, similar to the method of Warburg and Christian (*9*), an apoenzyme was prepared which was inactive in the presence of reduced DPN and neotetrazolium (Fig. 2). When FAD was incubated with this apoenzyme, the system was reconstituted, and about 60% of the original activity was recovered. Neither reduced DPN nor FAD, alone or combined, could reduce the neotetrazolium.

By limiting the addition of flavin (Fig. 3), it was found that the amount of diformazan produced is a function of the concentration of added FAD. Experiments were performed in which the rate constant in

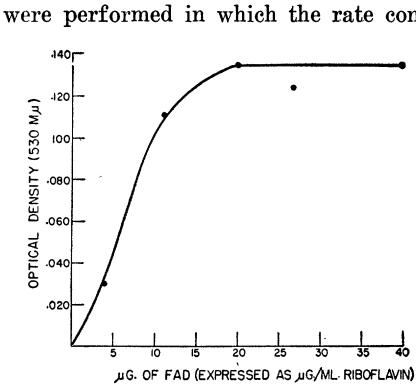

FIG. 3. Effect of various concentrations of FAD on bacterial oxidase after dialysis in dilute HCl. (Conditions same as in Fig. 2.)

mole^{-1} min^{-1} liter was determined for the oxidation of the bacterial enzyme with neotetrazolium and oxygen (*10*). The bacterial enzyme was found to have a greater turnover when neotetrazolium was used as an electron acceptor. The rate of reaction of neotetrazolium was similar to the rate of reaction of ordinary redox dyes such as methylene blue.

In bacteria the reduction of tetrazolium salts has been shown to occur in discrete granules (*11*). *In vitro* this reduction with isolated bacterial enzymes can be accomplished by flavin enzymes. Neither DPN-dehydrogenases nor reduced DPN, per se, can reduce these salts to the formazan state.

References

1. BRODIE, A. F., and GOTS, J. S. *Science*, **114**, 40 (1951).
2. KUN, E. *Proc. Soc. Exptl. Biol. Med.*, **78**, 195 (1951).
3. SHELTON, E., and SCHNEIDER, W. C. *Anat. Record*, **112**, 61 (1952).
4. MATTSEN, A. M., JENSEN, C. O., and DUTCHER, R. A. *Science*, **106**, 294 (1947).
5. JENSEN, C. O., SACKS, W., and BALDAUSKI, F. A. *Ibid.*, **113**, 65 (1951).
6. KORKES, S., *et al. J. Biol. Chem.*, **193**, 721 (1951).
7. RACKER, E. *Ibid.*, **184**, 313 (1950).
8. BRODIE, A. F., and GOTS, J. S. *Federation Proc.*, **11**, 191 (1952).
9. WARBURG, O., and CHRISTIAN, W. *Biochem. Z.*, **298**, 150 (1938).
10. BRODIE, A. F. *J. Biol. Chem.*, **199**, 835 (1952).
11. MUDD, S., *et al. J. Bact.*, **62**, 729 (1951).

Manuscript received May 19, 1952.

The Grafting of Large Monocotyledonous Plants

Thomas J. Muzik and Carl D. La Rue

Federal Experiment Station, Mayagüez, Puerto Rico,
and *Department of Botany,*
University of Michigan, Ann Arbor

The grafting of plants has been a common horticultural practice for many centuries. For almost as great a stretch of time two ideas have been associated with this practice: first, that monocotyledonous plants cannot be grafted; and second, that leaves cannot be grafted. As late as 1947, Transeau, Sampson, and Tiffany (*1*) stated, "Neither twig grafting nor budding is possible in stems without cambiums." For successful union it was stated by Eames and McDaniels (*2*) that the cambium of the scion must be united with that of the stock. Similar expressions may be found in dozens of textbooks.

In 1946 La Rue and Reissig (*3*) reported that leaves of numerous species could be grafted and found that fleshy leaves required a technique different from that used with thin ones.

Calderini (*4*) reported, more than a hundred years ago, that he had made successful grafts of rice on barnyard grass. He claimed that by this means he had produced a new strain of rice with superior characteristics which, thereafter, were transmitted by the seeds. The latter statement, however, brings into question the validity of the entire paper. Plotnikov (*5*) made grafts in grains, and Monakima and Sölevey (*6*) grafted