An Assay for Azo-Reductase Activity in Micro-organisms

By R. WALKER and R. GINGELL. [Department of Biochemistry, University of Surrey, Guildford, Surrey, U.K., and National College of Food Technology (University of Reading), St George's Avenue, Weybridge, Surrey, U.K.]

Several microbial species commonly found in the mammalian gastrointestinal tract possess azo-reductase activity, and in the cases of *Proteus vulgaris* (Roxon, Ryan & Wright, 1967) and *Streptococcus faecalis* (Scheline, Nygaard & Longberg, 1970) the enzymes involved have been characterized as NAD(P)H-dependent flavoproteins. We have developed an assay system which allows for continuous measurement of the rate of the azo-reduction reaction and applied this to studies of cofactor requirements and mechanisms of reduction in several microbial species.

Cell-free extracts of the microbial species under investigation were used. These were prepared by ultra-sonication and centrifugation and the protein content was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

The assay was performed in 3 ml stoppered 10 mm-path-length cuvettes in a double-beam recording spectrophotometer (Unicam SP.800) equipped with thermostatically controlled cuvette holders and programme control unit (SP.825). The test cuvette contained an electron-generating system [ethanol + alcohol dehydrogenase (EC 1.1.1.1) + NAD, or glucose 6-phosphate + glucose 6-phosphate dehydrogenase (EC 1.1.1.49) + NADP], flavin (FMN FAD or riboflavin) and azo-substrate (normally Red 2G), the latter being omitted from the reference cuvette. All cofactors and substrate were made up in 0.1 M-phosphate buffer at an appropriate pH and were deoxygenated by bubbling oxygen-free nitrogen for 10 min before the assay. To each cuvette was added the appropriate amount (typically 0.05 ml) of cell-free extract, the volume was made up to 3 ml with deoxygenated buffer and, after sweeping out the headspace with nitrogen, the stopper was inserted. The reduction of the azo-compound was followed continuously, or intermittently at pre-determined time-intervals, by monitoring the E_{525} ($\lambda_{\max}$ for Red 2G).

Optimum conditions of temperature, pH and cofactor concentrations have been determined for *Strep. faecalis*, *Escherichia coli* and *Saccharomyces cerevisiae* and the cofactor and substrate specificities studied in relation to the mechanism of azo reduction.

Despite rigorous exclusion of oxygen the reaction was not immediately initiated on addition of the enzyme preparation; there was a time-lag, which varied inversely with the activity of the enzyme.

The lag has been shown to be associated with the production of reduced cofactors.

Assay systems previously described (e.g. Roxon *et al.* 1967; Scheline *et al.* 1970; Williams, Grantham, Yamamoto & Weisburger, 1970) have not taken into account the lag phase and hence may have underestimated azo-reductase activity. Preliminary work (R. Walker & B. G. Lake, unpublished work) indicates that the assay system can be adapted to the measurement of flavin-dependent azo-reductase of liver microsomal preparations, where a similar lag is observed.

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Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Roxon, J. J., Ryan, A. J. & Wright, S. E. (1967). *Fd Cosmet. Toxicol.* **5**, 645.

Scheline, R. R., Nygaard, R. T. & Longberg, B. (1970). *Fd Cosmet. Toxicol.* **8**, 55.

Williams, J. R., Grantham, P. H., Yamamoto, R. S. & Weisburger, J. H. (1970). *Biochem. Pharmac.* **19**, 2523.

Kinetics and Inhibition by Adenosine Phosphates and Nitrite of Nitrate Reductase from *Spinacea oleracea* L.

By A. R. J. EAGLESHAM and E. J. HEWITT. (University of Bristol Research Station, Long Ashton, Bristol BS18 9AF, U.K.)

Nitrate reductase (NADH-nitrate oxidoreductase, EC 1.6.6.1) from tomato leaf is inhibited by ADP (Nelson & Ilan, 1969) and possible allosteric control was suggested. Kinetic aspects of spinach leaf enzyme were investigated in this context. Enzyme was extracted in 0.1 M-tris-HCl buffer, pH 7.5, precipitated by 50% saturated ammonium sulphate, desalted on Sephadex G-25 and either stored at -18°C (aged) or used immediately (fresh). Nitrate-dependent oxidation of NADH at 27°C recorded spectrophotometrically was stoichiometric with nitrite production.

Plots of $1/v$ against $1/[\text{NADH}]$ for different fixed [nitrate] and vice versa produced parallel slopes indicating a Ping Pong (Bi Bi) mechanism (Cleland, 1963a). Secondary plots of $1/V_{\max}$ against $1/[\text{fixed substrate}]$ (Florini & Vestling, 1957) gave theoretical K_m values (NADH, $4.4 \mu\text{M}$; nitrate, $180 \mu\text{M}$) for aged enzyme with phosphate (10 mM) as activator (cf. Hewitt & Nicholas, 1964). Phosphate requirement appeared to be co-operative, with Hill (1910) plot values of $n \geq 4$. For reduced benzyl viologen (BV^+) as electron donor (Cresswell, Hageman, Hewitt & Hucklesby, 1965) K_m was $3 \mu\text{M}$.