relationships and various matters of detail are similarly difficult to account for, and are the subject of further studies.

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Studies on the Reaction of Glutamate Dehydrogenase with Ethoxyformic Anhydride

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The use of ethoxyformic anhydride for studying the acylation of histidine residues has considerable advantages over other similar reagents. The extent of the reaction can be followed spectrophotometrically by measuring the increase in absorption at 242nm due to the formation of ethoxyformylhistidine residues (Ovadi *et al.*, 1967). The reagent is, however, not specific for histidine residues, but is susceptible to nucleophilic attack from tyrosine, cysteine, lysine and tryptophan residues (Rosen & Fedorcsak, 1966; Mühlrád *et al.*, 1967), although at pH6.1 it reacts preferentially with histidine residues. The reagent has proved particularly useful in the present studies on glutamate dehydrogenase.

Reaction of the reagent at pH6.1 in 0.1M-sodium acetate–acetic acid buffer containing 0.1M-NaCl produced a rapid loss of about 40% of the initial activity as a result of the acylation of a single histidine residue. Over a longer period of time two further residues could be modified without any further loss of activity. When the enzyme was treated with the reagent at pH7.5 in 0.1M-tris–HCl buffer containing 0.1M-NaCl complete loss of enzymic activity occurred after modification of only two histidine residues. When the enzyme was first allowed to react at pH6.1 and the pH then adjusted to 7.5 only a single residue reacted at the latter pH, although this resulted in the complete loss of the enzymic activity. If the excess of ethoxyformic anhydride was removed after the pH6.1 treatment by gel filtration loss of activity was not observed on adjusting the pH to 7.5 unless further anhydride was added.

The reagent appeared to react only with histidine residues at both pH6.1 and 7.5. Analysis of the difference spectrum produced on modification of the enzyme showed none of the characteristics associated with ethoxyformylation of the aromatic residues. In addition, no decrease in the number of cysteine residues present was observed. It was not possible to exclude reaction with all the lysine residues present in the molecule, although lysine-97, which reacts specifically with pyridoxal phosphate (Piszkiewicz *et al.*, 1970) and *N*-(*N'*-acetyl-4-sulphamoylphenyl)maleimide (Holbrook & Jeckel, 1969), was not acylated.

Both the loss of activity at pH7.5 and the extent of ethoxyformylation were decreased by the presence of glutamate. This protection was not observed at pH6.1, suggesting that the residue protected was important for activity but was not available for acylation at pH6.1. At pH7.5 NAD^+ did not affect the loss of activity but it decreased the extent of modification of the enzyme.

The implications of these results will be discussed.

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The Reduced Nicotinamide–Adenine Dinucleotide Phosphate- and Oxygen-Dependent *N*-Oxygenation of Trimethylamine by *Pseudomonas aminovorans*

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Extracts of *Pseudomonas aminovorans* grown on trimethylamine contain an enzyme catalysing the NADPH- and oxygen-dependent conversion of trimethylamine into trimethylamine *N*-oxide. The specific activity of the enzyme in cells grown on other methylamines or succinate was only about 2–3% of the activity in trimethylamine-grown cells.

The enzyme was assayed spectrophotometrically by measuring the rate of trimethylamine-dependent oxidation of NADPH spectrophotometrically at 340nm. A number of other tertiary amines were active substrates, including ethyldimethylamine, diethylmethylamine, *NN*-dimethylethylenediamine, *NN*-dimethylethanolamine, triethylamine, *NN*-dimethylpropylenediamine, 3-dimethylaminopropan-1-ol, 3-dimethylaminopropan-2-ol and *NN*-dimethyl-2-chloroethylamine. All substrates showed inhibition at concentrations above about 1mM. NADPH was

the preferred electron donor (K_m 16μM compared with 2mM for NADH). The enzyme was not inhibited by CO, cyanide or SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride), nor by chelating agents. No metal ion requirement could be demonstrated, and Co^{2+}, Ca^{2+} and Zn^{2+} were inhibitory. The enzyme was rather unstable (half-life in the crude extract 20min at 35°C) and a sixfold purification is the best so far obtained. Such preparations are free of cyanide-sensitive secondary-amine oxidase activity (Eady *et al.*, 1971) and trimethylamine *N*-oxide demethylase activity (Large, 1971), and have an absorption spectrum suggesting the presence of flavin. The enzyme had optimum pH7.7 and was stable at 0°C for 30min at any pH value in the range 4–9. The reaction product was identified as trimethylamine *N*-oxide by its identical mobility with the authentic material in t.l.c., by its identical behaviour on ion-exchange chromatography (Blau, 1961), and by showing that it was converted into formaldehyde by (*a*) SO_2 (Mitchell & Ziegler, 1969) and (*b*) partially purified preparations of trimethylamine *N*-oxide demethylase (Large, 1971; Myers & Zatman, 1971). The stoicheiometry of the reaction agrees with the equation:

$$(CH_3)_3N + NADPH + H^+ + O_2 \rightarrow (CH_3)_3NO + NADP^+ + H_2O$$

It differs from the secondary-amine mono-oxygenase present in the same extracts (Eady *et al.*, 1971) by its lack of sensitivity to CO and cyanide, and by its specificity for NADPH. We have also found it present in trimethylamine-grown *Hyphomicrobium vulgare* NQ.

No evidence for a phenazine methosulphate-linked trimethylamine dehydrogenase has been found in *Ps. aminovorans*. Since other bacteria that can grow on trimethylamine contain such an enzyme (Colby & Zatman, 1971), it appears that a diversity of pathways for amine oxidation occur in bacteria (Large, 1972).

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Resistance of Alkylated Deoxyribonucleic Acid to Nuclease Attack

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We have investigated the ability of some deoxyribonucleases to degrade methylated DNA in connexion with studies on carcinogenesis, first for their potential as analytical tools and secondly for the possible significance of these enzymic reactions in the metabolism of DNA methylated *in vivo* by carcinogens. In view of the suggested importance of DNA synthesis to the carcinogenic process (Craddock, 1971; Stewart & Magee, 1971), enzymes known to exhibit increased activity during DNA replication in partially synchronized systems (Brody & Balis, 1959; O'Connor, 1971) are of special interest.

In these initial studies, calf thymus DNA was hypermethylated by using dimethyl sulphate (Michelson & Pochon, 1966) and the amount of methylation was determined by ion-exchange chromatography of acid hydrolysates of the DNA. Endonuclease and exonuclease activity was measured by using acid-soluble nucleotide assays, and paper chromatography was used to verify the exonuclease assays.

The endonucleases studied were pancreatic deoxyribonuclease I, an alkaline deoxyribonuclease from rat liver (O'Connor, 1969), and hog spleen acid deoxyribonuclease (Bernardi, 1966); the exonucleases were from snake venom (*Crotalus adamanteus*) and from hog spleen (Bernardi & Bernardi, 1968). The deoxyribonucleases from pancreas and from rat liver showed only slightly lower activity with hypermethylated DNA compared with normal DNA, and the venom exonuclease readily digested methylated DNA to mononucleotides, indicating the usefulness of these enzymes for the base analysis of chemically methylated DNA.

On the other hand the activity of the spleen acid deoxyribonuclease with methylated DNA was greatly diminished. This effect was obtained with commercial samples and the enzyme purified as described by Bernardi (1966). Results from initial reaction rates obtained with purified enzyme and DNA methylated to various extents suggested that the enzyme would be virtually inactive when about half of the guanine residues were converted into 7-methylguanine.

The preferred substrate for the spleen exonuclease is the 3′-oligodeoxyribonucleotide produced from DNA by the action of the spleen deoxyribonuclease. However, when hypermethylated DNA oligonucleotides were prepared, either by digestion of methylated DNA or by methylation of the oligonucleotides from a digest of normal DNA, and subjected to