

The Microbial Oxidation of Methanol

THE PROSTHETIC GROUP OF THE ALCOHOL DEHYDROGENASE OF *PSEUDOMONAS* SP. M27: A NEW OXIDOREDUCTASE PROSTHETIC GROUP

BY C. ANTHONY* AND L. J. ZATMAN
Department of Microbiology, University of Reading

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1. The purified alcohol dehydrogenase of *Pseudomonas* sp. M27, whose action is independent of nicotinamide nucleotides, has absorption peaks at 280 m μ and at 350 m μ with little or no absorption at or above 450 m μ . 2. It does not fluoresce, but green-fluorescent material, diffusible on dialysis, is produced when the enzyme is treated with acid or alkali or when it is boiled. 3. Evidence is presented that the enzyme is not a flavoprotein. 4. Kinetic studies show a correlation between enzyme inactivation by acid, alkali or heat and liberation of the fluorescent material. 5. Some purification of the fluorescent material was achieved, but definite identification was not possible; the major component has a fluorescence maximum at about 460 m μ with excitation maxima at about 260 m μ and 365 m μ . 6. Data are given (including absorption and fluorescence spectra) that support the suggestion that the prosthetic group of the enzyme is a pteridine derivative. 7. Possible mechanisms of action of the enzyme are discussed.

Anthony & Zatman (1964*b*, 1965, 1967*b*) have described some of the properties of the unusual alcohol dehydrogenase of *Pseudomonas* sp. M27, a pink organism capable of growth on methanol as sole source of carbon and energy (Anthony & Zatman, 1964*a*). This alcohol dehydrogenase oxidizes methanol at least as fast as ethanol, it does not react directly with oxygen and it contains insignificant amounts of metal; further, the spectrum (Fig. 1) precludes the presence of cytochrome, of haem, or of cobalamin derivatives. Enzyme activity is completely independent of nicotinamide nucleotides (Anthony & Zatman, 1964*b*) and phenazine methosulphate is the only substance tested that acts as primary hydrogen acceptor for methanol oxidation. This alcohol dehydrogenase cannot therefore be included in any known class of enzymes (Dixon & Webb, 1964; *Enzyme Nomenclature*, 1965) with the possible exception of the flavine-dependent oxidoreductases. Evidence that this enzyme is not a flavoprotein is presented below together with the results of studies on the prosthetic group of the enzyme. In the present paper the term 'prosthetic group' refers to the green-fluorescent material that is produced from the non-fluorescent enzyme by boiling, or by lowering the pH to about 3, or by raising the pH to about 12.

* Present address: Department of Physiology and Biochemistry, University of Southampton.

A preliminary report of this work has been published (Anthony & Zatman, 1967*a*).

MATERIALS AND METHODS

Unless otherwise stated materials were obtained from the sources given by Anthony & Zatman (1965).

Buffer solutions. Buffer solutions were prepared according to the data of Dawson, Elliott, Elliott & Jones (1959).

Spectrophotometry. All spectrophotometric measurements were made with a Unicam SP. 800 recording spectrophotometer.

Standard spectrophotometric enzyme assay. The cuvettes (10 mm. light-path) contained the following, in a total volume of 3 ml.: 0.3 m-mole of tris-HCl, pH 9.0; 20 μ moles of methanol; 3.3 μ moles of *N*-methylphenazonium methosulphate (phenazine methosulphate); 0.13 μ mole of 2,6-dichlorophenol-indophenol; 45 μ moles of NH₄Cl. The reference cuvette contained deionized water. Enzyme solution was blown in from a pipette and the initial rate of dye reduction taken as twice the change in E_{600} occurring between 15 and 45 sec. after addition of enzyme. The amount of enzyme was always adjusted to give a rate of change of E_{600} of less than 0.6/min.

Enzyme units. One unit of enzyme activity as measured by the spectrophotometric assay is defined as the amount of enzyme that produces a change in E_{600} of 0.01/min. between 15 and 45 sec. after addition of the enzyme. Assuming that the molar extinction coefficient of 2,6-dichlorophenol-indophenol at 600 m μ is 1.91×10^7 cm.²mole⁻¹ (Basford & Huennkens, 1955), 570 of these units are equivalent to 1 standard unit as defined in *Enzyme Nomenclature* (1965).

Ion-exchange cellulose. Ion-exchange cellulose was

obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; it was prepared and columns were set up according to instructions in the Company's manual. Fractions were collected with a Central fraction collector made by Aimer Products Ltd., London, N.W. 1.

Preparation of purified enzyme. Crude extracts were prepared, and enzyme was purified as described by Anthony & Zatman (1967b).

Kinetic studies on the liberation of the prosthetic group from the enzyme

Purified enzyme was used in all these experiments and sodium pyrophosphate buffer was substituted for tris-HCl buffer in the enzyme assays; the initial rate in 0.1 M-pyrophosphate buffer, pH 9.0, is about $1\frac{1}{2}$ times that in tris-HCl buffer, pH 9.0 (A. Ladner, unpublished work from this Laboratory). The enzyme assay mixture contained in a total volume of 3 ml.: 0.3 M-mole of sodium pyrophosphate, pH 9.0; 20 μ moles of methanol; 45 μ moles of NH_4Cl ; 3.3 μ moles of phenazine methosulphate; 0.13 μ mole of 2,6-dichlorophenol-indophenol. The measurement of fluorescence was made with the SP. 860 fluorimeter accessory made by Unicam Ltd. for use with the SP. 800 recording spectrophotometer. The reference solution was riboflavine (50 μ g./ml.) and the pale-yellow Kodak 2B filter was used; the excitation wavelength was 360 μ . As a logarithmic factor is involved in measurements with this accessory, calibration curves were prepared for quantitative measurement of fluorescence. The arbitrary fluorescence units in Figs. 2-6 are directly proportional to the amount of fluorescent material present. The total fluorescence produced from a given amount of enzyme was the same whether it was produced by very low pH (0.1 N-HCl), by high pH (0.1 N-NaOH) or by boiling. When, however, enzyme was inactivated as carefully as possible in milder conditions the total amount of fluorescence that was produced differed according to the conditions; in Figs. 2-6 fluorescence is expressed as a percentage of that eventually produced by the treatment under investigation.

Inactivation of enzyme and production of fluorescence as a function of low pH values. Purified enzyme (3 mg.) was dissolved in 3 ml. of deionized water and the pH very carefully lowered to a value between 2.6 and 4.0 with 0.01 N-HCl. After 40 min. the pH was raised to 7.5 by addition of 0.45 ml. of 0.5 M-sodium phosphate buffer, pH 7.5, the volume was made up to 4.5 ml. in a graduated tube and enzyme activity and fluorescence were determined.

Inactivation of enzyme and production of fluorescence as a function of high pH values. Purified enzyme (3 mg.) was dissolved in 3 ml. of deionized water and the pH raised to a value between 9.0 and 12.5 with 0.1 N-NaOH. After 10 min. the pH was lowered to 7.5 with 0.1 N-HCl, 0.45 ml. of 0.5 M-sodium phosphate buffer, pH 7.5, was added, the volume was made up to 4.5 ml. in a graduated tube and enzyme activity and fluorescence were determined.

Inactivation of enzyme and production of fluorescence at low pH values as a function of time. Purified enzyme (2 mg.) was dissolved in 40 ml. of deionized water and the pH lowered to either 3.25, 3.10 or 2.90 as rapidly as possible with 0.01 N-HCl. At suitable time-intervals 2 ml. samples were removed and mixed with sodium phosphate buffer, pH 7.5, and made to a final volume of 3 ml. (final buffer concn. 50 mM, final pH 7.5). For enzyme assay, 0.5 ml. was re-

moved; this was replaced with 0.5 ml. of deionized water and the fluorescence measured.

Inactivation of enzyme and production of fluorescence as a function of temperature. Samples (2 ml.) of purified enzyme solution (1 mg. of protein/ml. of deionized water) were rapidly heated to the required temperatures. After the samples had been kept at the elevated temperatures for exactly 5 min., each was poured into 2 ml. of 10 mM-sodium phosphate buffer, pH 7.5, in an ice bath. The final temperature of the 100° sample after this treatment was 50°, at which temperature the enzyme is stable. Any precipitate was removed by centrifugation and the final volume adjusted to 4 ml. (to compensate for any water loss); 0.25 ml. was removed for assay of enzyme activity and the remainder was used for the measurement of fluorescence.

Inactivation of enzyme and production of fluorescence by high temperature as a function of time. Purified enzyme (10 mg.) was dissolved in 1 ml. of deionized water; at zero time this 1 ml. of enzyme solution was added to 9 ml. of sodium phosphate buffer, pH 7.5 (final concn. 50 mM), at the required temperature. At suitable time-intervals 2 ml. samples were withdrawn, immediately added to 2 ml. of ice-cold deionized water and centrifuged, and the supernatants assayed for enzyme activity and fluorescence.

Microbiological assay of the purified enzyme. We thank Dr J. E. Ford of the National Institute for Research in Dairying, Shinfield, Reading, for doing these assays. *Lactobacillus casei* was used for the assay of folic acid and derivatives and for riboflavine; *Lactobacillus arabinosus* was used for nicotinic acid, biotin and pantothenic acid; *Kloekera brevis* was used for vitamin B₆; *Lactobacillus fermenti* was used for thiamine and *Lactobacillus leichmanii* was used for vitamin B₁₂. The methods of Barton-Wright (1963) were used except for folic acid (Herbert, 1961) and vitamin B₁₂ (Skeggs, Hepple, Valentik, Huff & Wright, 1950). The forms of folic acid to which *L. casei* responds are given by Eigen & Shockman (1963) and by Harding (1966), and the forms of vitamin B₁₂ to which *L. leichmanii* responds are given by Guttman (1963). A stock solution for the assays was prepared by dissolving 100 mg. of purified enzyme in 25 ml. of deionized water; portions of this stock solution were pretreated in different ways before the microbiological assays. For riboflavine, thiamine, nicotinic acid, vitamin B₆ and biotin, 1 ml. of N-H₂SO₄ was added to 12 ml. of the stock solution and the mixture autoclaved at 10 lb./in.² for 10 min.; after cooling and neutralization the solution was made up to 50 ml. and assayed. For vitamin B₁₂, 1 drop of aq. 1% (w/v) NaCN solution was added to 2 ml. of stock solution and the pH adjusted to 5.5; this solution was steamed for 20 min., cooled and diluted to 20 ml. for assay. For pantothenic acid, 10 ml. of 50 mM-potassium phosphate buffer, pH 6.6, was added to 4 ml. of stock solution; this mixture was steamed for 10 min., cooled and diluted to 20 ml. for assay. For the folic acid group, 15 mg. of ascorbic acid were added to 4 ml. of stock solution and the pH adjusted to 3.0; the solution was then neutralized and diluted to 20 ml. for assay. This diluted mixture was autoclaved at 10 lb./in.² for 10 min. in the assay medium.

Fluorescence (emission) and excitation spectra. We thank Miss B. G. Gunlack for help with this work at the Marie Curie Research Foundation, Caterham, Surrey. Spectra (uncorrected) were measured and recorded on an Aminco-Bowman spectrophotofluorimeter at room temperature (about 20°). A xenon lamp and a type IP21 photomultiplier

were used with slit arrangement no. 4 and gain no. 5. (The spectra shown in Fig. 9 were obtained by subtracting the spectrum given by the solvent from that given by the sample.)

RESULTS

Evidence against a flavine prosthetic group. (1) Flavoproteins typically show a spectrum with two absorption bands in the region of 330–500m μ , usually with peaks at about 370m μ and 450m μ . The latter peak is often diminished by reduction of the flavoprotein with substrate or sodium dithionite. In this region, the spectrum of this alcohol dehydrogenase has only one peak, at 345–350m μ (Fig. 1). Continuation of the spectra shown in Fig. 1 up to 700m μ shows no peaks and the extinction is never higher than the value at 450m μ (E 0.05). The spectrum is unchanged by addition of methanol, sodium borohydride or sodium dithionite.

(2) One or more of the following substances can act as hydrogen or electron acceptor for the oxidation of reduced flavines: ferricyanide, methylene blue, tetrazolium salts, NAD(P), 2,6-dichlorophenol-indophenol, quinones, cytochromes, oxygen; however, none of these substances reacts with this alcohol dehydrogenase.

(3) When the purified enzyme was assayed microbiologically for vitamin content, no riboflavin was detected and no other microbial growth factor was present in significant quantity (Table 1). If riboflavin had been present in the proportion of 1 mole of riboflavin to 1 mole of enzyme (146000g.) there would have been at least 2.75mg. of riboflavin/g. of enzyme.

(4) On acid treatment or boiling, flavoproteins

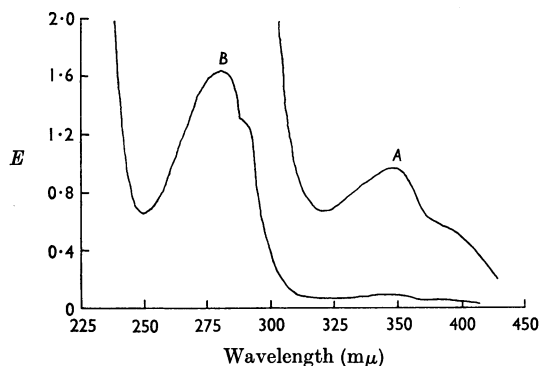


Fig. 1. Absorption spectrum of the purified enzyme in deionized water at pH 7.0. Silica cuvettes of 10mm. light-path were used and the reference cuvette contained deionized water. Continuation of the spectra up to 700m μ shows no peaks and the extinction is never higher than the value at 450m μ (E 0.05). A, 18.0mg. of protein/ml.; B, 1.8mg. of protein/ml.

typically liberate a riboflavin derivative that is fluorescent (yellow-green) and that exhibits a characteristic absorption spectrum with peaks at about 265, 370 and 450m μ . On similar treatment, however, this dehydrogenase liberates a green-fluorescent substance that shows no significant absorption above 400m μ .

The fluorescence maximum of riboflavin derivatives is in the region of 530m μ and the excitation spectrum is similar to the absorption spectrum with peaks at about 265, 370 and 450m μ . The green-fluorescent material from this enzyme, however, has excitation maxima at about 260m μ and 365m μ with the fluorescence maximum at about 460m μ (Figs. 7, 8 and 9, and Table 2).

(5) The classical type of resolution and reconstitution experiment done with flavoproteins (Warburg & Christian, 1938) was not successful with this dehydrogenase. Treatment with ammonium sulphate over the pH range 2–5 did not achieve reversible dissociation of the prosthetic group from the enzyme; enzyme activity was lost only when the pH was lowered to a value below 3.6. The presence of ammonium sulphate, methanol, FAD, FMN or riboflavin did not prevent this inactivation, and addition of high concentrations of these substances, after inactivation in their absence, did not achieve reactivation.

Kinetic studies on the liberation of the prosthetic group from the enzyme. In the experiments described above, the enzyme used was nearly 100% pure (Anthony & Zatman, 1967b). It is not fluorescent, but yields a fluorescent material, diffusible on dialysis, when treated with acid or alkali, or when it is boiled. The following experiments were designed to show whether or not there was a correlation between loss of enzyme activity and production of the fluorescent material from the enzyme preparations. Should such a correlation be shown to exist, it would suggest that either the fluorescent material itself, or a chemically related substance from which it was derived by the preparative treatment, is the prosthetic group of the enzyme.

In all the experiments in this section it was observed that whenever enzyme inactivation occurred some visible fluorescence was produced; in some cases this amount was too low to be recorded as a positive value on the fluorescence scale used in Figs. 2–6. Fig. 2 shows the result of incubating the enzyme at various pH values for 40min. At pH 3.3 there was 60% loss of enzyme activity while only about 8% of the maximum fluorescence was produced. At pH 3.2 almost complete loss of enzyme activity occurred, but the maximum fluorescence was not produced until the pH value was lowered to 2.9. Fig. 3 shows similar results obtained by using alkali instead of acid for inactivation of the enzyme with concomitant release of

Table 1. *Microbiological assay of the purified enzyme*

Purified enzyme (100 mg.) was dissolved in 25 ml. of deionized water and assayed for vitamins as described in the Materials and Methods section. For the vitamin content of the enzyme to be significant there would have to be at least 1 mg. of vitamin/g. of enzyme protein.

Vitamin	Test organism	Form of the vitamin to which the organism will respond	Maximum amount of vitamin that could be present in 1g. of enzyme ($\mu\text{g.}$)
Riboflavine	<i>Lactobacillus casei</i>	Riboflavine	2.5
Folic acid group	<i>Lactobacillus casei</i>	Wide range of folic acid derivatives including oxidized and reduced forms, C ₁ -substituted derivatives and polyglutamates (see the text for reference)	0.01
Vitamin B ₁₂	<i>Lactobacillus leichmanii</i>	Vitamin B ₁₂ , pseudovitamin B ₁₂ , factor A etc. (see the text)	0.04
Nicotinic acid	<i>Lactobacillus arabinosus</i>	Nicotinic acid and nicotinamide	2.5
Vitamin B ₆	<i>Kloekera brevis</i>	Pyridoxine, pyridoxal and pyridoxamine	0.1
Biotin	<i>Lactobacillus arabinosus</i>	Biotin, oxybiotin, biotin (+)-sulphoxide and <i>N</i> -biotinyl-lysine	0.0008
Thiamine	<i>Lactobacillus fermenti</i>	Thiamine	0.5

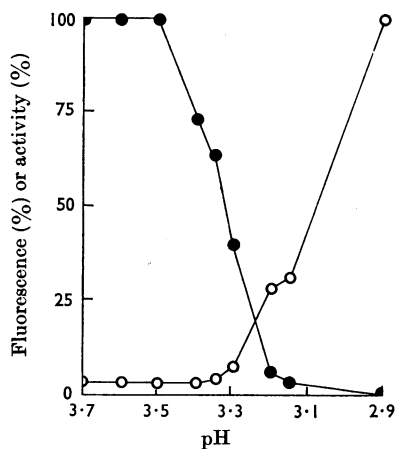


Fig. 2. Correlation of the production of fluorescence with inactivation of the enzyme by acid. Purified enzyme was incubated at room temperature for 40 min., the pH was raised to 7.5, the total volume was made up to a standard volume and activity and fluorescence were measured. ●, % of original enzyme activity; ○, % of maximum fluorescence.

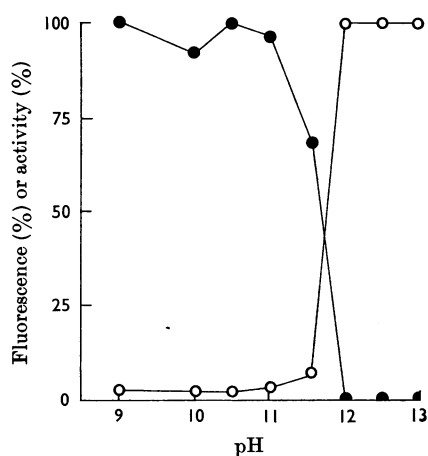


Fig. 3. Correlation of the production of fluorescence with inactivation of the enzyme by alkali. The conditions were similar to those described in Fig. 2 except that the incubation period at the high pH was 10 min. and the incubation mixtures were neutralized with acid before the addition of buffer. ●, % of original enzyme activity; ○, % of maximum fluorescence.

fluorescent material. Fig. 4 shows that enzyme inactivation at low pH values occurred at a slightly greater rate than did the production of fluorescent material. More significant is the similar enormous increase in rate of the two phenomena with small decreases in pH value (from 3.25 to 2.90; Fig. 4).

A further demonstration of the correlation between enzyme inactivation and the production of fluorescence was obtained by incubating the enzyme at various temperatures between 60° and 90° for 5 min. and removing samples into cold buffer for measurement of fluorescence and enzyme activity.

The rate of production of the fluorescent material was again lower than the rate of inactivation, as shown in Figs. 5 and 6; Fig. 6 also shows that the change with temperature of the rate of enzyme inactivation is again similar to the change with temperature in rate of production of the fluorescent material.

Although these results do not show whether or not the green-fluorescent material is part of the active site they do indicate that it arises from the enzyme protein and that it is associated in some way with

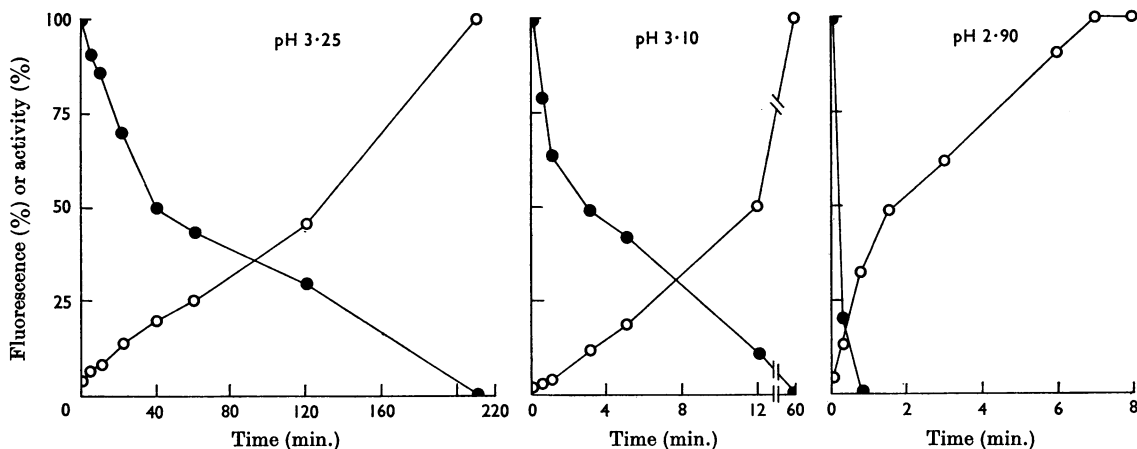


Fig. 4. Inactivation of the enzyme with production of fluorescence at low pH values as a function of time: 2 mg. of purified enzyme was dissolved in 40 ml. of deionized water and the pH lowered to the appropriate value as rapidly as possible. At suitable time-intervals 2 ml. samples were removed and mixed with sodium phosphate buffer, pH 7.5, to a final volume of 3 ml. (final buffer concn. 50 mM): 0.5 ml. was removed for enzyme assay, replaced with 0.5 ml. of deionized water and the fluorescence measured. ●, % of original enzyme activity; ○, % of maximum fluorescence.

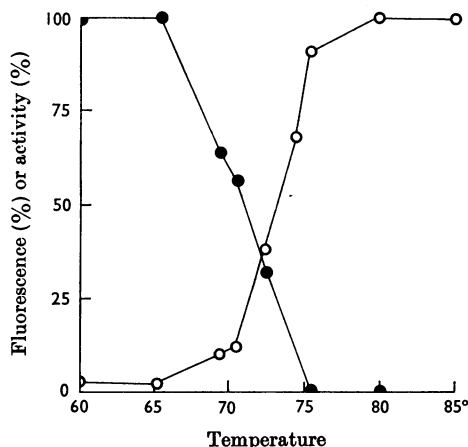


Fig. 5. Inactivation of enzyme as a function of temperature. Samples of enzyme were heated to the required temperature and maintained at that temperature for exactly 5 min. After dilution with ice-cold buffer the samples were assayed for activity and fluorescence. See the Materials and Methods section for full details. ●, % of original enzyme activity; ○, % of maximum fluorescence.

solution of the purified enzyme (about 10 mg./ml.) was boiled for 2 min., cooled and the heavy precipitate removed. The green-fluorescent material in the supernatant liquid was then adsorbed on a small column of TEAE-cellulose* (2 cm. \times 1 cm. diam.) equilibrated with 50 mM-ammonium acetate buffer, pH 6.0. The column was washed with the same buffer to remove protein and then with 0.5 M-ammonium acetate buffer, pH 6.0, to remove any trace amounts of blue-fluorescent material. The adsorbed green-fluorescent material, visible as a red-brown band, was eluted from the column with a linear gradient of 0.7–1.2 M-ammonium acetate buffer, pH 6.0. All the green-fluorescent fractions had the same u.v. absorption characteristics. These fractions were pooled, diluted five- to six-fold and adsorbed on a small pad of TEAE-cellulose (10 mm. diam., 5 mm. thick). All of the green-fluorescent material was then eluted from the pad with the minimum volume (usually less than 1 ml.) of 10.0 M-ammonium acetate buffer, pH 6.0. This concentrated material was freeze-dried to remove the ammonium acetate. The dried material, red-brown in colour, is referred to below as 'G-F fraction'. Although purified enzyme was used for all the work described in this paper, when using the technique for prosthetic group purification described above it was not necessary to start with pure enzyme. When the last stage in purification of the enzyme (i.e. removal of cytochrome *c* by gel filtration on

* Abbreviation: TEAE-cellulose, triethylaminoethyl-cellulose.

the activity of the enzyme. That the loss of enzyme activity and production of fluorescent material are not related in a perfectly linear manner indicates that heat or extreme pH values affect the structure of the enzyme in more than one way.

Purification of the prosthetic group. An aqueous

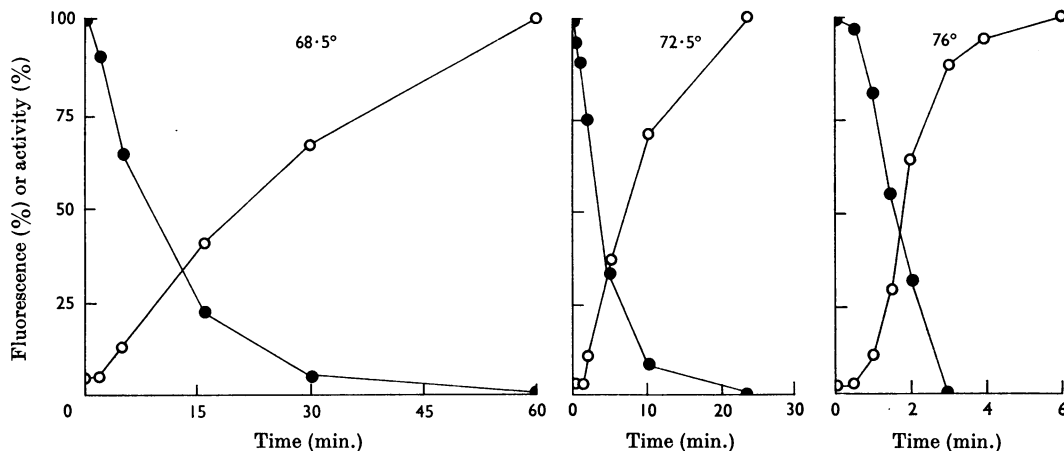


Fig. 6. Inactivation of enzyme by heat as a function of time. Enzyme (10 mg.) was dissolved in 1 ml. of deionized water and the solution added at zero time to 9 ml. of sodium phosphate buffer, pH 7.5 (final concn. 50 mM) at the required temperature. Samples (2 ml.) were removed at suitable time-intervals into 2 ml. of ice-cold deionized water, centrifuged and assayed for activity and fluorescence. ●, % of original enzyme activity; ○, % of maximum fluorescence.

Sephadex G-150) was omitted, the cytochrome *c* that remained in the boiled enzyme preparation was not adsorbed on the TEAE-cellulose and so passed completely through the column during washing with 50 mM-ammonium acetate buffer, pH 6.0.

Properties of the prosthetic group. At room temperature the purified prosthetic group, G-F fraction, is freely soluble in water, but not in methanol, ethanol, acetone, diethyl ether, light petroleum (b.p. 40–60°), hexane, cyclohexane or chloroform. When aqueous solutions of G-F fraction (at pH 3.0, 7.0 and 10.4) were shaken with water-immiscible solvents, the absorption spectrum and the fluorescence of the aqueous phase were unchanged. Methanol, ascorbic acid, sodium borohydride and hydrogen peroxide had no effect on fluorescence at pH 6.0 (in 20 mM-ammonium acetate). Sodium dithionite in large amounts quenched fluorescence, but this was restored on addition of hydrogen peroxide; as noted above, sodium dithionite has no effect on the absorption spectrum of this enzyme. The fluorescence of G-F fraction in water at pH 7.0 was green, in 0.1 N-hydrochloric acid it was blue-green and in 0.1 N-sodium hydroxide fluorescence was quenched; these effects were reversible in that fluorescence of the usual colour was restored when the pH was readjusted to 7.0.

Fluorescence and excitation spectra of G-F fraction in water at pH 7.0, in 0.1 N-hydrochloric acid and 0.1 N-sodium hydroxide are given in Figs. 7, 8 and 9. These spectra indicate the presence of two fluorescent compounds in G-F fraction. The major component (as indicated by amount of fluorescence) has excitation peaks at about 255 m μ

and about 365 m μ with a fluorescence maximum at about 460 m μ that is reversibly quenched in alkali. A summary of the spectra of the two components is given in Table 2. The absorption spectra of G-F fraction (Fig. 10) are difficult to interpret because of the probability, indicated by the fluorescence spectra, of two substances being present. If, as is usually the case, the excitation and absorption spectra of a given substance correspond, then the absorption spectra shown here can be considered to be the addition spectra of two substances, one absorbing under all conditions at about 255 m μ and 365 m μ , and the other absorbing at 310 m μ at pH 7.0 and in alkali but not in acid. This would account for the lower absorption at 310 m μ shown by G-F fraction in acid.

The properties of the prosthetic group described here are not sufficient for an identification, but they do provide considerable circumstantial evidence that the prosthetic group is a pteridine derivative (possibly related to pteroylglutamic acid). The apparent absence of a folate (or derivative) from the purified enzyme according to the microbiological assay results (Table 1) does not exclude this; many folate derivatives are unstable and such a prosthetic group may have been inactivated as a growth factor for *Lactobacillus casei* by the method used for its removal from the enzyme. Absorption spectra of many pteridine derivatives exhibit absorption maxima in the region 350–370 m μ (Mason, 1954; Rabinowitz, 1960), but the absorption spectra presented here do not conform with any of the published spectra seen by the authors; this is possibly because the spectra given in Fig. 10 are those of

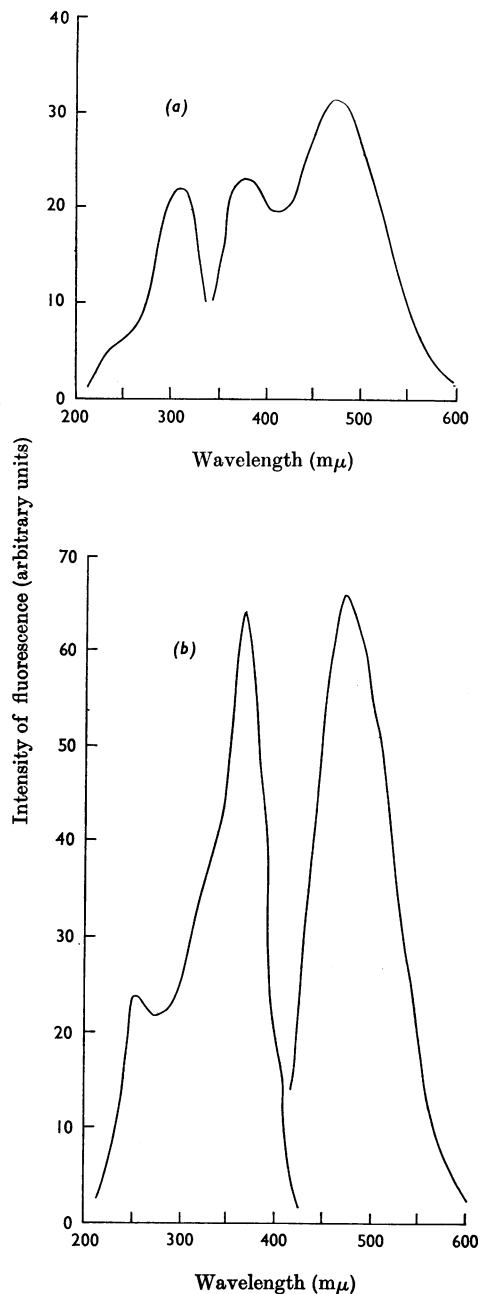


Fig. 7. Excitation spectra (left) and fluorescence spectra (right) of the prosthetic group (after some purification) in water at pH 7.0. (a) Excitation spectrum, with fluorescence measured at $370\text{ m}\mu$; fluorescence spectrum, with excitation at $310\text{ m}\mu$. (b) Excitation spectrum, with fluorescence measured at $470\text{ m}\mu$; fluorescence spectrum, with excitation at $365\text{ m}\mu$. The concentration of the fluorescent material and the arbitrary units of intensity of fluorescence are the same in Figs. 7, 8 and 9.

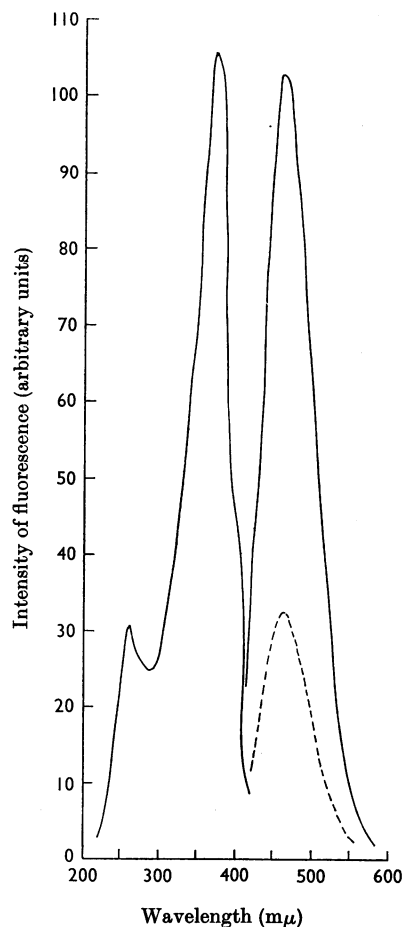


Fig. 8. Excitation spectrum (left) and fluorescence spectra (right) of the prosthetic group (after some purification) in 0.1 N-HCl . —, Excitation spectrum, with fluorescence measured at $470\text{ m}\mu$; fluorescence spectrum, with excitation at $360\text{ m}\mu$. ----, Fluorescence spectrum, with excitation at $260\text{ m}\mu$. The concentration of fluorescent material and the arbitrary units of intensity of fluorescence are the same in Figs. 7, 8 and 9.

more than one substance. It should be noted that the untreated purified enzyme has a characteristic absorption peak in the region of $350\text{ m}\mu$ (Fig. 1), as one might expect from a protein-bound pteridine. The fluorescence (excitation) and emission spectra presented here conform with published spectra for pteridine derivatives (for references see below). For purposes of comparison the fluorescence spectra of a range of folic acid derivatives that had been left at room temperature in the light at various pH values were obtained and all of these showed excitation peaks between $355\text{ m}\mu$ and $375\text{ m}\mu$ with emission

peaks at 450–470 μ . Fluorescence spectra of a total of about 200 pharmaceutical chemicals and chemicals of biological origin were published by Duggan, Bowman, Brodie & Udenfriend (1957), the American Instrument Co. Inc. (1963) and Williams & Bridges (1964). Only 15 of these emitted light between 445 μ and 490 μ and had excitation maxima between 355 μ and 385 μ ; only four of these compounds showed maximum fluorescence at a pH value below 9.0 and these were pteroyl-

glutamate, pterioic acid, 2,4-diaminopteroylglutamate (aminopterin) and 2,4-diamino-10-methylpteroylglutamate (amethopterin). Uyeda & Rabinowitz (1963) have published fluorescence data on 18 folate and pteridine derivatives. They have shown that the following all show maximum fluorescence below pH 9.0 and have excitation spectra with peaks between 355 μ and 385 μ , and emission peaks between 450 μ and 470 μ : pteroylglutamate, pterioic acid, 10-formylpteroylglutamate, 5,10-methylidynetetrahydropteroylglutamate, 4-amino-4-deoxypteroylglutamate, 2-amino-4-hydroxypteridine, 2-amino-4-hydroxy-6-methylpteridine, 2-amino-6-formyl-4-hydroxypteridine and 2-amino-6-carboxy-4-hydroxypteridine. It is noteworthy that the following reduced folate derivatives all have similar fluorescence characteristics to those of the minor components shown in Table 2, i.e. maximum fluorescence in neutral or alkaline solution with excitation peaks between 300 μ and 320 μ and emission peaks between 360 μ and 380 μ (Uyeda & Rabinowitz, 1963):

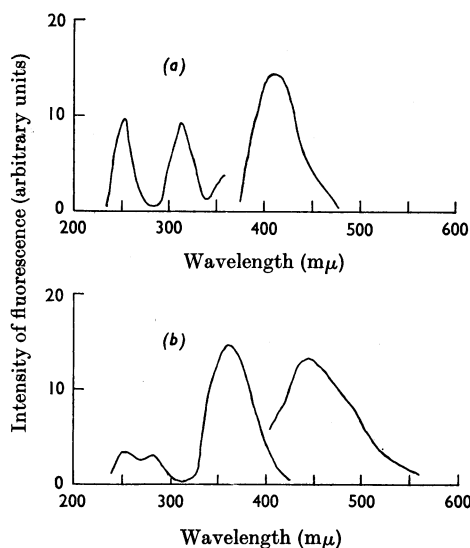


Fig. 9. Excitation spectra (left) and fluorescence spectra (right) of the prosthetic group (after some purification) in 0.1 N-NaOH. (a) Excitation spectrum, with fluorescence measured at 405 μ ; fluorescence spectrum, with excitation at 310 μ . (b) Excitation spectrum, with fluorescence measured at 450 μ ; fluorescence spectrum, with excitation at 365 μ . The concentration of fluorescent material and the arbitrary units of intensity of fluorescence are the same in Figs. 7, 8 and 9.

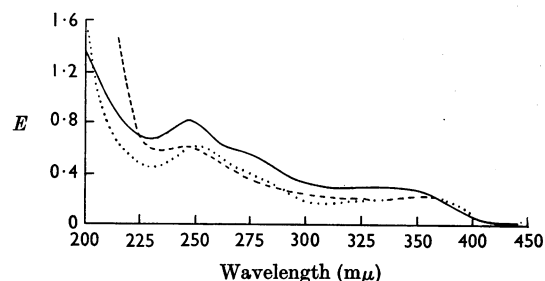


Fig. 10. Absorption spectra of the prosthetic group (after some purification). Silica cuvettes of 10 mm. light-path were used and the reference cuvettes contained solvent (water, acid or alkali). The concentration was the same for each spectrum but the actual concentration was not known. —, Water at pH 7.0; ·····, 0.1 N-HCl; ----, 0.1 N-NaOH.

Table 2. Summary of the fluorescence characteristics of the green-fluorescent prosthetic group

Spectra were determined as described in the Materials and Methods section. These data were summarized from Figs. 7, 8 and 9.

Conditions	Excitation maxima (μ)	Fluorescence maxima (μ)	Maximum intensity of fluorescence (arbitrary units)
Major component			
Water, pH 7.0	255, 365	470	66
0.1 N-HCl	260, 370	460	104
0.1 N-NaOH	250, 280, 360	450	13
Minor component			
Water, pH 7.0	310	375 (470)	23 (at 375 μ)
0.1 N-NaOH	250, 310	410	14.5

tetrahydropteroylglutamate, 5-formyltetrahydropteroylglutamate, 10-formyltetrahydropteroylglutamate, 5-formimidoyltetrahydropteroylglutamate, 4-amino-4-deoxytetrahydropteroylglutamate and *p*-aminobenzoic acid. The behaviour of the green-fluorescent material is very like that of degraded folic acid derivatives in general. For example, when tetrahydrofolic acid solution is left in air at room temperature in the light for more than a few minutes a reddish-brown derivative (structure unknown) is formed that is green-fluorescent and that adsorbs very strongly on TEAE-cellulose (Dr N. G. L. Harding and Dr M. A. Foster, personal communication); its fluorescence spectrum is very similar to that of the prosthetic group described here, having peaks at 365m μ (excitation) and 460m μ (emission).

An attempt to elucidate the structure of G-F fraction by mass spectrometry was unsuccessful because of the low volatility of the material.

DISCUSSION

That the alcohol dehydrogenase from *Pseudomonas* sp. M27 described here is not a flavoprotein and that it appears to contain a bound pteridine derivative makes the mechanism of action a matter of considerable interest. Of the two mechanisms discussed below the first hypothesizes that an enzyme-bound pteridine acts in a manner analogous to FAD or FMN in flavoproteins, whereas the other mechanism involves a folic acid type of function with binding of the alcohol to the N₍₅₎ atom of the pteridine ring. The nature of the natural hydrogen acceptor that is replaced in the assay system by phenazine methosulphate is at present unknown.

Pteridine derivatives have been implicated in a number of enzymic oxidizing systems (for references see below), but this alcohol dehydrogenase differs from these systems in that the pteridine hydrogen acceptor (if such it is) is bound tightly to the enzyme protein. Reactions that involve pteridine hydrogen-transfer coenzymes include the hydroxylation of phenylalanine to tyrosine (Kaufman, 1964a), the hydroxylation of tyrosine to dihydroxyphenylalanine (Kaufman, 1964b) and the oxidation of batyl alcohol (*D*- α -mono-octadecyl ether of glycerol) to glycerol and stearaldehyde (Tietz, Lindberg & Kennedy, 1964). In all of these cases the reaction mixture must contain a pteridine derivative, a reducing system, e.g. reduced NADP, and also molecular oxygen. The reduced pteridine acts as a hydrogen donor and an oxygen atom is introduced into the molecule undergoing oxidation. In the *Pseudomonas* alcohol dehydrogenase, however, molecular oxygen is not involved, a reducing system is not required and the bound pteridine is

presumably acting as a hydrogen acceptor. In the hydroxylation of phenylalanine a number of naturally occurring or synthetic 2-amino-4-hydroxy-pteridines can be used as cofactors, and Kaufman (1964a), using the synthetic pteridine 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, has shown that the oxidized (dihydro) form of this substance during the reaction is the quinonoid form. The bound form of a similar quinonoid intermediate might be involved in the mechanism of the 'pteridoprotein' oxidoreductase described in the present paper.

An alternative reaction mechanism can be postulated for this enzyme which links the function of pteridine derivatives as hydrogen-transfer coenzymes with their role as C₁ carriers (as folate derivatives). *Pseudomonas* sp. M27 (Anthony & Zatman, 1964a) is very similar to *Pseudomonas* AM1 (Peel & Quayle, 1961) and much less similar to *Pseudomonas methanica* (Peel & Quayle, 1961) or to the non-pigmented *Pseudomonas* sp. that grows on methylamine as sole source of carbon and energy but not on methanol (Shaw, Tsai & Stadtman, 1966). It is reasonable to suppose that the reactions involved in the assimilation of methanol by *Pseudomonas* sp. M27 are similar to those described in *Pseudomonas* AM1 (Large, Peel & Quayle, 1962; Large & Quayle, 1963), i.e. methanol is oxidized to the level of formaldehyde and is assimilated as 5,10-methylenetetrahydropteroylglutamate by addition to glycine, giving serine (mediated by serine hydroxymethylase). It is conceivable that the pteridine that is bound to the alcohol dehydrogenase responsible for the oxidation of methanol to the level of formaldehyde is in the form of a folate. The methyl group of the methanol might be bound to the N₍₅₎ atom of the pteridine ring and then oxidized to the 5,10-methylene derivative by using the added phenazine methosulphate as hydrogen acceptor; this would be essentially the reverse of the reactions involved in methionine biosynthesis (Woods, Foster & Guest, 1965). Alternatively, the methanol might be bound as the 5-methyldihydro derivative, followed by intramolecular rearrangement of the hydrogen atoms to give the 5,10-methylenetetrahydro derivative (Anthony, 1963), followed by oxidation, in the presence of the phenazine methosulphate, to the dihydro derivative; this could occur before or after transfer of the C₁ residue. This would be essentially the reverse of the reaction involved in the methylation of deoxyuridine to thymidine (Huennekens, 1963).

The 5,10-methylene derivative produced during the above reactions might be utilized as a substrate for serine hydroxymethylase, or be hydrolysed to free formaldehyde, or be oxidized further to the 5,10-methyldiyne derivative and thence to formate. The finding that formaldehyde is oxidized by the

purified alcohol dehydrogenase at about the same rate and with a similar K_m as that for methanol (A. Ladner & L. J. Zatman, unpublished work) lends support to the possibility that the methanol can be oxidized completely to the level of formate while bound as a folate derivative. The hypothesis, involving binding of the alcohol to the N₍₅₎ atom of a pterate ring followed by methylene-bridge formation, might also account for the unusual and well-defined substrate specificity of the purified enzyme (Anthony & Zatman, 1965). Secondary and tertiary alcohols are not oxidized and these would not be able to form the 5,10-methylene bridge. Further, it can be seen (by using molecular models) that substituted 5,10-methylene derivatives of all the alcohols that are oxidized are stereochemically possible; the 5,10-methylene derivatives of those alcohols that are not oxidized (except the amino alcohols) are, however, stereochemically impossible.

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