Control of 5-Aminolaevulinate Synthetase Activity in *Rhodopseudomonas spheroides*

BINDING OF PYRIDOXAL PHOSPHATE TO 5-AMINOLAEVULINATE SYNTHETASE

By RICHARD C. DAVIES and ALBERT NEUBERGER

Department of Biochemistry, Charing Cross Hospital Medical School, London W.6, U.K.

(Received 1 June 1978)

1. Pyridoxal 5'-phosphate is a cofactor essential for the enzymic activity of aminolaevulinate synthetase from *Rhodopseudomonas spheroides*. It also aids activation of the low-activity enzyme by trisulphides such as cystine trisulphide, whereas inactivation of enzyme is facilitated by its absence. 2. The fluorescence spectrum of purified high-activity enzyme is that expected for a pyridoxal phosphate-Schiff base, but the firmly bound cofactor does not appear to be at the active centre. In dilute solutions of enzyme this grouping is inaccessible to nucleophiles such as glycine, hydroxylamine, borohydride and cyanide, at pH 7.4. 3. An active-centre Schiff base is formed between enzyme and added pyridoxal phosphate, which is accessible to nucleophiles. Concentrated solutions of this enzyme-Schiff base on treatment with glycine yield apo- and semi-apoenzyme, which can re-bind pyridoxal phosphate. 4. Two types of binding of pyridoxal phosphate are distinguishable in dilute solution of enzyme, but these become indistinguishable when concentrated solutions are treated with cofactor. A change occurs in the susceptibility towards borohydride of the fluorescence of the 'structural' pyridoxal phosphate. 5. One or two molecules of cofactor are bound per subunit of mol.wt. 50000 in semiapo- or holo-enzyme. The fluorescence of pyridoxamine phosphate covalently bound to enzyme also indicates one to two nmol of reducible Schiff base per 7000 units of activity in purified and partially purified samples of enzyme. 6. Cyanide does not convert high-activity into low-activity enzyme, but with the enzyme-pyridoxal phosphate complex it forms a yellow fluorescent derivative that is enzymically active.

Purified samples of the high-activity form of 5-aminolaevulinate synthetase of *Rhodopseudomonas spheroides* had a specific activity of 170000 nmol of 5-aminolaevulinate formed/h per mg of protein, contained 5–6 cysteine residues per protein subunit of mol.wt. 49000, and showed an intrinsic yellow fluorescence under u.v. light (Davies & Neuberger, 1979). This fluorescence was not introduced from pyridoxal 5'-phosphate that was added to stabilize the enzyme, and must have been present in the native enzyme. A similar yellow fluorescence had been reported for the enzyme-pyridoxal phosphate complex isolated from *R. spheroides* strain Y (Fanica-Gagnier & Clement-Metral, 1973a) and seemed to belong to pyridoxal phosphate bound to the active centre of 5-aminolaevulinate synthetase.

Nearly all examples of pyridoxal phosphate-dependent enzymic reactions proceed via an intermediate Schiff base formed between the aldehydic group of the cofactor and an amine group of a substrate or an enzyme. For 5-aminolaevulinate synthetase, enzyme-bound pyridoxal phosphate has

been shown to bring about the exchange with the solvent of one hydrogen atom of the substrate glycine or of the product aminolaevulinate (Laghai & Jordan, 1976, 1977; Akhtar *et al.*, 1976). This agrees with a mechanism of action that involves the subsequent condensation of a thiol ester, succinyl-CoA, with the activated nucleophilic carbanion of glycine bound to pyridoxal phosphate, i.e. $-\text{CH}=\text{N}-\text{CH}^--\text{CO}_2^-$ (see Neuberger, 1961; Jordan & Shemin, 1972). Activation of glycine is brought about by the electron-withdrawing effect of an enzyme-bound conjugated Schiff base. Since the aldehydic group is involved in the enzymic reaction, the other groups of pyridoxal phosphate must also play a significant part in binding to the protein. But the enzyme-cofactor link cannot be very stable during the reaction since excess pyridoxal phosphate is always required for enzymic activity. The original enzyme-pyridoxal phosphate linkage is most likely to involve a protein lysine ϵ -amino group (Davis & Metzler, 1972) and to be an aldimine, an aldamine or a carbinolamine, although Scholnick *et al.* (1972)

have proposed an alternative thiohemiacetal grouping. The aldamine is probably formed from an amino group and XH of the protein, where X is an unknown nucleophilic group that is often considered to be a protein thiol group. Pyridoxal phosphate seems to protect an essential thiol group from reaction with thiol-specific reagents in the 5-aminolaevulinate synthetase from rat liver (Scholnick *et al.*, 1972) and initial formation of a thiohemiacetal or a thiazolidine-type of grouping has been proposed (Fanica-Gaignier & Clement-Metral, 1973a). Group X may, of course, be an amino group of another lysine or yet another nucleophilic group, such as histidine (Hathaway & Noltman, 1977). It is unlikely that most of the pyridoxal phosphate is bound in a non-covalent complex, although the participation of such a complex has been detected in the reaction of ornithine transcarbamoylase with pyridoxal phosphate (Marshall & Cohen, 1977).

Pyridoxal phosphate has been used to stabilize 5-aminolaevulinate synthetase obtained from several sources. The absorption spectra in the 420–450 nm region indicated that an enzyme–pyridoxal phosphate complex is definitely formed with the enzymes isolated from chick embryo liver mitochondria (Whiting & Granick, 1976) and from *R. spheroides* strain Y. For the latter enzyme Fanica-Gaignier & Clement-Metral (1973a) have proposed that an equilibrium exists between Schiff base and aldamine forms that is sensitive to pH and to the concentration of thiol compounds. These workers suggested that the mode of binding of pyridoxal phosphate is similar to that found in phosphorylase. If this were so, then it is interesting that a similar linkage of pyridoxal phosphate to protein plays a catalytic role in the activity of 5-aminolaevulinate synthetase, but probably has only a structural function in phosphorylase (Steiner *et al.*, 1977). It is assumed that in enzyme from *R. spheroides* strain Y the bound pyridoxal phosphate is at the active site and is not reduced by borohydride at pH 7, but is reduced at pH 5.6. Jordan & Shemin (1972), on the other hand, reported that the active centre is modified by reduction with borohydride at pH 7, but only when an excess of pyridoxal phosphate was present. These results suggest that two populations of pyridoxal phosphate-binding sites may well be present in native 5-aminolaevulinate synthetase.

By studying samples of 5-aminolaevulinate synthetase in the presence and absence of pyridoxal phosphate, it may be possible to differentiate between the two populations of pyridoxal phosphate-binding sites, and to clarify the role played by pyridoxal phosphate in the enzymic reaction and in the inter-conversion of enzymic forms. We have therefore examined the binding of pyridoxal phosphate to our samples of high-activity enzyme by gel disc electro-

phoresis, absorption spectra of form Ia samples, fluorescence, estimation of bound pyridoxal phosphate by phenylhydrazine, reduction of Schiff bases with borohydride and by reaction of Schiff bases with cyanide. We show that the fluorescence belongs to a bound molecule of pyridoxal phosphate, but does not seem to be associated directly with enzymic activity. A structural function for pyridoxal phosphate in 5-aminolaevulinate synthetase may be indicated.

Materials and Methods

NaBH₄ was from BDH Chemicals, Poole, Dorset, U.K. Phenylhydrazine hydrochloride was treated with charcoal and was re-crystallized three times from dilute HCl. Other chemicals and methods used for the investigation of 5-aminolaevulinate synthetase were as given in the previous paper (Davies & Neuberger, 1979), e.g. preparation of crude extracts of enzyme, enzyme assays, gel disc electrophoresis in three buffer systems, detection of enzyme activity and fluorescence on polyacrylamide gels and gel filtration on Sephadex G-100.

Protein was measured in solutions that were dialysed against 1 mM-potassium phosphate, pH 7.4, by the method of Lowry *et al.* (1951), with bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard.

Enzyme

Samples of the purified high-activity form of 5-aminolaevulinate synthetase (130 000–170 000 nmol of 5-aminolaevulinate formed/h per mg of protein at 37°C) were prepared from *R. spheroides* grown semi-anaerobically in light by the method reported by Davies & Neuberger (1979). Partially purified preparations of enzyme (fraction Ia; sp. activity 50 000–90 000 nmol of 5-aminolaevulinate formed/h per mg of protein) were the fractions that were eluted first from a column of DEAE-Sephadex A-25 with a gradient of potassium phosphate, pH 7.4, 5 mM-mercaptoethanol (Sandy *et al.*, 1975). One unit of activity corresponds to the formation of 1 nmol of 5-aminolaevulinate/h at 37°C.

Absorption and fluorescence spectra

These were recorded with a Unicam SP.800 spectrophotometer and a Perkin-Elmer MPF-3 spectrofluorimeter. Fluorescence spectra were recorded at 0°C for the native enzyme and at room temperature for the modified enzymes. Emission spectra were recorded at 10–100% sensitivity settings with a band width of 8 nm (1 mm slits) at excitation wavelengths of 420 and 330 nm, and were uncorrected for photomultiplier sensitivity and spectral efficiency of the monochromator.

Other methods

Treatment with hydroxylamine. Concentrated solutions of enzyme (fraction Ia) in 0.15M-potassium phosphate, pH7.4, containing 5mM-2-mercaptoethanol, were divided into two portions. One half served as a control, whereas the other half (1.4ml) was treated with 0.1M-hydroxylamine (0.15ml) in 0.15M-potassium phosphate buffer, pH7.3, at 0°C for 30min. The mixture was dialysed against three changes of 300ml of the same buffer containing 10mM-hydroxylamine over 4h and was dialysed against 1mM-hydroxylamine in buffer overnight. Finally, the solution was dialysed against several changes of buffer (1 litre) over 24h. The solution still showed a yellow fluorescence under u.v. light.

Treatment with borohydride and reduction of Schiff bases. Solutions of enzyme in 0.15M-phosphate buffer, pH7.3, 5mM-2-mercaptoethanol, were pre-incubated with various concentrations of pyridoxal phosphate (0–200 μ M) at 0°C for at least 1h. The solutions (2.5–2.7ml) were treated with 5mM-sodium borohydride (25 μ l, 0.5M-NaBH₄ in 1mM-NaOH) at room temperature for 30min, and then dialysed against three changes of the same buffer (500ml) at 0°C over 24h away from bright light. They were then dialysed against several changes of buffer (2 litre) over 2 days. For examination of the changes in the enzymic activity and in the gel-disc-electrophoretic pattern, the solutions were dialysed against 1mM-potassium phosphate, pH7.4, containing 0.1mM-pyridoxal phosphate and 2.5mM-2-mercaptoethanol. For experiments concerned with the changes in fluorescence and enzymic activity the solutions were dialysed against 0.1M-potassium phosphate buffer, pH7.4, containing 5mM-2-mercaptoethanol. The fluorescence intensity of pyridoxamine phosphate bound covalently to protein was compared with a range of standard solutions (0.5–2.0 μ M) of pyridoxamine phosphate in the same buffer solution.

Results

Partially purified preparations of enzyme in the Ia form have been routinely stabilized by the addition of pyridoxal phosphate. Fánica-Gaignier & Clement-Metral (1973a) reported that pyridoxal phosphate could replace 2-mercaptoethanol very effectively, whereas we found both were needed to stabilize our samples of fraction Ia. Glycerol (10% v/v) was without beneficial effect over the pH range 5.6–8.0, and enzyme samples (3000units/ml) were unstable outside this range in dilute solution. Enzyme was readily precipitated at pH values less than 6. Consequently we have examined the enzyme mainly at pH7.3–7.5 and in the presence of 5mM-2-mercaptoethanol.

Requirement for pyridoxal phosphate in the assay system

The substrates and cofactors for 5-aminolaevulinic synthetase activity are glycine, succinyl-CoA and pyridoxal phosphate. In all our experiments succinyl-CoA was generated from succinate, CoA and ATP by the addition of succinic thiokinase. Enzymes were assayed in Tris buffer (25mM), pH7.8, containing 4.1mM-ATP and 6mM-Mg²⁺. They were not inhibited more than 30% by 12mM-ATP, apparently in contrast with the enzyme isolated from *R. sphaeroides* strain Y and studied by Fánica-Gaignier & Clement-Metral (1971, 1973b), which was inhibited about 75% by 1mM-ATP. No marked effects were observed on the activity of enzyme fraction Ia on doubling or trebling the concentration of buffer. Activities in phosphate buffer were only 65% of the activities in Tris, which were in turn about 10% higher than the activities in veronal buffers, so that phosphate seemed to inhibit the activity a little. The K_m values for glycine increased from 12.5mM in veronal to 15mM in Tris and to 32mM in phosphate buffer, so that phosphate ions seem to interfere with the binding of glycine. Assay solutions containing Tris and glycine have most of the added cofactor, pyridoxal phosphate, bound as Schiff bases to the non-protein bases, which give a deep-yellow colour to the solutions. Enzyme must compete with these buffer-pyridoxal phosphate and substrate-pyridoxal phosphate complexes for reaction with pyridoxal phosphate. The apparent K_m values (3–5 μ M) for pyridoxal phosphate were not significantly different in phosphate or Tris buffer, but these concentrations were 50 times greater than the concentration of enzyme used in the assay.

Enzyme that was dialysed exhaustively against buffer at pH7.4, or which was treated with 10mM-hydroxylamine in buffer for 30min and dialysed, had only 1% activity in assays lacking added pyridoxal phosphate, but had full activity in the presence of 0.17mM-pyridoxal phosphate. Similarly, enzyme treated with 1M-glycine at pH6.1 or 7.5 and dialysed exhaustively against glycine buffer had only 5% activity without pyridoxal phosphate and full activity with pyridoxal phosphate added, even in assays lasting only 10min. Hence, if treatment with the nucleophilic molecules had dissociated pyridoxal phosphate from the active centre, then the apoenzyme could re-combine with added pyridoxal phosphate very quickly.

Assays of crude samples of low-activity enzyme from oxygenated cells with and without cystine trisulphide (5 μ M), and assays of high-activity enzyme, all showed a similar dependence of enzymic activity on the concentration of pyridoxal phosphate, so that the differences in the activities of the low-activity

and high-activity forms were not due to a deficiency of pyridoxal phosphate.

Role of pyridoxal phosphate in activation

Previous work has shown that crude samples of low-activity enzyme and some partially purified preparations were activated by extracts of the natural activator only in the presence of pyridoxal phosphate, succinate or the complete substrate mixture (Neuberger *et al.*, 1973). We have confirmed this with cystine trisulphide as the 'activator' molecule. In the absence of these cofactors the trisulphides evidently reacted with the enzyme, inactivated it, and prevented subsequent activation by trisulphide and substrates. Similar findings have been observed with the low-activity enzyme (100000g supernatant) and the selenodisulphide analogue of glutathione trisulphide (0.5 μ M; Sandy *et al.*, 1975), but only pyridoxal phosphate and not succinate was needed to activate the enzyme fully before it was assayed (Table 1). In the absence of 0.1 mM-pyridoxal phosphate low concentrations (1–10 μ M) of selenodisulphide left the basic activity unchanged, but prevented the subsequent activation by trisulphide and substrates. As the concentration was increased (from 10 to 100 μ M), the

basic activity was decreased as well. With aged samples of low-activity enzyme, which was present in solutions centrifuged at 400000g, activation was enhanced by small amounts (10–100 μ M) of 2-mercaptoethanol added together with trisulphide or selenodisulphide, suggesting that thiol groups were also required for the interaction with trisulphides. The role of pyridoxal phosphate may be to direct the trisulphides to particular groups on the enzyme, and perhaps to the cross-linking of specific thiol groups. The key to understanding the interconversion of the various forms of 5-aminolaevulinate synthetase may lie in the conformation of the protein around the cofactor-binding regions, which might be affected by both the number and location of the thiol and disulphide groups. Hence the binding of pyridoxal phosphate to 5-aminolaevulinate synthetase was examined.

Absorption and fluorescence spectra

Samples of fraction Ia were prepared in the absence of pyridoxal phosphate (Davies & Neuberger, 1979) and at enzyme concentrations of 55000 units/ml. The absorption spectrum in phosphate buffer, pH 7.4, showed peaks at 420 and 340 nm, that appeared similar to those peaks reported for other samples of 5-aminolaevulinate synthetase and that were generally considered to indicate the presence of pyridoxal phosphate-Schiff bases (Fanica-Gaignier & Clement-Metral, 1973a; Whiting & Granick, 1976). This fraction Ia solution in the absence of added pyridoxal phosphate had a fluorescence-emission spectrum

Table 1. Effect of some aminolaevulinate synthetase substrates on the activation of enzyme by glutathione selenodisulphide

Tubes containing crude low-activity enzyme (2.5 mg of protein) were mixed with the compounds listed and water to make a total volume of 500 μ l and were incubated at 37°C. After the initial incubation, samples (100 μ l) were added to assay tubes containing complete substrate mixture (1 ml) and were assayed for aminolaevulinate synthetase activity at 37°C for 60 min. Concentrations were: pyridoxal phosphate (0.09 mM), succinate (0.05 M). Concentrations of glutathione selenodisulphide activator were: (a) and (b) 10^{-5} M; (c) 10^{-4} M. In column (b) all samples were assayed with 10^{-5} M-activator added to the assay solution. Times for initial incubation were 15 min for (a) and (b) and 30 min for (c).

Addition to enzyme for initial incubation	Aminolaevulinate synthetase activity (nmol/h per mg of protein)		
	(a)	(b)	(c)
None	30	73	30
Activator	25	28	21
Pyridoxal phosphate	33	67	
Pyridoxal phosphate and activator	62	66	57
Succinate			28
Succinate and activator			30
Pyridoxal phosphate, succinate and activator	72	72	64

Fig. 1. Fluorescence-emission spectra of purified aminolaevulinate synthetase

Samples of aminolaevulinate synthetase (130000 units/mg of protein) were concentrated to 11600 units/ml by DEAE-Sephadex A-25 in 0.15 M-potassium phosphate, pH 7.4, containing 5 mM-2-mercaptoethanol. Spectra were uncorrected and were recorded at 1°C with 8 nm slit widths, 30% sensitivity, and were excited by light of wavelength 330 nm (—) and 420 nm (---).

with a peak at 495 nm and a shoulder at 510 nm when excited at 420 nm, and had a fluorescence spectrum with a peak of approximately similar intensity at 380 nm when excited at 330 nm (Fig. 1). The excitation maxima were at 330 and 420 nm. The fluorescence spectra were not those expected for NADH, riboflavin, oxidized or reduced flavins (Guilbault, 1973; Ghisla *et al.*, 1974), but were probably those of a pyridoxal phosphate derivative. The spectra resembled those reported for 5-aminolaevulinic synthetase by Fanica-Gaignier & Clement-Metral (1973a). The fluorescence was removed by acidification of the solutions to pH 5.6 and was not restored by readjustment to pH 7. However, interpretation is complicated by precipitation of the protein at pH values less than 6.

The yellow fluorescence at 495 nm was unchanged by treatment of the enzyme at 30000 units/ml with hydroxylamine. This procedure should have removed any loosely bound pyridoxal phosphate and produced apoenzyme. Apparently a semi-apoenzyme was present that still had a bound molecule of pyridoxal phosphate [cf. forms of the aminotransferases studied by Yonaha *et al.* (1975) and by Misono & Soda (1977)] that was unable to react with hydroxylamine.

The absorption spectra of dilute solutions of enzyme were unobtainable in the presence of pyridoxal phosphate (10–100 μ M), but the long-wavelength fluorescence excited at 420 nm could be examined. The fluorescence peak at 495 nm in dilute solutions of enzyme (8000 units/ml) was hardly affected by the addition of 0.1 mM-pyridoxal phosphate as the fluorescence of the enzyme and pyridoxal phosphate solutions were additive. No new fluorescent band has been observed in the presence of pyridoxal phosphate in dilute solution. Gel disc electrophoresis clearly showed the presence of the yellow fluorescent protein in concentrated solutions and the fluorescent band was associated with enzymic activity.

In one type of experiment the fluorescence and enzymic activity were partially separated. Concentrated samples of fraction Ia (150000 units; 4 ml) were subjected to slab-gel electrophoresis in glycine buffer, pH 7.5 (Davies & Neuberger, 1979). It was found that 35% of the enzyme activity was associated with the fluorescent species ($R_F = 0.28$ – 0.30) and 65% with the non-fluorescent form that ran just behind it ($R_F = 0.26$ – 0.28). The enzymes were extracted with phosphate buffer containing pyridoxal phosphate and gel-filtered on Sephadex G-100. The apparent molecular weights were similar and the specific activities were 180000 units/mg for the apoenzyme, and 125000 units/mg for the fluorescent enzyme. The samples in pyridoxal phosphate were concentrated with dry Sephadex G-200, dialysed and

re-examined by gel disc electrophoresis. The enzyme that was originally non-fluorescent ran as a single band of protein and was now fluorescent in the Tris system, pH 8.9, but did not remain fluorescent in the glycine system, pH 7.4. The protein that was originally fluorescent behaved as a single fluorescent protein in both systems. Thus glycine had removed some pyridoxal phosphate from the enzyme and electrophoresis had partially resolved holo- and apoenzymes. The resolved apoenzyme could re-bind pyridoxal phosphate to give a fluorescent protein from which pyridoxal phosphate was not removed by Tris, but was removed by glycine. The intrinsic fluorescence observed on polyacrylamide gels was therefore almost certainly due to the binding of pyridoxal phosphate. Imidazole/veronal buffers, pH 7.5, were used in preparative slab-gel electrophoresis to avoid this partial resolution of forms and to avoid a decrease in the already small amounts of enzyme available for analysis. The intrinsic or firmly bound molecule of pyridoxal phosphate probably lies outside the active centre and appears to differ from the pyridoxal phosphate that has to be added for the enzymic reaction to occur.

The addition of excess pyridoxal phosphate (0.1 mM) to concentrated solutions of fraction Ia (40000 units/ml) caused the fluorescent protein to migrate slightly faster than it did in the absence of pyridoxal phosphate during gel electrophoresis in imidazole buffer, and the enzymic activity was spread over both bands of protein. This clearly showed that an interaction had occurred between enzyme and added pyridoxal phosphate. At 40000 units/ml perhaps a dimeric form of enzyme-pyridoxal phosphate was present in samples with excess pyridoxal phosphate (Nandi & Shemin, 1977). At lower concentrations of enzyme only one active fluorescent band was ever observed.

Samples of fraction Ia obtained from enzyme that was present in oxygenated cells, but that had become activated during gel-filtration on Sephadex G-200 also showed the intrinsic fluorescence. Compared with samples of fraction Ia from semi-anaerobically grown cells the fluorescence maximum was of approximately the same intensity, but was associated with only half the enzymic activity. This is consistent with the findings that the low-activity form of the enzyme in oxygenated cells had a specific activity that was approximately half that of the low-activity form present in semi-anaerobically grown cells (Sandy *et al.*, 1975), and with the finding that only half the enzymic activity was associated with the amount of radioactive pI 5.55 species isolated from oxygenated cells when compared with the amount isolated from anaerobic cells (Davies & Neuberger, 1979). This suggests that a modified and inactive form of the enzyme may also have been formed on oxygenation.

Table 2. *Effect of borohydride and pyridoxal phosphate on aminolaevulinate synthetase*

Samples of partially purified enzyme (fraction Ia) and samples treated with hydroxylamine (prepared as described in the Materials and Methods section) were divided into two. One half was treated with 0.1 mM-pyridoxal phosphate in 0.15M-potassium phosphate buffer, pH7.3, 1.5mM-2-mercaptoethanol, and the other half was mixed with buffer. Total volume was 2.7ml. All samples were incubated at 0°C for 60min and treated with 5mM-NaBH₄ at 20°C for 30min. Solutions were dialysed exhaustively against 0.15M-potassium phosphate buffer containing 5mM-2-mercaptoethanol, and the yellow fluorescence was either measured at 495 nm (λ excitation 420nm) or observed under u.v. light. Samples were withdrawn and assayed for aminolaevulinate synthetase activity at 37°C. Protein concentrations were: Expt. (a), about 0.1 mg of protein/ml; Expt. (b), about 0.4mg of protein/ml. The yellow fluorescence was strong (++), moderately strong (+) or negligible (-). N.d., not determined.

	Aminolaevulinate synthetase activity (nmol/h per ml of solution)		
	Expt. (a)		Expt. (b)
	Before reduction	After reduction	After reduction
Native enzyme	4810 (+)	4620 (+)	32700 (++)
Native enzyme and pyridoxal phosphate	5580 (N.d.)	850 (+)	7100 (-)
Hydroxylamine-treated enzyme	7420 (+)	6440 (+)	25500 (++)
Hydroxylamine-treated enzyme and pyridoxal phosphate	8160 (N.d.)	1030 (+)	12000 (-)
Pyridoxal phosphate	0 (-)	0 (-)	0 (-)

Fig. 2. *Effect of borohydride and pyridoxal phosphate on aminolaevulinate synthetase activity*

Enzyme fraction Ia (6000nmol of aminolaevulinate/h per ml) was incubated with increasing concentrations of pyridoxal phosphate in 0.15M-potassium phosphate buffer, pH7.4, containing 2.5mM-2-mercaptoethanol, and treated with 5mM-NaBH₄ at 20°C for 15 min. The solutions were dialysed against buffer and assayed for enzymic activity. Solutions to which enzyme was added last to mixtures of pyridoxal phosphate (0.1mM) and borohydride showed unchanged enzymic activity (6000units/ml).

tion of cell cultures, and this form was not activated by trisulphide (Sandy *et al.*, 1975).

Reduction with borohydride

Treatment of enzyme either at 40000units/ml or at 8000units/ml in the absence of added pyridoxal

phosphate at pH7.5 with 5mM-NaBH₄ altered neither the enzymic activity nor the yellow fluorescence of the enzyme when this was either measured directly in solution or was observed after gel disc electrophoresis. Neither had borohydride any effect on samples of semi-apoenzyme prepared by treatment with hydroxylamine (Table 2). Samples were fully active after exhaustive dialysis when assayed with pyridoxal phosphate, but were inactive (less than 3%) without added pyridoxal phosphate.

However, reductions of enzyme and semi-apoenzyme carried out in the presence of increasing concentrations of pyridoxal phosphate led to a marked decrease in the enzymic activity in agreement with the work of Jordan & Shemin (1972). Half the activity was lost with approx. 22μM-pyridoxal phosphate with enzyme at both the above concentrations (Fig. 2), and pyridoxamine phosphate was bound covalently to the enzyme. Hence the added pyridoxal phosphate had interacted with the enzyme and a reducible Schiff-base grouping had been formed that was not present in the original preparation. This Schiff base is considered to involve pyridoxal phosphate at the active centre.

On the other hand, the intrinsic fluorescent grouping is thought to involve a structural molecule of pyridoxal phosphate lying outside the active centre, since the intrinsic yellow fluorescence was unaltered when enzyme samples at 10000units/ml were treated with borohydride in either the presence or the absence of pyridoxal phosphate. When borohydride was added to the more concentrated solutions of enzyme (32000units/ml) in the presence of added pyridoxal phosphate the intrinsic yellow fluorescence was lost along with the enzymic activity. Treatment

of enzyme with pyridoxal phosphate had altered the mode of binding of the intrinsic fluorescent grouping and made it accessible to reduction. This only occurred at high concentrations of enzyme when dimeric molecules were probably present (Nandi & Shemin, 1977). Perhaps the addition of pyridoxal phosphate and the probable dimerization of the enzyme affected the environment of the intrinsic cofactor, i.e. caused a conformational change of protein structure around the region of the marker group and now masked differences between the two types of binding of pyridoxal phosphate.

Covalently bound pyridoxamine phosphate

After reduction of fraction Ia to which 200 μ M-pyridoxal phosphate had been added, the fluorescence intensity of the bound pyridoxamine phosphate (λ_{max} , emission 392 nm, λ excitation 330 nm) was about 26 times greater than the intrinsic yellow fluorescence (λ_{max} , emission 495 nm, λ excitation 420 nm) and could be measured quite easily in dilute solution (Fig. 3).

Samples of this modified enzyme were eluted from DEAE-Sephadex at pH 7.4 just behind the residual (3%) unchanged active enzyme. The species that had fluorescence-emission maxima at 395 and 495 nm moved together. Thus the modified proteins were slightly more acidic than untreated 5-aminolaevulinate synthetase and both fluorescent groupings were present in the same protein.

Fig. 3. Fluorescence emission spectra of aminolaevulinate synthetase reduced in the presence of pyridoxal phosphate

Enzyme fraction Ia (12600 units/ml) in 0.1 M-pyridoxal phosphate, 0.15 M-phosphate buffer, pH 7.4, containing 5 mM-2-mercaptoethanol, was treated with NaBH_4 (5 mM) and was dialysed. For details see the text. Spectra were uncorrected and were recorded with 8 nm slit widths, 30% sensitivity. The excitation wavelength was 330 nm (A) and 420 nm (B). —, Sample treated with pyridoxal phosphate and borohydride; ----, sample treated either with borohydride alone or sample to which enzyme was added last to a mixture of borohydride and pyridoxal phosphate.

The amount of cofactor covalently bound to the enzyme by treatment with borohydride and the accompanying loss of enzymic activity were measured for enzyme fraction Ia and for samples of highly purified 5-aminolaevulinate synthetase. The fluorescence intensity recorded at 395 nm relative to free pyridoxamine phosphate at pH 7.5 was not assumed to be quenched. Solutions of bovine serum albumin plus pyridoxal phosphate were treated similarly. The results of two experiments are shown in Table 3.

For samples of highly purified enzyme, originally at 5000 units/ml, but only 2500 units/ml after extensive dialysis in the absence of pyridoxal phosphate at the end of the experiment, half of the activity was lost at 25 μ M-pyridoxal phosphate and approx. 14000 units of activity were lost per nmol of pyridoxamine phosphate bound. In the presence of 0.1 mM-pyridoxal phosphate about 8 nmol of cofactor was bound per mg of protein at pH 7.5 and no loss of the intrinsic fluorescence was observed.

For samples of fraction Ia at 8000 units/ml, 6000 and 10000 units of activity were lost from the aged and original samples respectively for each nmol of cofactor bound, again with no loss of intrinsic fluorescence. The instability of the control enzyme after extensive dialysis without added pyridoxal phosphate made exact correlation of activity and pyridoxamine phosphate difficult, hence the wide spread of values reported in the present paper. For enzyme at 7000 units/ml and 12000 units/ml treated with 100 μ M- and 200 μ M-pyridoxal phosphate respectively, 12 and 3% activity remained after reduction, and in the final dialysed solution between 4400 and 5600 units of activity were lost per nmol of bound cofactor. This corresponded to a loss of 6000–11000 units from the original samples for every nmol of pyridoxal phosphate bound. Thus the equivalent of 1 nmol of free pyridoxamine phosphate seemed to be bound to enzyme for every 6000–14000 units of activity, i.e. 1 nmol per 40–85 μ g of enzyme protein, if no quenching of fluorescence had occurred in the protein-bound derivative.

Samples of bovine serum albumin and pyridoxal phosphate mixed in a 1:2.3 ratio seem to give maximum formation of the Schiff base (Cabantchik *et al.*, 1975; Dempsey & Christensen, 1962). Samples of bovine serum albumin (15 μ M) were dialysed against 0.1 M-phosphate, pH 7.4, 35 μ M-pyridoxal phosphate, and gave a molar absorption coefficient at 320 nm of $\epsilon = 7300 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Reduction at pH 7.4 with borohydride, followed by extensive dialysis against phosphate pH 7.4, removed the absorption at 420 nm and gave a peak at 320 nm [$\epsilon = 7200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$; literature value 8300 (Churchich, 1965)], suggesting 1 mol of pyridoxamine phosphate was bound. This derivative had an initial fluorescence intensity at 395 nm which was 45% that of the same concentration of free pyridoxamine phosphate.

Table 3. *Estimation of pyridoxal phosphate covalently linked to aminolaevulinate synthetase*

Samples of either highly purified or partially purified enzyme (fraction Ia) were dialysed for 60h against 0.15M-potassium phosphate buffer, pH7.3, 5mM-2-mercaptoethanol, and were mixed with various amounts of pyridoxal phosphate and buffer in a total volume of 1 ml. After incubation for 30 min, the samples were treated with 5mM-NaBH₄ at 20°C for 15 min, diluted to 2.5 ml and dialysed exhaustively against 0.15M-phosphate and 0.05M-phosphate buffer, containing 5mM-2-mercaptoethanol, over 4 days. The enzyme activity and the fluorescence emitted at 395 nm (λ excitation 330 nm) relative to the fluorescence of unbound pyridoxamine phosphate were recorded. For purified enzyme [Expt. (a)] the final solution was dialysed against 1 mM-potassium phosphate, pH7.4, for 24h and the protein concentration was determined. For partially purified enzyme [Expt. (b)] samples of enzyme were also added last to mixtures of pyridoxal phosphate and borohydride. N.d., not determined.

	Concentration of pyridoxal phosphate (μ M)	Activity (nmol/h per ml)	Concentration of protein (μ g/ml)	Concentration of bound pyridoxamine phosphate (μ M)
(a) Purified enzyme	0	2010	18	0.01
	25	940	22	0.09
	100	350	15	0.12
Untreated enzyme	0	2500	N.d.	0
(b) Partially purified enzyme	0	5400	N.d.	0
	10	3970	N.d.	0.21
	50	1200	N.d.	0.47
	100	850	N.d.	0.75
Enzyme added after reduction	100	5120	N.d.	0

Hence about 50% quenching of fluorescence had occurred. Data that do not involve assumptions about quenching are usually obtained by examination of the absorption spectrum rather than the fluorescence spectrum, but much more material than was available would be needed. For the reduced serum albumin-pyridoxal phosphate complex and the reduced 5-aminolaevulinate synthetase-pyridoxal phosphate samples at pH7.5 the ratios of the fluorescence intensities measured at 392 nm to the absorbance measured at 320 nm were of similar size, suggesting that the quenching of fluorescence of bound pyridoxamine phosphate was similar in both these proteins.

For the isolated subunits of aspartate transcarbamoylase containing covalently bound pyridoxamine phosphate about 50% quenching of fluorescence was also observed at pH7.5 (Kempe & Stark, 1975), and for phosphorylase *b* only 20% fluorescence was obtained for the covalently bound cofactor unless the enzyme was deformed by urea or salts, when 100% fluorescence was obtained (Jones & Cowgill, 1971).

Hence it is not unreasonable to assume, as a first approximation, that there was 50% quenching of the fluorescence of the bound pyridoxamine phosphate in modified 5-aminolaevulinate synthetase at pH7.4. Therefore samples of pure enzyme would appear to bind 1 nmol of pyridoxal phosphate as a Schiff base for every 7000 units of activity, and Ia samples would seem to bind 1 nmol of pyridoxal phosphate for every 3000–5000 units of activity. This corresponds to the

binding of one, or at the most two, extra molecules of cofactor per enzyme subunit of mol.wt. 50000, i.e. 1 or 2 nmol of pyridoxal phosphate per 8000 units of activity (Davies & Neuberger, 1979).

Binding of pyridoxal phosphate

In addition to the active-site pyridoxal phosphate a 'structural' molecule of pyridoxal phosphate may also be present in the high-activity form of 5-aminolaevulinate synthetase. Pyridoxal phosphate was measured in dialysed samples (0.24 mg of protein; 16800 units of activity) of untreated and hydroxylamine-treated samples of fraction Ia, which was stored with pyridoxal phosphate. The method of Wada & Snell (1961) was used with phenylhydrazine in acid solution (total volume 1.06 ml). Small amounts (4.5 and 2.3 nmol) of pyridoxal phosphate were found in each sample, which corresponded to 39 and 20 nmol of pyridoxal phosphate per 165000 units of activity respectively for each of the two forms. These were equivalent to 2 molecules of pyridoxal phosphate per subunit (mol.wt. 50000) of the holoenzyme and 1 molecule of pyridoxal phosphate per subunit of the semi-apoenzyme. These amounts were for every 123000 g of protein in fraction Ia that, with a specific activity of 70000 units/mg, contained only 45% 5-aminolaevulinate. Although the amount of pyridoxal phosphate was low, it is clear that treatment with hydroxylamine had removed 1 molecule of pyridoxal phosphate from the subunit of the holoenzyme that was stored in pyridoxal phosphate. This supports the previous conclusion that two types of

binding of pyridoxal phosphate (one structural and one active-centre molecule) are present in our stock samples of the high-activity form of 5-aminolaevulinate synthetase.

Reaction with cyanide

Cyanide and sulphite effectively stopped the activation of the low-activity enzyme by trisulphide, but did not affect the activity of crude samples of enzyme. If high-activity enzyme contained a trisulphide group, then it should have been sensitive to cyanide and sulphite (Wider de Xifra *et al.*, 1976) and should have decreased its activity.

Partially purified samples of fraction Ia (6000 units/ml) in the absence of pyridoxal phosphate were treated with 5mM-potassium cyanide in 0.15M-phosphate buffer, pH 7.4, containing 5mM-2-mercaptoethanol, for 50 min at room temperature and were dialysed first against cyanide and then against 25mM-phosphate containing 5mM-2-mercaptoethanol at 0°C. The treated and untreated enzymes were fully active after dialysis and were not activated further by cystine trisulphide. No low-activity enzyme had been formed by this treatment and the intrinsic fluorescence was unchanged.

No change in enzymic activity was observed when samples either of fraction Ia (6000 units/ml) or pure enzyme (11000 units/ml) were treated first with pyridoxal phosphate (0.1 mM) for 60 min and then with cyanide and were subsequently dialysed. Again, the intrinsic yellow fluorescence was not affected. However, there was an additional marked change in the fluorescence that was observed under u.v. light. A new yellow-green fluorescence was seen and the modified protein was stable to dialysis and to gel filtration. The new fluorescence was obtained by reaction of cyanide with both highly and partially purified samples of 5-aminolaevulinate synthetase that had been preincubated with pyridoxal phosphate. It was not formed by the addition of pyridoxal phosphate to mixtures of enzyme and cyanide, nor by the addition of enzyme to mixtures of pyridoxal phosphate and cyanide. The new fluorescence maximum was at 480–490 nm when excited at 330 nm (λ_{max} excitation, 350 nm) and was not excited at 420 nm, in contrast with the intrinsic fluorescence.

The new fluorescence was removed by dialysis of the protein against pyridoxal phosphate (0.1 mM). Therefore the modified grouping was probably displaced by pyridoxal phosphate in the assay mixture, so restoring full activity to the enzyme. Assay of the modified enzyme in the absence of excess pyridoxal phosphate again showed only 1% activity. The fluorescence was not destroyed by reduction with borohydride and probably belonged to a saturated addition product of either the Schiff base or of the quinone form of an enzyme-bound pyridoxal phosphate molecule.

The fluorescent protein had the same apparent molecular weight as native 5-aminolaevulinate synthetase on Sephadex G-100 and ran as the same enzymically active fluorescent band on gel disc electrophoresis at pH 7.5, and so was identified with 5-aminolaevulinate synthetase.

A similar stable intense yellow-green fluorescence was produced when cyanide reacted with the Schiff base formed between bovine serum albumin and pyridoxal phosphate. With the glycine-pyridoxal phosphate Schiff base the yellow-green fluorescent derivative was only observed as an intermediate reaction product that decomposed over a few hours to give a product that had an absorption spectrum similar to that of the reaction product of pyridoxal phosphate and cyanide at pH 7.3, i.e. pyridoxic acid [3-hydroxy-5-(hydroxymethyl)-2-methylpyridine-4-carboxylic acid], λ_{max} 320 nm (Ohishi & Fukui, 1968), but with λ_{max} displaced 8 nm to longer wavelengths, i.e. it may have been a cyano-substituted pyridoxic acid.

Thus the results obtained with nucleophilic cyanide ion have complemented the results obtained with borohydride in showing the formation of a Schiff base between 5-aminolaevulinate synthetase and added pyridoxal phosphate. Unlike the formation of the covalently bound pyridoxamine phosphate the formation of the cyanide derivative does not destroy the enzymic activity since the modified grouping can be displaced by the addition of pyridoxal phosphate. Consequently if it is bound strongly enough the modified grouping may be useful as a reporter group for reflecting changes occurring near the active centre.

As for experiments with hydroxylamine and borohydride, treatment of dilute solutions of the high-activity form of 5-aminolaevulinate synthetase with cyanide did not affect the intrinsic fluorescence, which may be regarded as an integral part of the high-activity 5-aminolaevulinate synthetase.

Conclusion and General Discussion

The purified high-activity enzyme has an intrinsic yellow fluorescence and in solutions containing 2-mercaptoethanol at pH 7.5 the fluorescent grouping is inaccessible to the nucleophiles hydroxylamine, glycine, borohydride and cyanide. The fluorescence excitation and emission spectra suggest that it is present as a phenolate or protonated iminium form of an inaccessible pyridoxal phosphate-Schiff base in a relatively polar environment [cf. studies with phosphorylase (Jones & Cowgill, 1971)]. When excess pyridoxal phosphate is added to dilute solutions of enzyme, an additional molecule of pyridoxal phosphate is bound per subunit and another type of Schiff base is formed. This second Schiff base reacts with borohydride and cyanide and appears to be mainly

in a phenolic or in an imine form. This form would be expected to show an absorption maximum at 330 nm rather than at 420 nm (Feldmann & Helmreich, 1976) and not be expected to have a fluorescence excitation maximum at 420 nm. This second Schiff base appears to involve the active centre of the enzyme since extra pyridoxal phosphate is always needed for the assay of enzymic activity and an inactive enzyme is formed when the pyridoxal phosphate-enzyme complex is reduced with borohydride.

Partially purified samples of the high-activity enzyme have only one fluorescent protein present and behave in the same way as the purified enzyme towards nucleophiles. The intrinsic fluorescence is not affected by treatment with nucleophiles, but the more weakly bound molecules of added pyridoxal phosphate can be removed by this treatment. One or two molecules of cofactor can be covalently linked to the enzyme by treatment with borohydride with a consequent loss of enzymic activity, but since the partially purified sample was a mixture of enzyme species, interpretation of the spectral changes was not possible. Up to four active enzymes were detected when the original sample was isoelectrically focused in the presence of ampholytes (Davies & Neuberger, 1979). Half the enzyme molecules contained the firmly bound intrinsic fluorescent grouping and half the enzyme molecules contained the resolvable pyridoxal phosphate. Thus, in dilute solutions of enzyme, two types of binding of pyridoxal phosphate to 5-aminolaevulinate synthetase are apparent. However, differentiation of the two types of pyridoxal phosphate molecules is lost when an excess of pyridoxal phosphate is added to concentrated solutions of enzyme, which probably contain dimeric molecules rather than monomeric molecules of 5-aminolaevulinate synthetase. In concentrated solutions of enzyme the intrinsic fluorescent grouping is still present and is not affected by treatment with borohydride, but when pyridoxal phosphate is added to the enzyme a change in the structure or the environment of the Schiff base seems to occur since the intrinsic fluorescence is now lost on subsequent treatment with borohydride. Pyridoxal phosphate must have caused a change in the conformation of the protein around the intrinsic molecule of pyridoxal phosphate. With such concentrated solutions of enzyme and pyridoxal phosphate a more accessible form of the Schiff base must have been formed, since it has also proved possible to resolve some apoenzyme from holoenzyme by electrophoresis in glycine buffer. In this instance a non-fluorescent protein was formed that was capable of re-binding pyridoxal phosphate with moderate firmness, and in concentrated samples of fraction Ia three forms of the enzyme could be recognized.

Since it is possible that the two sets of sites may have become similar in concentrated solutions of enzyme treated with pyridoxal phosphate, they may

have gone undetected in previous investigations. For example, the absorption and fluorescence spectra of the enzymes isolated from strain Y of *R. sphaeroides* are similar in several respects to the enzyme in dilute solution reported in the present paper, but the enzymes apparently behave differently with respect to the rate of re-combination of resolved apoenzyme with pyridoxal phosphate and to the mode of binding the catalytic molecule of pyridoxal phosphate. Alternatively, of course, the enzyme from strain Y may also have contained two types of Schiff base in a mixture of either phenol and phenolate forms or aldamine and aldimine forms (Fanica-Gaignier & Clement-Metral, 1973a).

The part played by pyridoxal phosphate in a large number of enzymes has been elucidated by studies of the absorption and fluorescence spectra of native enzymes and of the reduced derivatives (Davis & Metzler, 1972). Many details are known about the structure adopted by the active-centre molecule of pyridoxal phosphate in amino acid transferases and about the structure of the 'structural' pyridoxal phosphate in phosphorylase *b* (Johnson *et al.*, 1970; Shaltiel & Cortijo, 1970). Uncertainty exists about the precise role played by pyridoxal phosphate in the latter enzyme, though a structural function appears most likely. Both catalytic and structural functions for pyridoxal phosphate also seem possible for 5-aminolaevulinate synthetase. To understand the mechanism of action of the high-activity form of 5-aminolaevulinate synthetase the contribution of each pyridoxal phosphate molecule to the action of the enzyme must be individually assessed. The structural type must be determined for the various concentrations and conformations of the protein in the native enzyme, in the complex with cofactor and in the enzyme-cofactor-substrate complex.

There seems to be a complicated inter-relationship between the conformation of the protein, the enzymic activity, the binding of the two types of pyridoxal phosphate, dimerization of the enzyme and the state of the cysteine and cystine residues in 5-aminolaevulinate synthetase. The regulation of 5-aminolaevulinate synthetase activity may be affected by any of these factors.

We have been concerned with the conversion of the Ib form into the Ia form of the enzyme, which is achieved by cystine trisulphide in cell extracts. The activity of the low-activity form of the enzyme is increased maximally by reaction with cystine trisulphide and partially by oxidation. The activation process is aided by the presence of pyridoxal phosphate and thiol compounds. The final activity achieved is probably also determined by the number and location of thiol and disulphide groups on the enzyme. Pyridoxal phosphate may function in several ways; it may protect an essential thiol group at the active centre from oxidation or cross-linking, or it may hold

the enzyme in a conformation required for specific pairing of thiol groups under the influence of cystine trisulphide. It may also be incorporated into the enzyme at sites different from the catalytic centre. Combinations of disulphide groups other than those required for maximal activity might lead to enzymes of intermediate activity that are not activated by trisulphide. While it is difficult to determine directly which active form of the enzyme is present within the growing cell because of the changes brought about by harvesting and disrupting the organisms, it is probable that an enzyme is present in cells that can convert the high-activity a-form enzyme into the low-activity b-form enzyme in the presence of thiol compounds, as this enzyme has been detected in cell extracts (Tuboi & Hayasaka, 1973). Such an enzyme may be capable of rearranging or reducing all 'correctly' or 'incorrectly' paired disulphide bonds in 5-aminolaevulinate synthetase, so that the low-activity b form of the enzyme is always detected in cell extracts immediately on disruption. If this occurs within cells, then this may serve as a fine control of 5-aminolaevulinate synthetase activity.

It may prove that thiol, disulphide or trisulphide structures of different forms of the enzyme, and hence the enzymic activity, are affected to some extent by the binding of different molecules of pyridoxal phosphate to the enzyme, e.g. no labile sulphur has yet been detected in high-activity 5-aminolaevulinate synthetase, but the intrinsic molecule of pyridoxal phosphate was still present in the samples tested. Pyridoxal phosphate protects an essential thiol group in the enzyme obtained from rat liver and probably also does so in *R. sphaeroides*. Clement-Metral & Fanica-Gaignier (1975) have suggested that ATP and iron, which also effectively mask thiol groups on two forms of the enzyme from strain Y of *R. sphaeroides*, also play a crucial role in the regulation of 5-aminolaevulinate synthetase activity. Hence a multitude of small molecules and cofactors seem to influence the activity of 5-aminolaevulinate synthetase in this photosynthetic organism. Further investigation of the effects of these molecules will be possible when greater amounts of each enzyme become available.

We thank The Wellcome Trust for financial support. We also thank Professor Brenda Ryman, Head of the Department of Biochemistry, Charing Cross Hospital Medical School, for the hospitality she has given us and Mr. David Ashford for his skilled technical assistance.

References

- Akhtar, M., Abboud, M. M., Barnard, G., Jordan, P. M. & Zaman, Z. (1976) *Philos. Trans. R. Soc. London Ser. B* **273**, 117–136.
- Cabantchik, I. Z., Balshin, M., Breuer, W. & Rothstein, A. (1975) *J. Biol. Chem.* **250**, 5130–5136.
- Churchich, J. E. (1965) *Biachim. Biophys. Acta* **102**, 280–288.
- Clement-Metral, J. D. & Fanica-Gaignier, M. (1975) *Eur. J. Biochem.* **59**, 73–77.
- Davies, R. C. & Neuberger, A. (1979) *Biochem. J.* **177**, 649–659.
- Davis, L. & Metzler, D. E. (1972) *Enzymes 3rd Ed.* **7**, 33–74.
- Dempsey, W. B. & Christensen, H. N. (1962) *J. Biol. Chem.* **237**, 1113–1120.
- Fanica-Gaignier, M. & Clement-Metral, J. D. (1971) *Biochem. Biophys. Res. Commun.* **44**, 192–198.
- Fanica-Gaignier, M. & Clement-Metral, J. D. (1973a) *Eur. J. Biochem.* **40**, 13–18.
- Fanica-Gaignier, M. & Clement-Metral, J. D. (1973b) *Eur. J. Biochem.* **40**, 19–24.
- Feldmann, K. & Helmreich, E. J. M. (1976) *Biochemistry* **15**, 2394–2401.
- Ghisla, S., Massey, V., Lhoste, J.-M. & Mayhew, S. G. (1974) *Biochemistry* **13**, 589–597.
- Guilbault, G. G. (1973) in *Practical Fluorescence*, pp. 312–322, Marcel Dekker Inc., New York.
- Hathaway, G. M. & Noltman, E. A. (1977) *Arch. Biochem. Biophys.* **179**, 24–33.
- Johnson, G. E., Tu, J.-I., Shonka-Bartlett, M. L. & Graves, D. J. (1970) *J. Biol. Chem.* **245**, 5560–5568.
- Jones, D. C. & Cowgill, R. W. (1971) *Biochemistry* **10**, 4276–4282.
- Jordan, P. M. & Shemin, D. (1972) *Enzymes 3rd Ed.* **7**, 339–356.
- Kempe, T. D. & Stark, G. R. (1975) *J. Biol. Chem.* **250**, 6861–6869.
- Laghai, A. & Jordan, P. M. (1976) *Biochem. Soc. Trans.* **4**, 52–53.
- Laghai, A. & Jordan, P. M. (1977) *Biochem. Soc. Trans.* **5**, 299–300.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
- Marshall, M. & Cohen, P. P. (1977) *J. Biol. Chem.* **252**, 4276–4286.
- Misono, H. & Soda, K. (1977) *J. Biochem. (Tokyo)* **82**, 535–543.
- Nandi, D. L. & Shemin, D. (1977) *J. Biol. Chem.* **252**, 2278–2280.
- Neuberger, A. (1961) *Biochem. J.* **78**, 1–10.
- Neuberger, A., Sandy, J. D. & Tait, G. H. (1973) *Biochem. J.* **136**, 491–499.
- Ohishi, N. & Fukui, S. (1968) *Arch. Biochem. Biophys.* **128**, 606–610.
- Sandy, J. D., Davies, R. C. & Neuberger, A. (1975) *Biochem. J.* **150**, 245–257.
- Scholnick, P. L., Hammaker, L. E. & Marver, H. S. (1972) *J. Biol. Chem.* **247**, 4132–4137.
- Shaltiel, S. & Cortijo, M. (1970) *Biochem. Biophys. Res. Commun.* **41**, 594–600.
- Steiner, R. F., Greer, L. & Gunther, C. (1977) *Biachim. Biophys. Acta* **494**, 233–244.
- Tuboi, S. & Hayasaka, S. (1973) *Enzyme* **16**, 86–93.
- Wada, H. & Snell, E. S. (1961) *J. Biol. Chem.* **236**, 2089–2095.
- Whiting, M. J. & Granick, S. (1976) *J. Biol. Chem.* **251**, 1340–1346.
- Wider de Xifra, E. A., Sandy, J. D., Davies, R. C. & Neuberger, A. (1976) *Philos. Trans. R. Soc. London Ser. B* **273**, 79–98.
- Yonaha, K., Misono, H., Yamamoto, T. & Soda, K. (1975) *J. Biol. Chem.* **250**, 6983–6989.