acetone), than found in earlier investigations. Mayer (1930) used a concentration of 66% for his determinations, but this is definitely inhibitory, though less so for insoluble preparations than for soluble.

The enzyme is remarkably stable before it becomes soluble and the activity of soluble preparations is retained well in the cold. The optimum temperature for enzyme action is much lower than for many enzymes, presumably because the temperature at which the enzyme is inactivated is comparatively low, particularly in the presence of acetone.

The rise in chlorophyllase activity when dark-grown pea seedlings are transferred to light is similar to that found by Hageman & Arnon (1955) for triphosphopyridine nucleotide-linked glyceraldehyde phosphate dehydrogenase. Marcus (1960) induced the formation of triose phosphate dehydrogenase, without the development of chlorophyll, in kidney-bean leaves by brief exposure to red light. Booth (1960) found that a carotene-destroying enzyme system was not normally present in potatoes and carrots, but exposure to light leading to chlorophyll formation in the surface layers also caused the enzyme to appear.

SUMMARY

1. Soluble chlorophyllase preparations were made from sugar-beet leaves, which have the highest activity of all species tested.

2. For the partly purified enzyme the optimum conditions for enzyme action are pH 7.7 at 25° and an acetone concentration of 40%.

3. When dark-grown pea seedlings with very low chlorophyllase activity were transferred to daylight the activity rose in 48 hr. to a level comparable with that of light-grown seedlings.

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Pyridoxamine Phosphate-Oxidase and Pyridoxal Phosphate-Phosphatase Activities in *Escherichia coli*

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Beechey & Happold (1955) first demonstrated the conversion of pyridoxamine phosphate into the aldehyde form by cell-free extracts of *Escherichia coli*. The nature of the product was established by its ability to activate apotryptophanase, an enzyme also present in the aqueous extracts. Optimum conditions for enzyme assay were studied, and simple kinetic studies were carried out by spectrophotometric methods, by Beechey & Happold (1957), who found that *E. coli* extracts

catalysed the disappearance of pyridoxal phosphate and that light-absorption at the wavelength characteristic of pyridoxamine phosphate increased during the reaction. The reaction involved thus appeared to be reversible, although only the conversion of pyridoxamine phosphate into the pyridoxal ester required Mg^{2+} ions for maximum activity.

The possibility that more than one enzyme was involved was recognized, but the apparent reversibility of the reaction, and other data, led these workers to assume that a transamination reaction was involved. Unlike Meister, Sober & Tice (1951),

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who presented evidence for transamination between pyridoxamine phosphate and pyruvic acid in extracts of *Clostridium welchii*, Beechey & Happold (1957) were unable to identify amino-group acceptor-donors in their system.

The present paper suggests that the apparent interconversion of pyridoxamine phosphate and pyridoxal phosphate in extracts of *E. coli* is due to the presence of two separate enzymes. These enzymes have been identified as pyridoxamine phosphate oxidase and a pyridoxal phosphate phosphatase. Pogell (1958) has reported the existence of similar enzyme activities in mammalian tissues, also in yeast and *Streptococcus faecalis*.

EXPERIMENTAL

Materials

The preparation and storage of cell-free extracts of acetone-ether-dried *E. coli* were carried out as described by Beechey & Happold (1957). Glutamic-aspartic transaminase was prepared from heart muscle by the method of Cammarata & Cohen (1951), the fraction 'Ppt. WFT-II' being used. An alkaline-phosphatase preparation (Intestinal Phosphatase) was obtained from Armour Laboratories, Chicago, Ill., U.S.A., and a soluble enzyme reagent (Polidase-S), known to contain acid phosphatase, was obtained from Schwartz Laboratories Inc., New York, N.Y., U.S.A. Pyridoxal phosphate and pyridoxamine phosphate were from Hoffmann-La Roche and Co. Ltd., keto acids from British Drug Houses Ltd. and adenosine triphosphate (ATP) from L. Light and Co. Ltd.

Assay of vitamin B₆ compounds and associated enzyme activities

The assay of vitamin B₆ compounds was carried out spectrophotometrically with a Unicam spectrophotometer, model SP. 500. Preliminary experiments were carried out to determine maximum absorption wavelengths for pyridoxamine phosphate and pyridoxal phosphate, also pyridoxal under the conditions in which these compounds were to be assayed.

Composition of standard reaction mixture. For interconversions of vitamin B₆ compounds, reactions were carried out in a total volume of 4.0 ml. The standard composition was: buffer, 1.0 ml.; 0.02M-MgSO₄, 0.5 ml.; concentrated cell extract, approx. 10.0 mg. of protein/ml., 2.0 ml.; vitamin B₆ compound (200 µg./ml.) 0.5 ml., i.e. approx. 0.4 µmole of pyridoxamine phosphate and pyridoxal phosphate. Water replaced substrate in the blank and replaced MgSO₄ solution when pyridoxal phosphate was used as substrate. When the effect of various additions was investigated, cell extract was made up in buffer, and the additions were contained in 1.0 ml. Where necessary, reaction-mixture components were adjusted to the pH of the reaction.

Measurement of the conversion of pyridoxamine phosphate into the aldehyde form. The buffer used was 0.05M-NaHCO₃-Na₂CO₃, pH 10.1 (Delory & King, 1945). Blank, and reaction mixture without substrate, were equilibrated at 37° in 1 cm. quartz cells, the latter being placed in a

thermostatically controlled cell-holder. Addition of substrate to the equilibrated reaction system, with stirring, was timed. Extinction values were then read alternatively at 312.5 and 390 mµ, at timed intervals over the period of the reaction. Overall changes in extinction were calculated after extrapolation of readings to zero time.

As the thermostatically regulated cell-holder had positions for only two cells, the complete reaction mixture was used as a blank when the activity of reaction mixture plus addition was of interest. In this case inhibition or activation was related to enzyme activity measured immediately beforehand as described above. When inhibition was observed, readings were made with reaction mixture plus inhibitor in the blank position.

Alternatively, reaction mixtures minus substrate were made up in thin-walled test tubes and equilibrated in a water bath at 37°. Substrate was then added at timed intervals to all but the controls, and at the end of the reaction 1.0 ml. of 2.5N-NaOH was added to all tubes. Substrate was then added to controls. Pyridoxamine phosphate is stable during incubation under the above-mentioned conditions in the absence of enzyme preparation. Precipitated protein was removed by centrifuging at 10 000 rev./min. for 30 min. (12 000 g at the bottom of the tube). The extinction of tube contents was read against the corresponding blank in order to assay the product formed. In later work, the extinction of the control was read against the reaction mixtures in order to assay the substrate utilized. The wavelengths used were the wavelengths of maximum absorption ($\lambda_{\max}$), determined for pyridoxamine phosphate and pyridoxal phosphate in 0.5N-NaOH, i.e. 307.5 and 390 mµ respectively.

Measurement of the dephosphorylation of pyridoxal phosphate. The same spectrophotometric methods were used as described for the measurement of the conversion of pyridoxamine phosphate into the aldehyde form at pH 10.1. Here, however, product formation was measured by the increase in extinction at 300 mµ, i.e. $\lambda_{\max}$ for pyridoxal at pH 10.1.

Methods used for converting measured changes in extinction into changes in vitamin B₆-compound concentration are described in the Results section. Specific activities of the preparations are expressed in terms of µmoles/30 min./mg. of protein/ml. of reaction mixture.

Isolation and identification of vitamin B₆ compounds. A Dowex-1 formate column, described for the separation of pyridoxine metabolites by Rodwell, Volcani, Ikawa & Snell (1958), was used to isolate vitamin B₆ compounds from reaction mixtures. Deproteinization was by the addition of 100% trichloroacetic acid. Eluate samples were examined spectrophotometrically at two pH values, and vitamin B₆ compounds were detected by the blue colour produced with 2,6-dichloroquinonechloroimide reagent (Snyder & Wender, 1953). Identification was carried out by paper chromatography (Rodwell *et al.* 1958; Fasella & Baglioni, 1956).

Measurements of acid- and alkaline-phosphatase activities

The same composition of reaction mixture was used as for the spectrophotometric assay for interconversion of vitamin B₆ compounds. The protein concentration of *E. coli* extracts used was 2 mg./ml. and that of phosphatase preparations from other sources was usually 1 mg./ml.

E. coli extracts were dialysed overnight against a large volume of the appropriate buffer, before use, to remove traces of inorganic phosphate. In all cases the substrate was disodium phenyl phosphate (0.1 M). Deproteinization was accomplished by addition of 100% (w/v) trichloroacetic acid until no further precipitation of protein occurred, and, after centrifuging to remove protein, 1 ml. of supernatant was used for phosphate assay. The method of Gomori (1941-42) was used to determine inorganic phosphate.

Measurements of tryptophanase activity and indole assay

These were carried out as described by Beechey & Happold (1957).

RESULTS

Conversion of pyridoxamine phosphate into pyridoxal phosphate

Progress of the reaction and product identity. Spectrophotometric data showing the disappearance of pyridoxamine phosphate used as substrate, and the formation of product, are shown in Fig. 1. The absorption spectrum of the product, with peak absorption at 390 m μ (Fig. 2), confirms previous enzymic evidence on the identity of the product as pyridoxal phosphate (Beechey & Happold, 1957). Owing to the high light-absorbency of the cell extracts used, it was not possible to make measurements at wavelengths less than those indicated.

Effect of various compounds, also dialysis, on enzyme activity. In view of the previously reported

Fig. 1. Conversion of pyridoxamine phosphate into pyridoxal phosphate. Mixtures containing 1.0 ml. of 0.05 M-NaHCO₃-Na₂CO₃ buffer, pH 10.1, 0.5 ml. of 0.02 M-MgSO₄, 2.0 ml. of cell extract (10 mg. of protein/ml.) and 0.5 ml. of substrate (200 μ g./ml.) were incubated at 37°. At the indicated times, values of *E* at 312.5 m μ (●) and 390 m μ (○) were read against a control in which water replaced substrate.

suggestion that the reaction involved was a transamination, the effect of various potential amino-group acceptors on the activity of cell extracts was investigated. Glyoxylate, shown by Metzler, Ikawa & Snell (1954) to function as an amino-group acceptor in a number of transamination systems, inhibited the above-mentioned reaction when used as the sodium salt at a number of concentrations (Table 1). Dialysis of extracts against NaHCO₃-Na₂CO₃ buffer, pH 10.1, decreased their activity by between 41.0 and 42.5%. This activity loss was calculated by comparison with the activity of an extract sample allowed to stand under the

Fig. 2. Absorption spectrum of reaction product with pyridoxamine phosphate as substrate. Reaction conditions were as described in Fig. 1. Δ , Difference spectrum of product; ●, pyridoxamine phosphate (approx. 25 μ g./ml.) incubated under the same conditions but without enzyme; ○, pyridoxal phosphate (approx. 25 μ g./ml.). All spectra were plotted after an incubation time of approx. 120 min.

Table 1. Effect of sodium glyoxylate on the conversion of pyridoxamine phosphate into pyridoxal phosphate

Reaction mixtures contained: 1.0 ml. of 0.05 M-NaHCO₃-Na₂CO₃ buffer, pH 10.1; 0.5 ml. of sodium glyoxylate solution in buffer; 0.5 ml. of 0.02 M-MgSO₄; 2.0 ml. of cell extract (10 mg. of protein/ml.); 0.5 ml. of pyridoxamine phosphate (200 μ g./ml.; 0.4 μ mole). Buffer replaced sodium glyoxylate solution in the control, and water replaced substrate in the blank. At the end of incubation for 30 min. at 37°, 0.5 ml. of 2.5 N-NaOH was added, precipitated protein was removed by centrifuging and *E* at 390 m μ was read against the blank, in which cell extract was omitted until after the addition of NaOH.

Concn. of sodium glyoxylate (mm)	Increase in <i>E</i> at 390 m μ	Activity (%)
—	0.046	100
0.25	0.037	80
0.63	0.039	85
1.25	0.038	83

same conditions of buffer concentration and pH, but without dialysis; this sample was finally diluted to the same protein concentration as the dialysed preparation. The effect of a number of potential amino-group acceptors on the activity of dialysed cell extracts is shown in Table 2. With such extracts glyoxylate did not cause inhibition. Although the use of boiled cell-extract concentrates suggested that activity could be partially restored to dialysed cell extracts, the results were of little significance. Concentrated diffusate from the extract, observed to have an absorption maximum at 260 m μ at pH 7.0 and to exhibit green fluorescence in u.v. light, did not restore activity. The diffusate was obtained by dialysis of extracts against distilled water and was then concentrated by freeze-drying.

Table 2. *Effect of potential amino-group acceptors on the conversion of pyridoxamine phosphate into pyridoxal phosphate*

Reaction mixture and assay procedure details were as described for Table 1, except that dialysed cell extract was used where appropriate, and incubation was for 90 min.

Additions to dialysed extract (0.02 mM)	Increase in <i>E</i> at 390 m μ	Activity (%)
—	0.036	58
Pyruvate	0.020	35
α -Oxoglutarate	0.003	5
Oxaloacetate	0.008	13
Glyoxylate	0.036	58
Non-dialysed extract (no additions)	0.061	100

Table 3. *Effect of anaerobic conditions on the conversion of pyridoxamine phosphate into pyridoxal phosphate*

The reaction mixture contained in the main arm of Thunberg vessels: 1.0 ml. of 0.05M-NaHCO₃-Na₂CO₃ buffer, pH 10.1; 0.5 ml. of 0.02M-MgSO₄; 2.0 ml. of undialysed cell extract (approx. 10 mg. of protein/ml.). Either 0.5 ml. of water or pyridoxamine phosphate (200 μ g./ml.; 0.4 μ mole) was contained in the side arm. After repeated evacuation, and flushing with either air or nitrogen, vessels and contents were equilibrated at 37°. Reactions were started by tipping side-arm contents into the main arm. Incubation was for 90 min. Reactions were stopped by the addition of NaOH, and *E* at 390 m μ was read as described for Table 2.

Conditions of reaction (atmosphere)	Increase in <i>E</i> at 390 m μ	Activity (%)
Air	0.053	100
Nitrogen	0.011	19
Air	0.049	100
Nitrogen	0.015	30
Air	0.062	100
Nitrogen	0.018	29

Nature of the reaction. Since the conversion of pyridoxamine phosphate into the aldehyde form could be partly due to an oxidation process, the effect of anaerobic conditions was investigated (Table 3). At least 70–80 % of the pyridoxal phosphate was formed by an oxidation process. Previous indications suggested that the process might require a diffusible cofactor, but neither riboflavin nor riboflavin phosphate (0.01 mM) restored activity. The antimalarial mepacrine [6-chloro-9-(4-diethylamino-1-methylbutylamino)-2-methoxyacridine], however, at a concentration of 0.53 mM inhibited the activity of undialysed extracts by about 20 %. Higher mepacrine concentrations could not be employed at pH 10.1 owing to its insolubility. Various flavin-dependent oxidases have been shown to be inhibited by this compound (Hellerman, Lindsay & Bovarnick, 1946; Nygard & Thellwell, 1955; Doisy, Richert & Westerfield, 1955).

Method evolved to calculate changes in substrate and product concentration

In order to correlate observed changes in extinction with changes in substrate and product concentration, certain properties of the respective absorption spectra were found to be of importance. Thus, at pH 10.1, whereas pyridoxamine phosphate does not absorb light at wavelengths above 370 m μ , pyridoxal phosphate absorbs both at 390 m μ ($\lambda_{\max}$) and 312.5 m μ . Thus during the enzymic oxidation of pyridoxamine phosphate to its aldehyde form, any decrease in *E* observed at 312.5 m μ would be partly masked by an increase due to pyridoxal phosphate formation. General formulae were therefore constructed, relating observed *E* (1 cm. light path) to the concentration of both pyridoxamine phosphate (Pam.P) and pyridoxal phosphate (Pal.P) in terms of molecular extinction coefficients. During substitution of constants into such formulae, complications due to overlapping of absorption spectra at the wavelengths used are automatically allowed for. The general formulae are:

$$[\text{Pam.P}] = \frac{\epsilon_{\text{Pal.P}}^{390} E_{312.5}^{1 \text{ cm.}} - \epsilon_{\text{Pal.P}}^{312.5} E_{390}^{1 \text{ cm.}}}{\epsilon_{\text{Pal.P}}^{390} \epsilon_{\text{Pam.P}}^{312.5} - \epsilon_{\text{Pal.P}}^{312.5} \epsilon_{\text{Pam.P}}^{390}}$$

$$[\text{Pal.P}] = \frac{\epsilon_{\text{Pam.P}}^{312.5} E_{390}^{1 \text{ cm.}} - \epsilon_{\text{Pam.P}}^{390} E_{312.5}^{1 \text{ cm.}}}{\epsilon_{\text{Pam.P}}^{312.5} \epsilon_{\text{Pal.P}}^{390} - \epsilon_{\text{Pam.P}}^{390} \epsilon_{\text{Pal.P}}^{312.5}}$$

It was found that, at pH 10.1,

$$\epsilon_{\text{Pal.P}}^{312.5} = 1000; \quad \epsilon_{\text{Pal.P}}^{390} = 6000;$$

$$\epsilon_{\text{Pam.P}}^{312.5} = 5700; \quad \epsilon_{\text{Pam.P}}^{390} = 0.$$

Results supporting these values are included in Table 4, lines 1 and 5. Substituting these values in the general formulae,

$$[\text{Pam.P}] = (1.75 E_{312.5}^{1 \text{ cm.}} - 0.29 E_{390}^{1 \text{ cm.}}) \times 10^{-4} \text{ M}$$

$$[\text{Pal.P}] = 1.67 E_{390}^{1 \text{ cm.}} \times 10^{-4} \text{ M.}$$

In order to check the validity of the formulae, mixtures of standard solution of pyridoxamine phosphate and pyridoxal phosphate were prepared at two concentrations. The known concentration values for each component were then compared with values calculated from the values of E observed; good agreement was obtained.

Stoichiometry of the reaction. By following the progress of the reaction now deduced to be an oxidation of pyridoxamine phosphate, with time, as shown in Fig. 1, it was found that the relationship between the amounts of substrate utilized and product formed was variable. This non-equivalence was particularly marked when extracts of *E. coli* cells grown on a medium including 0.2% of glucose were used (Fig. 3). A quantitative examination of the results illustrated in Figs. 1 and 3, with the specially derived formulae, suggested a more rapid removal of pyridoxal phosphate from the system when extracts prepared from cells grown on glucose containing media were used. The calculated results

Fig. 3. Conversion of pyridoxamine phosphate into pyridoxal phosphate by extracts of cells grown on a medium containing 0.2% of glucose. Reaction conditions were as described in Fig. 1. ●, Measured at 312.5 mμ; ○, measured at 390 mμ.

Table 4. Comparison of known and calculated concentrations of pyridoxamine phosphate and pyridoxal phosphates

Standard solutions of pyridoxamine phosphate (Pam.P) and pyridoxal phosphate (Pal.P) were prepared, each at 0.12 mM and at one-half of this concentration, in 12.5 M-NaHCO₃-Na₂CO₃ buffer, pH 10.1. Mixtures of these solutions were made up as indicated, and values of E (1 cm. light-path) were measured at 312.5 and 390 mμ. Concentration values were calculated as described in the text.

Known concn. of mixture components (0.1 mM)		Content of Pal.P in mixture (%)	$E_{1\text{cm.}}^{1\text{mM}}$		Calc. concn. of mixture components (0.1 mM)	
Pam.P	Pal.P		312.5 mμ	390 mμ	Pam.P	Pal.P
1.20	0	0	0.685	0	1.20	0
0.90	0.30	25	0.540	0.180	0.890	0.300
0.60	0.60	50	0.400	0.360	0.595	0.600
0.30	0.90	75	0.265	0.535	0.305	0.890
0	1.20	100	0.120	0.720	0	1.20
0.60	0	0	0.340	0	0.595	0
0.45	0.15	25	0.270	0.090	0.445	0.150
0.30	0.30	50	0.200	0.180	0.280	0.300
0.15	0.45	75	0.135	0.267	0.159	0.445
0	0.60	100	0.070	0.360	0.017	0.600

Table 5. Apparent stoichiometric relationships between substrate and product during oxidation of pyridoxamine phosphate

The reaction mixture is described in the Experimental section of the paper; 5 mg. of protein was present/ml.

Source of data	Reaction time (min.)	Calc. changes in concn. (0.1 mM)		Ratio: $\Delta(a)/\Delta(b)$
		Pam.P (a)	Pal.P (b)	
Fig. 1 (Extract 1)	0	0	0	—
	30	0.10	0.09	1.11
	60	0.15	0.13	1.15
	90	0.18	0.16	1.12
Fig. 3 (Extract 2)	0	0	0	—
	30	0.19	0.07	2.70
	60	0.24	0.08	3.00

are given in Table 5. From Table 5, the specific oxidase activities of extracts 1 and 2 can be calculated as 2.0 and 3.8 $\mu\text{m-moles}$ of pyridoxamine phosphate/30 min./mg. of protein respectively.

Evidence for the removal of pyridoxal phosphate from reaction systems was also obtained when pyridoxamine phosphate was used as substrate and added apotryptophanase preparations were used to assay pyridoxal phosphate formation. The method used was that described by Beechey & Happold (1957). With either *E. coli* extracts or mammalian glutamic-aspartic-transaminase preparations it was found that an initial rise in the pyridoxal phosphate formation was followed by a rapid fall in the ability of samples to activate the apotryptophanase system.

In early experiments, designed to determine the equilibrium position for the reaction converting pyridoxamine phosphate into pyridoxal phosphate, it was observed that the initial decrease in E at 312.5 $\text{m}\mu$ was followed after several hours by a slow rise. A simultaneous slow decrease in E at 390 $\text{m}\mu$ was also observed. These results suggested, in retrospect, the conversion of pyridoxal phosphate into some compound having an absorption at 312.5 $\text{m}\mu$ similar to, but not identical with, that due to pyridoxamine phosphate.

Conversion of pyridoxal phosphate into free pyridoxal

Establishment of the nature of the reaction. Pyridoxal phosphate is readily hydrolysed to the free base and inorganic phosphate (Peterson, Sober & Meister, 1953), and it appeared likely that the disappearance of this compound from enzymic-reaction systems could be due to enzymic hydrolysis.

In order to distinguish between pyridoxal formation and the production of pyridoxamine phosphate as suggested by the data of Beechey & Happold (1957), the light-absorptions of reaction mixtures with pyridoxal phosphate as substrate were measured at 300, 312.5 and 390 $\text{m}\mu$, over a period of approx. 40 min. at 37°. At pH 10.1, the wavelengths quoted characterize the peaks of maximum absorption for pyridoxal, pyridoxamine phosphate and pyridoxal phosphate respectively. The results of one experiment are shown in Fig. 4. Absorption spectrum of the reaction product in a similar experiment is shown in Fig. 5, together with those of pyridoxal and pyridoxal phosphate incubated under the same conditions but in the absence of enzyme preparation. It can be seen that the cell extracts catalysed the breakdown of pyridoxal phosphate to the free base. As the extinction of pyridoxal at 312.5 $\text{m}\mu$ is appreciable compared with that at 300 $\text{m}\mu$, the apparent formation of pyridoxamine phosphate previously reported is thus explained.

Column chromatography of reaction mixtures in which pyridoxal phosphate had been used as substrate and where extracts had been prepared from cells grown on media, with or without added glucose, failed to show any pyridoxamine phosphate or free pyridoxamine. Only pyridoxal was detected in eluate samples.

Effect of various additions. In order to examine the possibility that the dephosphorylation of pyridoxal phosphate might be due to a reversal of the pyridoxal-kinase reaction (Hurwitz, 1953)

Fig. 4. Conversion of pyridoxal phosphate into free pyridoxal. Reaction conditions were as described in Fig. 1, except that pyridoxal phosphate (approx. 200 $\mu\text{g./ml.}$) was the substrate, and water replaced MgSO_4 solution. ●, Measured at 312.5 $\text{m}\mu$; Δ, measured at 300 $\text{m}\mu$; ○, measured at 390 $\text{m}\mu$.

Fig. 5. Absorption spectrum of reaction product with pyridoxal phosphate as substrate. Reaction conditions were as specified in Fig. 4; reaction time, 5 hr. Δ, Difference spectrum of product; ○, pyridoxal phosphate, approx. 25 $\mu\text{g./ml.}$; ●, pyridoxal, approx. 25 $\mu\text{g./ml.}$ Both pyridoxal and pyridoxal phosphate were incubated under the same conditions as when pyridoxal phosphate was used as substrate, except that no enzyme preparation was present.

rather than a hydrolysis, the effect of ATP, at a final concentration of 5 mM, on the dephosphorylation of pyridoxal phosphate was investigated. No unequivocal evidence was obtained that ATP affected the rate of dephosphorylation. Spectrophotometric results were complicated by the fact that the *E. coli* extracts appeared to catalyse a decrease in *E*, due to ATP, at 300 m μ , i.e. the wavelength used to assay pyridoxal formation. Under the conditions previously employed for the dephosphorylation of pyridoxal phosphate, no pyridoxal-kinase activity could be detected spectrophotometrically with pyridoxal and ATP as substrates, and with *E* at 390 m μ as the criterion of pyridoxal phosphate formation. This confirms the finding of Beechey & Happold (1957), who used enzymic methods for the detection of pyridoxal phosphate.

A study of the effect of Mg²⁺ ions at 2.5 mM on the dephosphorylation of pyridoxal phosphate showed a consistent 8% increase in activity. Ethylenediaminetetra-acetate, however, at a concentration of 0.62 mM, caused no inhibition when non-dialysed cell extract was used.

With either dialysed or non-dialysed *E. coli* preparations, no effect of 0.01 M-NH₄⁺ ions on the rate of pyridoxal phosphate utilization could be detected. This contrasts with the apparent 60% increase in rate observed by Pogell (1958), who reported a pyridoxal phosphate phosphatase to be present in mammalian-liver preparations, and suggested the formation of an imide type of intermediate in the presence of NH₄⁺ ions.

Phosphatase activities in extracts of Escherichia coli, and the effect of phosphatase preparations on pyridoxal phosphate. The fact that pyridoxal phosphate was dephosphorylated by *E. coli* extracts posed the question whether the enzyme involved was one specific for this substrate or possibly an alkaline phosphatase of broad specificity.

With disodium phenyl phosphate as substrate, and by measuring inorganic phosphate formation, cell extracts were found to have little activity at pH 10.1. At lower pH values, however, activity increased to an optimum at pH 5.3. Maximum activity was observed with 0.2 mM-Mg²⁺ ions. It thus appeared that cell extracts contained an acid phosphatase. The respective phosphatase activities at pH 5.3 and pH 10.1 were 12.20 and 0.27 μ moles of phosphate/30 min./mg. of protein.

With an alternative source of acid-phosphatase activity, i.e. Polidase-S, no dephosphorylation of pyridoxal phosphate could be detected at pH 10.1. When an alkaline phosphatase was used, i.e. Intestinal Phosphatase, rapid dephosphorylation of pyridoxal phosphate occurred, even at room temperature. These results suggest that, although *E. coli* extracts contain an acid phosphatase, this

enzyme is not responsible for the breakdown of pyridoxal phosphate at pH 10.1, but that an alkaline phosphatase present in smaller amounts is involved. Attempts to demonstrate pyridoxal phosphate-phosphatase activity by assay of inorganic phosphate formed were inconsistent, probably owing to the instability of the substrate under the conditions of the assay procedure.

Method evolved to calculate changes in substrate and product concentration. General formulae relating observed changes in *E* to the respective molar concentrations of the two components were derived for mixtures of pyridoxal phosphate and pyridoxal. Thus:

$$[\text{Pal.P}] = \frac{\epsilon_{\text{Pal}}^{300} E_{\text{Pal}}^{1 \text{ cm.}} - \epsilon_{\text{Pal}}^{390} E_{300}^{1 \text{ cm.}}}{\epsilon_{\text{Pal}}^{300} \epsilon_{\text{Pal.P}}^{390} - \epsilon_{\text{Pal}}^{390} \epsilon_{\text{Pal.P}}^{300}}$$

$$[\text{Pal.}] = \frac{\epsilon_{\text{Pal.P}}^{390} E_{300}^{1 \text{ cm.}} - \epsilon_{\text{Pal.P}}^{300} E_{\text{Pal}}^{1 \text{ cm.}}}{\epsilon_{\text{Pal.P}}^{390} \epsilon_{\text{Pal}}^{300} - \epsilon_{\text{Pal.P}}^{300} \epsilon_{\text{Pal}}^{390}}$$

It was found that, under the conditions used for enzymic dephosphorylation of pyridoxal phosphate, $\epsilon_{\text{Pal}}^{300} = 5000$; $\epsilon_{\text{Pal}}^{390} = 1600$; $\epsilon_{\text{Pal.P}}^{300} = 1080$; $\epsilon_{\text{Pal.P}}^{390} = 6000$. Thus the above formulae may be simplified to:

$$[\text{Pal.P}] = (1.77 E_{390}^{1 \text{ cm.}} - 0.56 E_{300}^{1 \text{ cm.}}) \times 10^{-4} \text{ M}$$

$$[\text{Pal.}] = (2.13 E_{300}^{1 \text{ cm.}} - 0.40 E_{390}^{1 \text{ cm.}}) \times 10^{-4} \text{ M.}$$

Applying these relationships to the data illustrated in Fig. 4, the specific activities for the dephosphorylation of pyridoxal phosphate by the cell extract obtained were 10.6 and 9.5 μ m-moles of pyridoxal and pyridoxal phosphate respectively/30 min./mg. of protein.

Relative pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities

The relative activities of pyridoxamine phosphatase oxidase and pyridoxal phosphatase were determined, with extracts prepared from a number of different batches of *E. coli* cells. The cells were grown with or without glucose. Methods used for the calculation of substrate concentration changes were those described above. In each case, the amount of substrate utilized was used as the criterion of activity measurements (Table 6). The pyridoxal phosphate-phosphatase activity of cells grown in a medium containing glucose is considerably in excess of the pyridoxamine phosphate-oxidase activity.

No pyridoxamine phosphate-phosphatase activity could be detected by the assay of inorganic phosphate production in any of the extracts examined.

DISCUSSION

The experimental measurement of pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities is complicated by the presence of

Table 6. *Relative pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities*

Reaction mixture composition and procedure were as described for Fig. 1, except that cell-extract concentration was 1.0 mg. of protein/ml. (final concn.), and MgSO_4 solution was replaced by water when Pal.P was used as substrate. Phosphatase activity was determined immediately after oxidase activity, with the same sample of cell extract.

Cell sample	Growth conditions for <i>E. coli</i>	Relative specific activities*		Ratio: (b)/(a)
		Pam.P oxidase (a)	Pal.P phosphatase (b)	
1	Without glucose	0.28	0.20	0.72
2		0.12	0.17	1.42
3	With 0.2% of glucose	0.48	0.98	2.05
4		0.20	0.79	3.95

* 10^{-2} $\mu\text{mole}/30 \text{ min.}/\text{mg.}$ of protein/ml. of reaction mixture.

both enzymes, in variable proportions, in the cell extracts used. If enzymic or chemical methods are used for the assay of pyridoxal phosphate when pyridoxamine phosphate is used as substrate, then the possible simultaneous breakdown of pyridoxal phosphate is difficult to allow for. On the other hand, spectrophotometric methods allow the precise determination of stoichiometric relationships between substrates and products. Spectrophotometric methods for the simultaneous determination of pyridoxamine phosphate, pyridoxal phosphate and pyridoxal concentrations in reaction mixtures are at present under development in these laboratories. Such methods allow exact measurement of pyridoxamine phosphate-oxidase activities even in the presence of pyridoxal phosphate phosphatase. The relative activities of pyridoxamine phosphate oxidase and pyridoxal phosphate phosphatase in biological systems is of intrinsic interest. Although Wada *et al.* (1959) claim that phosphatase activity towards pyridoxal phosphate can be removed from crude extracts of liver by precipitation at pH 5.0, Pogell (1958) was unable to remove this activity completely by such treatment.

In the present report, no explanation is given for the decrease in pyridoxamine phosphate-oxidase activity during dialysis of *Escherichia coli* extracts. In view of the linking of oxidase and pyridoxal phosphate-phosphatase activities, it is possible that the loss of some factor affecting the latter is responsible.

It has been reported that pyridoxamine phosphate is the predominating member of the vitamin B₆ group present in the cell (Rabinowitz & Snell, 1947; McNutt & Snell, 1948), and the possible significance of the enzymic conversion of pyridoxamine phosphate into pyridoxal phosphate in the control of amino acid metabolism has been pointed out (Beechey & Happold, 1957; Pogell, 1958). Pyridoxamine phosphate-oxidase activity has been shown to be responsible for the indirect

activation of tyrosine decarboxylase (Pogell, 1958), and is now thought to effect the observed activation by pyridoxamine phosphate of tryptophanase (Beechey & Happold, 1957) and kynureninase (Saran, 1958). The role played by pyridoxal phosphate in a large number of enzyme reactions has recently been reviewed by Snell (1958). The inhibition of pyridoxamine phosphate oxidase by α -oxo acids commonly involved in transamination reactions may be part of the mechanism used by cells for metabolic control. Of similar significance would be those factors affecting the activity of pyridoxal phosphate phosphatase. Both Pogell (1958) and Wada *et al.* (1959) have detected phosphatase activities for pyridoxal phosphate in rabbit-liver preparations. In the control of amino acid metabolism by coenzyme destruction, the strength of binding between pyridoxal phosphate and the respective apoenzymes would also be of importance. A number of pyridoxal phosphate-dependent enzymes lose their activity on aging under various conditions but can be reactivated by the addition of synthetic coenzyme (Kallio, 1951; Azarkh & Gladkova, 1952; Happold & Struyvenberg, 1954). The disappearance of bacterial lysine decarboxylase after induction (Mandelstam, 1954) also implicates coenzyme destruction (Pardee, 1958). If local intracellular control of either or both of the oxidase and phosphate enzymes dealt with here is possible, then differential regulation of individual enzymes or groups of enzymes involved in amino acid metabolism would occur. Horiuchi (1959) has recently reported that *Escherichia coli* cells grown on a phosphate-deficient medium exhibit an alkaline-phosphatase activity approximately 100 times as great as that of cells grown on a complete medium. In view of the fact that alkaline phosphatase rapidly destroys pyridoxal phosphate, these findings suggest that the amino acid economy of cells grown on deficient media may be affected by the inactivation of enzymes responsible for amino acid breakdown.

SUMMARY

1. Evidence is presented for the presence of pyridoxamine phosphate oxidase and a pyridoxal phosphate phosphatase in aqueous extracts of *Escherichia coli*.

2. Spectrophotometric methods have been developed for the precise measurement of substrate and product concentrations in both enzyme systems. Specific activities of the order of 10^{-2} μ -mole/30 min./mg. of protein/ml. of reaction mixture are reported.

3. Pyridoxamine phosphate oxidase was inhibited by dialysis, and further inhibition was caused by pyruvate, α -oxoglutarate and oxaloacetate at a concentration of 0.02 mM. Inhibition of activity by mepacrine suggests that a flavin coenzyme is involved.

4. Pyridoxal phosphate phosphatase was also found to be highly active in mammalian 'alkaline'-phosphatase preparations. The *Escherichia coli* extracts used exhibited high 'acid'-phosphatase and low 'alkaline'-phosphatase activities when disodium phenyl phosphate was used as substrate. No dephosphorylation of pyridoxamine phosphate could be detected.

5. The presence of glucose in *Escherichia coli* growth medium increased the relative amount of phosphatase compared with oxidase activity.

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The Sedimentation Characteristics of Deoxyribonucleic Acid from Human Tissues

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Polli & Shooter (1958) have presented the data obtained from sedimentation experiments with preparations of deoxyribonucleic acid from normal human spleen and leucocytes and from leucocytes

of patients with chronic myeloid and lymphatic leukaemia. It was found that the average sedimentation coefficients at infinite dilution for preparations from leucocytes of lymphatic leukaemia were higher than those observed for preparations from normal leucocytes. Different preparations

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