of DPNH[4]. The cause of the relatively small deviation from the DPNH spectrum may partly be the same as in yeast and other colorless cells: namely the binding of pyridine nucleotide to cell constituents; another cause of the deviation must be selective "self-absorption" of fluorescence light, for which no correction was applied. The relative smallness of the observed changes may be caused by a simultaneously increased rate of oxidation of pyridine nucleotide in photosynthesis, or by the inactivity of part of the fluorescing material in photosynthesis.

Our observations are consistent with the hypothesis that light drives the reduction of pyridine nucleotide in photosynthesis. The fluorescence method seems at present the most suitable to check this hypothesis in experiments on intact cells, since interference by other pigments is smaller than in absorption spectrophotometry.

SUMMARY

An increase in the blue fluorescence of suspensions of purple bacteria and of a blue alga was observed upon illumination with photosynthetically active infrared or red radiation. The spectrum obtained by subtracting the fluorescence spectrum in the "dark" from that in the "light" was similar to that of reduced pyridine nucleotide. This and other evidence obtained supports the hypothesis that during both algal and bacterial photosynthesis an accelerated reduction of pyridine nucleotide occurs.

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THE ISOLATION OF PYRIDOXAL-5-PHOSPHATE FROM CRYSTALLINE MUSCLE PHOSPHORYLASE*

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Phosphate analyses of crystalline muscle phosphorylase *a* were carried out on several occasions in the last 12 years during which time the method of preparation of the enzyme has been improved. The data are summarized in Table I. When the enzyme is recrystallized from cysteine-glycerophosphate buffer, followed by exhaustive washing of the crystals with 0.03*M* KCl in the cold (prep. 1, Table I), there still adheres to the crystals an impurity which gives a pentose reaction and which

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is presumably ribonucleic acid. This phosphorus-containing impurity can be removed by treatment of the dissolved crystals with Norit, followed by one or more crystallizations from cysteine–glycerophosphate buffer. When these recrystallizations are done from versene-glycerophosphate, a preparation (No. 3 in Table I) results which contains, on an average, 8 gram atoms of P per mole of enzyme. On precipitation of the enzyme with trichloroacetic or perchloric acid, 4 of the 8 moles of P are liberated. Phosphorylase *a* (mol. wt. 500,000) has been shown to be composed of 4 subunits, each of which has one combining site[4]. If the phosphorus were equally distributed, there would be present in phosphorylase *a* one firmly bound and one loosely bound organic phosphorus atom per subunit. The compound split off by acid from the enzyme has now been identified as pyridoxal-5-phosphate.

The identification rests on a comparison with a standard sample of synthetic pyridoxal-5-phosphate with respect to the following properties. (a) Absorption spectrum in acid and alkaline solution, (b) molar extinction coefficients, based on P analysis, (c) paper electrophoresis, (d) column chromatography, (e) activation of an apoenzyme preparation of aspartic-glutamic transaminase, (f) various color reactions based on the phenolic group and on diazotization. In all these properties the pyridoxal-5-phosphate isolated from phosphorylase was identical with the synthetic compound.

TABLE I

PHOSPHORUS CONTENT OF MUSCLE PHOSPHORYLASE

Values are given in gram atoms of phosphorus per mole of phosphorylase *a* (mol. wt. 500,000), rounded up to the nearest whole number.

No.	*Type of preparation*	*Total P in protein*	*P in TCA filtrate*			*P in protein precipitate*
			Inorg.	*Org.*	*Total*	
1	Recrystallized from cysteine-glycerophosphate. Crystals washed with 0.03 *M* KCl*	13			9	4
2	Same as (1), solution of crystals treated with Norit**	11	2	5	7	4
3	Same as (2), followed by crystallization from versene-glycerophosphate***	8	0	4	4	4

* Previous values[1], average of 6 preparations.

** Unpublished values by N. B. MADSEN, obtained by the micro-method of SOVENKOFF[2] on 3 different preparations. Precipitation with trichloroacetic acid (TCA) at 40 to 50°.

*** Present values, average of 5 preparations. One additional preparation gave higher values for total P and also contained inorganic P in the TCA filtrate. The phosphate analyses were done by the method of CHEN *et al.*[3].

Samples of synthetic pyridoxal-5-phosphate were obtained from Dr. GUNSALUS and from Merck and Co.*. The latter, a lyophilized preparation several years old, was purified by gradient elution with formic acid from a Dowex-1 column (8% cross-linked). The peak fractions were combined and precipitated as the barium salt from alkaline solution with 2 volumes of ethanol. The spectrum of this purified preparation,

* The authors are indebted to Dr. GUNSALUS and to Drs. UMBREIT AND FOLKERS for the supply of these samples.

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including both the position of peaks and the molar extinction coefficients, agreed with the values reported by PETERSON AND SOBER[5].

Isolation and identification of pyridoxal-5-phosphate

Batches of 100 to 150 mg of phosphorylase crystals (2 times crystallized from cysteine-glycerophosphate, followed by Norit treatment in solution, and 2 crystallizations from versene-glycerophosphate) were washed by centrifuging in the cold with successive portions of 0.03*M* KCl until calculations showed that the glycerophosphate would have been diluted beyond the limit of detection. The drained crystals were dissolved in about 5 ml of 0.1*M* $NaHCO_3$ and precipitated with 40% trichloroacetic acid (TCA, final concentration 7%) or with 70% perchloric acid (final concentration 7%). The acid was added dropwise with stirring to a solution kept in an ice bath. In other experiments the solutions were kept at room temperature. The immediate effect of the addition of the acid to these concentrated protein solutions is the appearance of a lemon yellow precipitate. The yellow color disappears in about 15 minutes in the cold and more rapidly at room temperature. Extraction in the cold requires stirring for about 20 minutes or longer, depending on the rate of fading of the color, and washing of the centrifuged precipitate with several portions of acid. Complete extraction is more easily accomplished at room temperature.

The combined TCA extracts were shaken with several portions of peroxide-free ether until the reaction was faintly acid to Congo Red paper. Perchloric acid was removed as the potassium salt in the cold. The extracts were adjusted to pH 8–8.5 with carbonate-free NaOH, whereupon they turned yellow. (As little as 4 μg of pyridoxal-5-phosphate per ml can be detected visually in alkaline solution.) A drop of 10% barium acetate was added, followed by the addition of 2 volumes of ethanol. On standing overnight in the cold a yellow precipitate formed. This was removed by centrifugation, extracted with water, and reprecipitated with ethanol. The yield was about 80%, based on spectrophotometric readings at 295 mμ in acid. In some cases a precipitate which formed rapidly in the cold on addition of one half to one volume of ethanol was removed by centrifugation, and the precipitate appearing after the addition of 2 volumes of ethanol was collected. This gave a product of higher purity at the expense of yield.

The material so isolated gave a negative orcinol reaction for pentoses and a positive Molisch test*. The presence of amino groups was indicated by tests with β-naphthoquinone-4-sulfonate[7] and ninhydrin[8]. When read against a tyrosine standard, less than one equivalent of amino N per pyridoxal-5-phosphate molecule was present. After purification by column-chromatography (see below), tests for carbohydrate and for amino groups were negative. Amino acids have been shown to adhere to phosphorylase after Norit treatment, repeated recrystallization and washing of the crystals[9]. A source of contamination with material giving carbohydrate tests are the cellophane dialyzing bags used in the preparation of the enzyme.

Column chromatography was carried out as follows. TCA extracts, prepared as indicated above, were made alkaline and adsorbed on a 1 × 9 cm Dowex-1 column (8% cross-linked, formate). An identical column was used for the adsorption and

* With the ferricyanide method of PARK AND JOHNSON[6] one observes an increase in reducing power after 10 minutes of hydrolysis in 1 *N* HCl at 100°. The same result is obtained with pyridoxal-5-phosphate.

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elution of similar small amounts of synthetic pyridoxal-5-phosphate*. Gradient elution was carried out with 2 *M* formic acid and a mixing volume of 500 ml of water. Pyridoxal-5-phosphate appeared in fractions (3 ml) 64 to 72, in which the formic acid concentration was about 0.5 *M*. The yield was similar in both cases (about 40%). No other material giving ultraviolet absorption, except pyridoxal-5-phosphate, could be obtained from the TCA extracts of phosphorylase. In particular, the presence of pyridoxamine-5-phosphate and of free pyridoxal or pyridoxamine could be excluded. In one case the elution was continued up to 4 *M* formic acid –0.4 *M* ammonium formate without any nucleotides being eluted.

Lyophilization of the fractions containing eluted pyridoxal-5-phosphate to dryness without previous neutralization of the formic acid resulted in decomposition, as shown by spectral changes and by enzymic tests with the aspartic-glutamic transaminase. It was possible, however, to concentrate the acid eluates *in vacuo* from the frozen state, and to precipitate the compound as the barium salt with alcohol without any decomposition. In Fig. 1 are shown the absorption spectra of the TCA extract

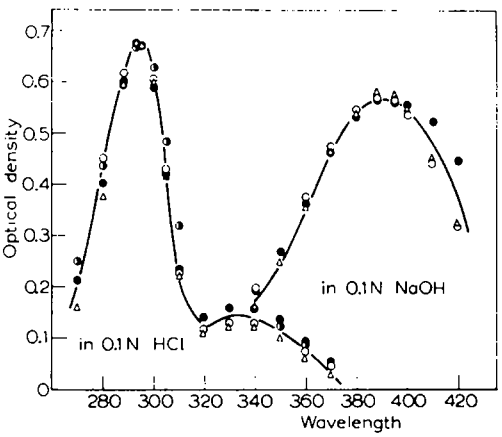

Fig. 1. A trichloroacetic acid (TCA) extract was prepared from 163 mg of phosphorylase and chromatographed as indicated in the text. △ Synthetic pyridoxal-5-PO_4, 0.1 μmole/ml based on P analysis; ○ TCA extract of phosphorylase *a*, 0.1 μmole/ml based on 295 mμ reading in acid; ● Column fraction, 0.1 μmole/ml based on 295 mμ reading in acid; ◑ Barium salt prepared from combined column fractions, 0.1 μmole/ml based on P analysis.

after removal of TCA with ether, of the column fractions containing pyridoxal-5-phosphate and of the barium salt prepared from the column fractions. The spectrum of synthetic pyridoxal-5-phosphate, purified by column chromatography, is included for comparison.

For paper electrophoresis the Model R—Series D apparatus and the paper strips supplied by Spinco were used. The buffer used was 0.015 *M* sodium acetate, pH 5.1. SILIPRANDI *et al.*[10] have shown that paper electrophoresis at pH 5.1 results in a complete resolution of mixtures of about 10 μg each of pyridoxal-5-phosphate, pyridoxamine-5-phosphate and the corresponding non-phosphorylated compounds. The ionization constants of these compounds have been determined[11,12]. At pH 5.1 pyridoxal-5-phosphate migrates to the anode, whereas pyridoxamine-5-phosphate remains close to the origin, and the non-phosphorylated compounds migrate to the cathode. The synthetic pyridoxal-5-phosphate, before purification by column chromatography, showed several spots with a blue fluorescence under ultraviolet light,

* The column was also used for the purification of large amounts (10 mg) of synthetic pyridoxal-5-phosphate and for the separation of artificial mixtures of pyridoxamine-5-phosphate, pyridoxal-5-phosphate and the corresponding non-phosphorylated compounds.

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as well as a yellow spot. After purification there was only a single, well-defined spot present which was poorly visible in ultraviolet light in the amounts used (5 to 10 μg), but which showed a yellow fluorescence when the paper was exposed to ammonia vapor. When the paper was sprayed with diazotized *p*-aminoacetophenone as described by SILIPRANDI *et al.*[10], only one spot appeared which corresponded in position to that previously marked under ultraviolet light. The presence of salts (and of formic acid) in the solution which is spotted on the paper, retards the migration of pyridoxal-5-phosphate, and in the presence of sufficient salt (*e.g.* when 10 μl are spotted containing 0.5 *M* NaCl) the compound remains at the origin. The material isolated from phosphorylase, either directly from the TCA extract or after column chromatography, gave one spot which migrated at the same rate as the authentic sample of pyridoxal-5-phosphate and which showed the characteristic yellow fluorescence when exposed to ammonia vapor.

In Table II is shown the standardization of a partially resolved transaminase preparation from pig heart* with known amounts of synthetic pyridoxal-5-phosphate. The concentration of the stock solution of pyridoxal-5-phosphate was calculated from phosphate analysis and from the molar extinction coefficients given by PETERSON AND SOBER[5]**, with good agreement (*cf.* Fig. 1). The concentration of the "pyridoxal-5-phosphate" isolated from phosphorylase was similarly determined. In enzymic tests carried out in conjunction with the experiment in Fig. 1, at a concentration of $2 \cdot 10^{-7} M$ pyridoxal-5-phosphate, the rates per minute were as follows. TCA extract of phosphorylase: standard pyridoxal-5-phosphate 0.115, unknown 0.125; column fraction before concentration *in vacuo* and conversion to barium salt: standard 0.120, unknown 0.125; after isolation as barium salt: standard 0.120, unknown 0.120. Similar results were obtained with a number of other preparations, *i.e.* there was close agreement between the concentration of pyridoxal-5-phosphate calculated from spectrophotometric readings and that calculated from enzymic tests.

TABLE II

ACTIVATION OF GLUTAMIC-ASPARTIC TRANSAMINASE

The reaction mixture consisted of 0.1 *M* tris(hydroxymethyl)aminomethane buffer, pH 8, 0.01 *M* α-ketoglutarate and L-aspartate, and 60 μg of enzyme per ml. The reaction was started by the addition of aspartate and the increase in optical density at 280 mμ was read at 15 second intervals in the Beckman DU spectrophotometer. The rates were linear over a 2 minute period. Temperature, 25°.

Addition of pyridoxal-5-P M	*Increase in optical density per min*
none	0.045
$1 \cdot 10^{-7}$	0.067
$2 \cdot 10^{-7}$	0.111
$1 \cdot 10^{-6}$	0.215
∞	0.286§

§ Calculated from Lineweaver-Burk plot; $K_m = 3.2 \cdot 10^{-7} M$.

* This preparation was carried out by Dr. SIDNEY VELICK according to an unpublished procedure. The authors are indebted to Dr. VELICK for making this enzyme preparation available.

** These values are 6700 at 295 mμ in 0.1 *N* HCl and 6600 at 388 mμ in 0.1 *N* NaOH. From Fig. 1 one obtains 6700 and 5700 respectively.

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The 4 moles of pyridoxal-5-phosphate while in combination with phosphorylase are not removed by dialysis or by adsorption on Norit, and are not split during incubation with intestinal phosphatase[1]. The pyridoxal-5-phosphate prepared from phosphorylase is adsorbed on Norit and can be eluted with Na_2CO_3 or with ammonia. The isolated compound is completely split by intestinal phosphatase and the spectral changes are those which would be expected to result from a conversion of pyridoxal-5-phosphate to free pyridoxal.

VELICK AND WICKS[13] used a microbiological assay for the determination of members of the vitamin B_6 group in samples of phosphorylase hydrolyzed in 0.1 *N* HCl -- 10 *N* formic acid. They reported 0.5 to 1 mole of "pyridoxine" per mole of enzyme. As indicated above, in the present work, the presence of other members of the vitamin B_6 group, except pyridoxal-5-phosphate, could not be detected in phosphorylase. The isolation of pyridoxal-5-phosphate from a natural source has not previously been reported. It is indeed strange that this natural source should now prove to be a crystalline enzyme from muscle. It should be mentioned that phosphorylase crystals do not contain glutamic-aspartic transaminase.

The role of pyridoxal-5-phosphate in combination with phosphorylase and the nature of the phosphate groups which remain attached to the enzyme after the removal of pyridoxal-5-phosphate are under investigation.

SUMMARY

Dialyzed and Norit-treated muscle phosphorylase *a*, after recrystallization from versene-glycerophosphate, contains 8 organic phosphate groups per mole or 2 phosphate groups per subunit of molecular weight of 125,000. Four of these phosphate groups are extracted by precipitation of the enzyme with trichloroacetic or perchloric acid. The extracted phosphate compound was isolated as the barium salt and identified as pyridoxal-5-phosphate by its spectrum and by specific enzymic tests. Column chromatography of the trichloroacetic acid extract and paper electrophoresis did not reveal the presence of other phosphorylated compounds. In particular, pyridoxamine-5-phosphate, adenylic acid and other nucleotides could not be detected. Free pyridoxal, pyridoxamine or pyridoxine were also absent.

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