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# Photosynthetic Reactions with Pyridine Nucleotide Analogs.
# III. *N*-Methylpyridinium Iodides*

Peter Böger, Clanton C. Black, and Anthony San Pietro

ABSTRACT: *N*-Methylpyridinium iodides with certain substituent groups on the pyridine ring actively catalyze photophosphorylation with spinach chloroplast fragments. During photophosphorylation neither reduction nor tritium incorporation could be detected. The reduced forms of the pyridinium salts and the free bases were inactive as catalysts. Photophosphorylation supported by the pyridinium salts was insensitive to anaerobic conditions but was sensitive to *o*-phenanthroline and substituted ureas. Desaspidin influenced photophosphorylation supported by the pyridinium salts in a manner comparable to that supported by triphosphopyridine nucleotide (TPN) plus spinach ferredoxin.

The reaction was not affected by the addition of soluble chloroplast enzymes such as ferredoxin. We suggest (in connection with previous findings with pyridine nucleotide analogs) that electron flow during photophosphorylation is mediated by a radical form of these compounds which does not accumulate but is rapidly quenched, presumably by some component of the chloroplast system or oxygen.

In previous papers (Böger *et al.*, 1966a,b) we reported that chloroplast fragments utilized as a cofactor of photophosphorylation not only TPN[1] but also a number of analogs of DPN and TPN. These studies demonstrated that the quaternary pyridinium portion of the molecule is necessary for photophosphorylation activity. The following observations support this conclusion. Certain pyridine nucleotide analogs could be reduced enzymatically and were unable to support photophosphorylation. The phosphoadenosine portion of the oxidized analogs could be removed with phosphodiesterase (dinucleotide nucleotidohydrolase, EC 3.6.1.9) without substantial loss in activity. The phosphoribose portion could be replaced by a methyl group and the N-methylated pyridinium iodides could serve as model compounds for the pyridine nucleotide analogs. Under appropriate experimental conditions, the rates of photophosphorylation were approximately the same.

Therefore, a number of substituted quaternary pyridinium iodides were synthesized and tested as catalysts for photophosphorylation. This approach to the study of chloroplast reactions is particularly useful since it is not possible to obtain all pyridine nucleotide analogs because DPNase (NAD glycohydrolase, EC 3.2.2.5) does not catalyze an exchange reaction with all substituted pyridines (*e.g.*, Lamborg *et al.*, 1958).

N-Alkylated pyridinium compounds have been studied intensively as model substances for DPN (Karrer *et al.*, 1938; Rafter and Colowick, 1954; van Eys and Kaplan, 1957; Wallenfels and Gellrich, 1959; Wallenfels and Schüly, 1959a,b) but were found not to function as reversible hydrogen carriers in enzyme reactions. Most of these investigations, however, were confined to the 3-carbamylpyridinium salt. The experiments described in this paper suggest that *N*-methylpyridinium salts, with certain substituent groups on the pyridine ring, function as redox carriers in the multienzyme system present in chloroplast material.

## Methods

*Preparation of Pyridinium Iodides.* The *N*-methylpyridinium iodides were synthesized in the following general manner. The pyridine compound with the

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[1] For abbreviations of the *N*-methylpyridinium iodides see Table I. Other abbreviations are: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; PMS, *N*-methylphenazinium methyl sulfate; Chl, chlorophyll; SCE, saturated calomel electrode; TPN and DPN, tri- and diphosphopyridine nucleotides; NAD, nicotinamide–adenine dinucleotide; ATP, adenosine triphosphate; NADP, nicotinamide–adenine dinucleotide phosphate.

TABLE I: Some Characteristics of the Synthesized *N*-Methylpyridinium Iodides.[q]

| *N*-Methyl Iodides of | Color of Solid Compd | Mp (cor) (°C) | Mp (°C) in Lit. |
|---|---|---|---|
| 2-Pyridinecarboxaldehyde (2-PA-$CH_3I$) | Orange | 175.5–177 | 174,[a] 178–179,[b] 180–183[c] |
| 3-Pyridinecarboxaldehyde (3-PA-$CH_3I$)[r] | Yellow | 172.5–173.5 | 173,[a] 173–175,[b] 174,[d] 164.5–166,[e] 90[f] |
| 4-Pyridinecarboxaldehyde (4-PA-$CH_3I$)[r] | Vermilion | 101–102 | 105–106[c] |
| 2-Hydroxymethylpyridine (2-$CH_2OH$-Py-$CH_3I$) | Grey-yellow | 148–149 | 153[g] |
| 3-Hydroxymethylpyridine (3-$CH_2OH$-Py-$CH_3I$) | Pale yellow | 80–81 | — |
| 4-Hydroxymethylpyridine (4-$CH_2OH$-Py-$CH_3I$)[r] | Grey-yellow | 96–98 | 96 and 98[h] |
| 2-Acetylpyridine (2-$COCH_3$-Py-$CH_3I$) | Light brown | 158–159 | 159–160[a] |
| 3-Acetylpyridine (3-$COCH_3$-Py-$CH_3I$) | Yellow | 163.5 | 163–164,[a] 154.4–155.2,[e] 162,[f] 160–163[i] |
| 4-Acetylpyridine (4-$COCH_3$-Py-$CH_3I$)[r] | Orange | 175 | 177–178[a] |
| 2-Cyanopyridine (2-CN-Py-$CH_3I$) | Yellow-orange | 175.5–177 | 183–184,[j] 175–176[k] |
| 3-Cyanopyridine (3-CN-Py-$CH_3I$) | Yellow | 200.5 | 194.5–195.5,[e] 196,[f] 198[l] |
| 4-Cyanopyridine (4-CN-Py-$CH_3I$)[r] | Orange | 197.5–198.5 | 197–198.5[m] |
| 4-Carbamylpyridine (INA-$CH_3I$) (isonicotine amide) | Yellow | 263 | 258.6,[e] 255,[n] 255–257,[o] 255–256[p] |
| 4-Thiocarbamylpyridine (IThNA-$CH_3I$) (isothionicotine amide) | Dark brown | — | — |
| 4-Pyridinecarboxylic acid hydrazide (INH-$CH_3I$)[r] (isonicotinic acid hydrazide) | Yellow | 203–206 | 205.2–206.8[e] |

[a] Ginsburg and Wilson (1957). [b] Ellin and Kondritzer (1959). [c] Steinberg *et al.* (1961). [d] Pannizon (1941). [e] Lamborg *et al.* (1957). [f] Pfleiderer *et al.* (1960). [g] Ferles and Prystas (1959, 1960). [h] Youatt (1961). [i] Akagi and Paretsky (1959). [j] Ellin (1958). [k] Kosower *et al.* (1960). [l] Schenker and Druey (1959). [m] Kosower (1958). [n] Karrer *et al.* (1938). [o] Grob and Renk (1954). [p] Kosower and Bauer (1960). [q] For nomenclature see Handbook of Chemistry and Physics (1964). [r] Confirmed also by elemental analysis.

proper substituent was refluxed with ten times the molar equivalent of methyl iodide. The mixture generally became colored during the reaction and the product precipitated. After 3–4 hr, the yield was 75–98% for pyridine derivatives substituted in the 3 or 4 position. The reaction time required for pyridine derivatives substituted in 2 position was longer (1–4 days) and the yield was low (*e.g.*, 10% for 2-acetylpyridine and 22% for 2-cyanopyridine). It should be noted that with the hydroxymethylpyridines, a 95% yield was obtained with all three isomers. Pyridine reacted with methyl iodide without heating and the reaction was complete in less than 5 min.

After refluxing, the excess methyl iodide was removed *in vacuo* and the product was washed three times with anhydrous ethyl ether. The N-methylated 4-thiocarbamylpyridine and isonicotinic acid hydrazide were used without washing; the methylated carbamylpyridines were recrystallized from hot methanol. The products were then dried in a desiccator.

The melting points of the synthetic *N*-methylpyridinium salts are listed in Table I and compared with the melting points reported in the literature. All melting points were rather sharp and reproducible with different preparations of the same compound. The melting point for the 4-thiocarbamylpyridinium iodide, however, could not be determined because it seemed to decompose at temperatures near the melting point. Elemental analyses of some of the representative compounds synthesized (see Table I) agreed reasonably with the theoretical values, *e.g.*, deviation of carbon analyses were below 2% from the theoretical value, with the exception of the *N*-methyl iodides of 4-pyridinecarboxaldehyde and of 4-acetylpyridine for which the deviations were 3.4 and 4.2%, respectively. The analyses were performed by the Spang Laboratories, Ann Arbor, Mich.

*Reduction of Pyridinium Iodides.* *N*-Methyl-3-cyanopyridinium iodide (3-CN-Py-$CH_3I$,[1] 500 mg), was reduced with $Na_2S_2O_4$ essentially as described by Schenker and Druey (1959). The reduced form was extracted from the reaction mixture by repeated extraction (20 times) with 3-ml portions of chloroform. The pooled extracts were dried over $Na_2SO_4$, evaporated to dryness *in vacuo*, and the resultant yellow oil was dissolved in 4 ml of anhydrous ethyl ether to which 20 ml of petroleum ether (bp 30–60°) was added subsequently. Yellow crystals precipitated during storage at $-15°$ overnight. The melting point and absorption spectrum of the crystalline material were the same as

TABLE II: Rates of Photophosphorylation and Oxygen Uptake and the Wavelength of the Longest Charge-Transfer Band.

| Compound | Concn (M)[a] | Phosphorylation Rate[b] With Free Base | Phosphorylation Rate[b] With *N*-Methylpyridinium Iodide | Oxygen Uptake[b] with *N*-Methylpyridinium Iodide | Longest Charge-Transfer Band[c] (mμ) |
|---|---|---|---|---|---|
| 2-PA-$CH_3I$ | $5 \times 10^{-3}$ | 2.0 | 151 | 64 | 456 |
| 3-PA-$CH_3I$ | $1 \times 10^{-2}$ | 1.0 | 118 | 45 | 360 |
| 4-PA-$CH_3I$ | $2 \times 10^{-3}$ | 8.0 | 165 | 35 | 458 |
| 2-$COCH_3$-Py-$CH_3I$ | $1 \times 10^{-3}$ | 2.0 | 160 | 16 | 430 |
| 3-$COCH_3$-Py-$CH_3I$ | $1 \times 10^{-2}$ | 2.0 | 57 | 27 | 346 |
| 4-$COCH_3$-Py-$CH_3I$ | $1 \times 10^{-3}$ | 2.0 | 154 | 16 | 435 |
| 2-CN-Py-$CH_3I$ | $5 \times 10^{-3}$ | — | 133 | 22 | 450 |
| 3-CN-Py-$CH_3I$ | $5 \times 10^{-2}$ | 3.0 | 70 | 34 | 361 |
| 4-CN-Py-$CH_3I$ | $2 \times 10^{-3}$ | 4.0 | 153 | 11 | 461 |
| 2-$CH_2OH$-Py-$CH_3I$ | $2.5 \times 10^{-2}$ | 10.0 | 70 | 22 | — |
| 3-$CH_2OH$-Py-$CH_3I$ | $5 \times 10^{-3}$–$3 \times 10^{-2}$ | — | <5 | 27 | — |
| 4-$CH_2OH$-Py-$CH_3I$ | $1 \times 10^{-2}$ | 3.0 | 114 | 11 | — |
| INA-$CH_3I$ | $1 \times 10^{-2}$ | 2.0 | 109 | 13 | |
| IThNA-$CH_3I$ | $2.5 \times 10^{-3}$ | — | 135 | 42 | |
| INH-$CH_3I$ | $1 \times 10^{-2}$ | 2.0 | 81 | 344 | |

[a] Concentration for optimal photophosphorylation activity. [b] ATP (μmoles/mg of Chl per hr or μatoms of O/mg of Chl per hr). [c] *N*-Methylpyridinium iodides dissolved in acetone.

reported. The reduction with sodium borohydride was performed according to the same authors. The pooled chloroform extract was washed with water (one-tenth volume), thoroughly dried, and the chloroform was removed. The orange-yellow oil had an absorption maximum at 349 mμ in ethanol as reported by Schenker and Druey (1959). It was used without further treatment.

*N*-Methyl-3-carboxaldehydepyridinium iodide (3-PA-$CH_3I$) was reduced with $Na_2S_2O_4$ as described above for the 3-CN-Py-$CH_3I$. The yellow oil, which was obtained after removal of the chloroform, was crystallized twice from an ethyl ether–petroleum ether mixture (1:1, v/v) at −15° overnight. The material melted at 30–31° and exhibited an absorption maximum at 370 mμ in 0.04 M Tris–HCl buffer, pH 7.8 ($\epsilon$ $1.97 \times 10^4$). In chloroform the maximum was at 362 mμ (Lamborg *et al.*, 1958). All the reduced compounds were devoid of absorbance around 260 mμ.

The aldehyde group was unaffected by the reduction procedure; that is, reduction of *N*-methyl-3-hydroxymethylpyridinium iodide did not give the product described above. Furthermore, the aldehyde test was still positive with the reduced 3-PA-$CH_3I$ (see below). Reduction of a pyridinium iodide having a substituent in the 4 position was attempted with *N*-methylisonicotiniamide iodide (INH-$CH_3I$) and sodium dithionite. We did not obtain a product which could be characterized by melting point or spectrum as being different from the oxidized form. The failure to reduce pyridinium salts with the substituent group in the 4 position of the ring was reported previously by Karrer *et al.* (1938) and Lamborg *et al.* (1958).

The presence of an aldehyde group was determined as follows. The substance was treated with an excess of dinitrophenylhydrazine (Vogel, 1959) in slightly acid solution at 100° for 2 min. After cooling, the solution was made 0.3 N with NaOH. An intense red-violet color ($\lambda_{max}$ 544 mμ) was observed with *N*-methyl-4-carboxaldehydepyridinium iodide (4-PA-$CH_3I$). The reaction was unaffected by an excess of *N*-methyl-4-hydroxymethylpyridinium iodide (4-$CH_2OH$-Py-$CH_3I$). Similar results were obtained with other aldehyde reagents, *e.g.*, 3-methyl-2-benzothiazolone hydrazone hydrochloride and ferric chloride (Sawicki *et al.*, 1961).

*Charge-Transfer Bands.* The absorption maximum of the longest charge-transfer band of the pyridinium iodides was determined in acetone. The substances (approximately 100 mg) were suspended in acetone (50 ml), stored for 14 hr in the refrigerator, and the supernatant solutions were used for the measurements. Some compounds were very unstable (*e.g.*, 4-PA-$CH_3I$) and they could not be dissolved completely in a short time such that destruction was negligible (Kosower and Skorcz, 1960). Therefore, $\epsilon$ values for these com-

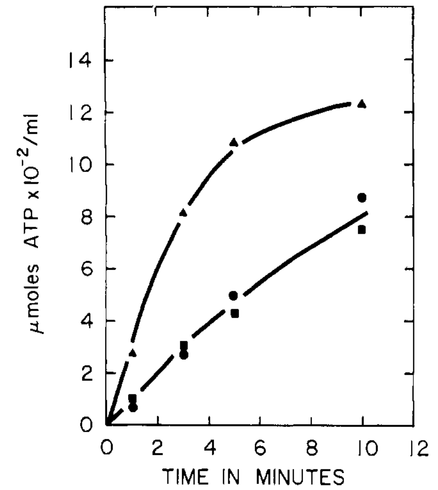


FIGURE 1: Time course of photophosphorylation catalyzed by 3-PA-$CH_3I$. The standard reaction mixture for photophosphorylation contained in micromoles: Tris–HCl buffer, pH 7.8, 40; $MgCl_2$, 2; $P_i$ + $^{32}P$ (0.4–1.5 μc of $^{32}P$), 1; ADP, 1; chloroplast fragments containing 20–30 μg of chlorophyll; total volume, 1 ml. In general, the reaction mixtures were illuminated for 2 or 3 min in cuvets of 1-cm light path with white light of 24,000 ergs/$cm^2$ sec. The gas phase was air unless mentioned otherwise. (●) reaction carried out in Thunberg cuvets which were evacuated and subsequently flushed with argon; (■) cuvets flushed with air; (▲) reaction carried out in Beckman cuvets aerobically without the previous evacuation and flushing procedure.

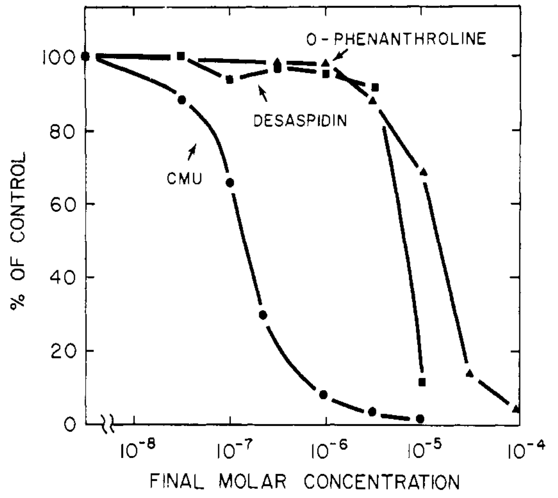


FIGURE 2: Action of inhibitors on photophosphorylation catalyzed by 3-PA-$CH_3I$. The experiments were performed under both aerobic and anaerobic conditions; Chl = 40 μg/ml.

pounds were not determined. The absorption bands mentioned in Table II were shifted to shorter wavelengths in more polar solvents (*e.g.*, ethanol) or by diluting the acetone with water. In water, no absorption bands in the visible region was detectable. This is in accord with the studies of Kosower (1958) on charge-transfer bands of pyridinium iodides.

*General Methods.* Spectra were determined with a Bausch and Lomb recording spectrophotometer Model 505; polarographic measurements were performed with a Sargent polarograph Model XXI at 22 ± 1° on $10^{-3}$ M pyridinium salt solutions in 0.1 M KCl and 0.04 M Tris–HCl buffer. The solutions were flushed with nitrogen prior to the measurement. Photophosphorylation was measured under both aerobic and anaerobic conditions as previously reported (Böger *et al.*, 1966a) by incorporation of $^{32}P$ (see Figure 1). Ferredoxin was not present in the reactions described, unless mentioned specifically. Only freshly prepared spinach chloroplast fragments were used. Oxygen uptake was measured polarographically. Tritium was counted with a Packard Tri-Carb liquid scintillation counter Model 314A. The counting solution contained in a final volume of 15 ml: 11 ml of toluene including 120 mg of 2,5-diphenyloxazole and 0.9 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene, 3.7 ml of ethanol, and 0.3 ml of the aqueous solution of material to be counted. Counting efficiency was 6.5%.

*Chemicals.* The substituted pyridines and the methyl-iodide were purchased from K & K Laboratories (Plainview, N. Y.) except the pyridinealdehydes, which were bought from Aldrich Chemical Co., Milwaukee, Wis. Desaspidin (a phlorobutyrophenone derivative; see Runeberg, 1962) was purchased from Oy Medica, Helsinki, Finland.

## Results

*Photophosphorylation.* The *N*-methylpyridinium iodides which catalyzed photophosphorylation are listed in Table II. The free bases were essentially inactive.

The *N*-methylpyridinium iodides of the following pyridine derivatives were found to be inactive, *i.e.*, the observed rate was less than 10 μmoles of ATP/mg of chlorophyll per hr: 3- and 4-pyridinecarboxylic acid, 3- and 4-methylpyridine, and 2- and 3-carbamylpyridine (each tested over the concentration range from $5 \times 10^{-3}$ to $3 \times 10^{-2}$ M); 3-thiocarbamylpyridine, 3-pyridinecarboxylic acid hydrazide, and 3- and 4-hydroxypyridine (concentration range tested was $5 \times 10^{-4}$–$10^{-1}$ M). The *N*-methyl iodides of pyridine and of *o*- and *p*-phenanthroline were also inactive.

There is an optimal concentration for phosphorylation activity for each active pyridinium salt as shown in Table II. The three isomers of the substituted pyridinium salts showed the same pattern of phosphorylation activity. The isomer with the substituent in the 3 position gave the lowest phosphorylation rate and the concentration required for optimal activity was highest. The rate was higher with the isomer containing the substituent in the 2 position of the ring and highest with the isomer containing the substituent in the 4 position; whereas, the optimal concentration required for optimal activity decreased corre-

TABLE III: Photophosphorylation with Oxidized and Reduced *N*-Methylpyridinium Iodides.

| Experiment[a] | Final Concn (M) | Reaction Time (min) | μmoles of ATP Formed: Oxidized | Reduced | Oxidized + Reduced[c] |
|---|---|---|---|---|---|
| 1. 3-PA-$CH_3I$ | $1 \times 10^{-2}$ | 3 | $1.4 \times 10^{-1}$ | $4.7 \times 10^{-3}$ | $8.3 \times 10^{-2}$ |
| | | 10 | $2.5 \times 10^{-1}$ | $6.1 \times 10^{-3}$ | — |
| 2. 3-CN-Py-$CH_3I$ | $4.5 \times 10^{-3}$ | 3 | $3.0 \times 10^{-2}$ | $4.7 \times 10^{-3}$ | — |
| | | 10 | $5.4 \times 10^{-2}$ | $4.9 \times 10^{-3}$ | — |
| 3. 3-CN-Py-$CH_3I$ | $4.5 \times 10^{-3}$[b] | 3 | | $5.1 \times 10^{-3}$ | — |
| | | 10 | | $6.1 \times 10^{-3}$ | $2.8 \times 10^{-2}$ |

[a] Experiments 1 and 2, reduced with $Na_2S_2O_4$; expt 3, reduced with sodium borohydride. [b] Concentration includes both *N*-methyl-3-cyano-1,6-dihydropyridine and *N*-methyl-3-cyano-1,2,5,6-tetrahydropyridine. [c] Each present in the concentration indicated in column 2.

TABLE IV: Influence of Chlorophyll Content on Desaspidin Inhibition.[a]

| Chl (μg) | Inhibition (%): 4-$COCH_3$-Py-$CH_3I$ | PMS |
|---|---|---|
| 13.7 | 93.2 | — |
| 22.9 | 62.4 | 99.5 |
| 45.7 | 41.0 | — |
| 91.5 | 31.5 | — |
| 183.0 | 28.0 | 98.0 |

[a] Final concentration for 4-$COCH_3$-Py-$CH_3I$ ($10^{-3}$ M) and for PMS ($10^{-5}$ M).

spondingly. The position of the longest charge-transfer band (in acetone) corresponded with the phosphorylation rate. The longer the wavelength, the higher was the phosphorylation rate (see discussion).

The phosphorylation rate was reproducible for a given concentration with different preparations, as checked with INH-$CH_3I$ and 3-PA-$CH_3I$. Use of tris-(hydroxymethyl)methylglycine buffer (Tricine, 0.04 M, pH 7.8) did not change the rates markedly.

As shown in Table III, neither 1,4-dihydro-3-PA-$CH_3I$ nor the reduced forms of 3-CN-Py-$CH_3I$ support photophosphorylation. The lack of activity is not due to a strong inhibitory effect of the reduced compounds (Table III, last column). This finding agrees with the data reported previously for the reduced pyridine nucleotide analogs. Practically no change in phosphorylation was observed when cuvets, previously flushed with argon (see Böger *et al.*, 1966b, for method), were opened to air and the rate was compared to that under complete anaerobic conditions. However, these rates were only 30–50% of that of the untreated aerobic control (Figure 1, upper curve).

*Inhibitors.* A strong inhibition of phosphorylation by CMU and *o*-phenanthroline was observed (Figure 2) in the concentration range which is known to inhibit photophosphorylation. The inhibition by desaspidin is similar to that reported for TPN plus ferredoxin supported phosphorylation (Arnon *et al.*, 1965; Böger *et al.*, 1966b). The degree of inhibition was the same under both air and argon. It was found, however, that the inhibition caused by desaspidin was inversely proportional to the amount of chloroplast material as shown in Table IV for the photophosphorylation supported by N-methyl-4-acetylpyridinium iodide (4-$COCH_3$-Py-$CH_3I$). Similar results were obtained with the phosphorylation supported by TPN plus ferredoxin. The effective inhibitory concentration of desaspidin may be less than that introduced since some of it could be removed by nonspecific binding to the chloroplasts in a noninhibitory manner as shown for DCMU inhibition by Izawa and Good (1965). It is interesting to note that the inhibition by desaspidin could always be relieved by adding bovine serum albumin (0.5%).

Photophosphorylation supported by the pyridinium compounds was unaffected by the addition of spinach ferredoxin, the antibody to ferredoxin-TPN reductase (reduced NADP:NAD oxidoreductase, EC 1.6.1.1), cyanide ($10^{-2}$ M), or aminotriazole ($10^{-2}$ M).

*Spectral or Chemical Changes during Photophosphorylation.* Under air no spectral changes were observed during photophosphorylation with pyridinium iodides. With 3-PA-$CH_3I$, the absorbance around 342 mμ increased slightly after illumination for some minutes in an atmosphere of argon (Figure 3). Inclusion of spinach ferredoxin in the reaction mixture resulted in an increase in this absorbance of approximately tenfold and an absorption maximum at 342 mμ was observed. Photophosphorylation was not increased under these conditions.

We did not investigate further the nature of the compound responsible for the absorbance at 342 mμ. It was noted that it was stable in air and its formation was completely inhibited by CMU. When a standard

TABLE V: Assay for Tritium Labeling.[a]

| Experiment | µmoles of ATP Formed | µmoles of *N*-Methyl-pyridinium Iodide in Counting Vial | Cpm | Cpm/ µmole of Pyridinium Iodide | Calcd Additional Cpm for $^3$H-Labeling/ µmole of Pyridinium Iodide |
|---|---|---|---|---|---|
| 4-COCH$_3$-Py-CH$_3$I, light | 8.89 | 1.71 | 1331 | 797 | 507 |
| 4-COCH$_3$-Py-CH$_3$I, dark | (0.203) | 1.71 | 1402 | 820 | — |
| 4-COCH$_3$-Py-CH$_3$I, control, dark | — | 1.45 | 1327 | 914 | — |
| 3-PA-CH$_3$I, light | 9.05 | 3.98 | 1216 | 306 | 307 |
| 3-PA-CH$_3$I, dark | (0.06) | 4.19 | 1277 | 305 | — |
| 3-PA-CH$_3$I, control, dark | — | 5.00 | 1137 | 228 | — |

[a] Reactions were carried out in open Warburg vessels in a water bath at room temperature under occasional shaking for 20 min; light intensity was approximately 150,000 ergs/cm$^2$ sec. Each reaction mixture contained in a final volume of 1 ml: 10 mc of $^3$H$_2$O (=0.346 µatom of $^3$H), 20 µmoles of Tris–HCl buffer (pH 8), 2 µmoles of MgCl$_2$, 606 µg of Chl, 14 µmoles of ADP, 14 µmoles of P$_i$, 4 µmoles of 4-COCH$_3$-Py-CH$_3$I, or 10 µmoles of 3-PA-CH$_3$I. Controls had no chloroplast material. For ATP determination the reaction vessel contained approximately 1 µc of $^{32}$P$_i$ instead of $^3$H$_2$O and the reaction was stopped by adding 0.1 ml of 4 N HClO$_4$. After the removal of the chloroplast fragments by centrifugation, 0.7 ml of the supernatant solution was withdrawn and diluted with 14 µmoles of 4-COCH$_3$-Py-CH$_3$I or 20 µmoles of 3-PA-CH$_3$I. This was placed on a Dowex 1 column (chloride form, 1 × 1.5 cm) and then eluted with water in the cold. Spectra of the effluents indicated neither a difference between the "light" and "dark" samples nor any contamination. Aliquots were dried *in vacuo* at room temperature over silica gel and the residue was redissolved in 0.5 ml of water and this drying procedure was repeated 11 times. The radioactivity (tritium) of the solid material was unchanged upon further recrystallization from acetone.

reaction mixture containing 3-PA-CH$_3$I was illuminated for 15 min under air or argon and the chloroplast particles were removed by centrifugation, the supernatant solution was as active in catalyzing photophosphorylation as that from a control kept in the dark.

During photophosphorylation with 4-CH$_2$OH-Py-CH$_3$I ($10^{-2}$ M, 28 µg of Chl, 6-min illumination) the formation of an aldehyde group could not be detected. The formation of 4-PA-CH$_3$I could have been detected easily in a concentration as low as $10^{-5}$ M even in the presence of the high concentration of the 4-hydroxymethylpyridinium salt.

*Lack of Tritium Incorporation.* The data from typical experiment are outlined in Table V. The samples kept in the dark, with and without chloroplast fragments, exhibited the same tritium incorporation as those in which photophosphorylation had occurred. The relatively high unspecific tritium labeling was dependent on the concentration of $^3$H$_2$O in the assay. The data in the last column represents the calculated theoretical additional counts per minute which were expected provided tritium incorporation had occurred during photophosphorylation. The calculation assumes an isotope effect of 10 (Rose and O'Connell, 1961; Simon *et al.*, 1964) and that one hydrogen (or tritium) would be incorporated into the *N*-methylpyridinium iodides per ATP formed. It is clear that this calculated theoretical additional $^3$H labeling was not observed.

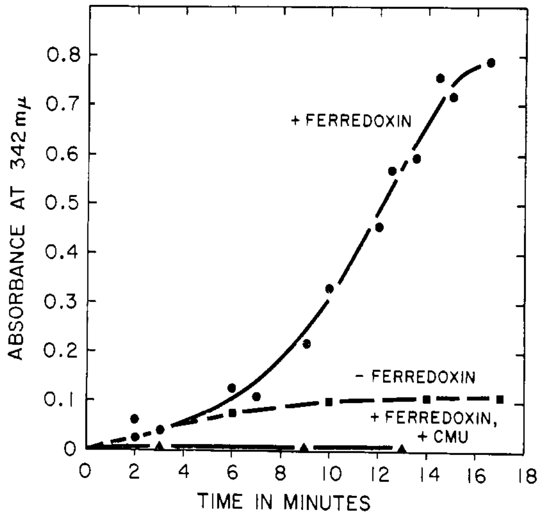
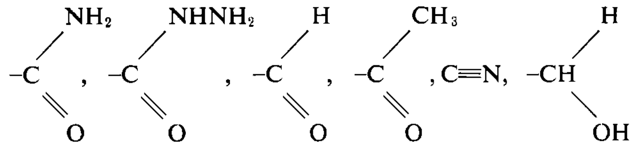


FIGURE 3: Increase of absorbance at 342 mµ during photophosphorylation catalyzed by 3-PA-CH$_3$I. Final concentration of: ferredoxin, 20 µg/ml; CMU, $10^{-5}$ M; 3-PA-CH$_3$I, $10^{-2}$ M; Chl, 30 µg/ml; gas phase, argon.

*Oxygen Uptake.* During photophosphorylation there was oxygen uptake but, as reported previously for the DPN analogs, it could not be related stoichimetrically to the amount of phosphate esterified (Table II). Furthermore, the data varied by a factor 2 or 3 with

TABLE VI: Substituent Groups in Synthesized Pyridinium Iodides.

Active



Nonactive

H, $CH_3$, OH, COOH

different chloroplast preparations and solutions of the *N*-methylpyridinium iodides. A high oxygen consumption was measured with the N-methylated isonicotinic acid hydrazide (INH-$CH_3$I). However, this compound was oxidized in the presence of chloroplast material even in the dark. Oxygen uptake was not influenced by adding KCN or aminotriazole; both are potent inhibitors of chloroplast catalase (Trebst and Eck, 1961).

*Electron Paramagnetic Resonance Studies.* Electron paramagnetic resonance (epr) signals could not be detected during photophosphorylation, or under the conditions described earlier (Böger *et al.*, 1966a), with 3-PA-$CH_3$I, 4-$COCH_3$-Py-$CH_3$I, or INH-$CH_3$I. In some experiments the sample was illuminated for 8 min, placed immediately into liquid nitrogen, and the epr measurements were performed at the temperature of liquid nitrogen. No epr signals were observed.

*Polarography.* 3-Carbamylpyridinium iodide gave two reduction waves of about equal height at −1.02 and −1.58 v *vs.* SCE at pH 8 which is consistent with the data reported by Burnett and Underwood (1965a). For the 2-, 3-, and 4-$COCH_3$-Py-$CH_3$I, half-wave potentials of −0.74 and −1.30, −0.86 and −1.28, and −0.61 and −1.30 v *vs.* SCE, respectively, were observed and, in each case, the height of each wave almost the same. The potentials of the first waves of the acetylpyridinium iodides may, therefore, represent the species which originate during photophosphorylation. Unfortunately, the results with other pyridinium ions were less clear. The first reduction waves for the 2-, 3-, and 4-PA-$CH_3$I were at −0.60, −0.72, and −0.50 v *vs.* SCE, respectively, but their height was much smaller than the second reduction waves (which appear at −0.90 v or higher). In these cases, we could not rule out conclusively the possibility that we were dealing with absorption waves. The hydroxymethylpyridinium iodides yielded only one reduction wave at approximately −1.38 v *vs.* SCE and the 4-carbamylpyridinium iodide gave only a single one at −1.50 v *vs.* SCE.

## Discussion

The free bases were inactive in photophosphorylation and had to be alkylated to observe catalysis of phosphorylation. In general, all the active pyridinium salts had a nonionic polar group, containing a carbon atom, as the substituent on the ring (Table VI). Activity appears to be connected closely with the presence of an electron-withdrawing substituent on the ring; inactivity appears to be correlated with electron-donating substituents. However, this rule is not strict. Introduction of the carbinol group resulted in phosphorylation activity, although it was lower than that observed with compounds containing electron-withdrawing substituents.

In addition to the character of the group, phosphorylation activity is related possibly to the position of the substituent. The relationship is position 4 > position 2 > position 3. This generalization is distorted by the extreme properties of some pyridinium salts. 3-$CH_2$OH-Py-$CH_3$-I is completely inactive and, of the carbamylpyridinium iodide isomers, only the 4 isomer supports phosphorylation. It is of particular interest that 3-carbamylpyridinium iodide is inactive in the phosphorylation system.

The positive ring charge seems to be necessary for phosphorylation activity as deduced from the lack of activity of the reduced forms. The carboxylic group abolishes activity, possibly due to the existence of this pyridinium ion as a zwitterion.

The easier it is to reduce a compound, the longer will be the wavelength of its absorption band. This was shown, for example, with carbonyl compounds (Brück and Scheibe, 1957) and nitrosobenzenes (Holleck and Schindler, 1956). In Kosower's laboratory it was demonstrated that the longest charge-transfer band corresponded with the one-electron reduction potential of pyridinium iodides (*e.g.*, for the *N*-methyl or *N*-ethyl iodides of 4-cyanopyridine and 4-acetylpyridine, Kosower and Skorcz, 1960; Schwarz *et al.*, 1961). For a theoretical approach, see Pullman *et al.* (1950). The correlation between photophosphorylation activity and the position of the wavelength of the longest charge-transfer bands suggests that the pyridinium isomers are reduced during the course of their catalytic activity. However, the lack of spectral changes during photophosphorylation indicates either that a reduced compound is not formed or that it is formed but does not accumulate in a measurable concentration because it is constantly reoxidized. If a reoxidation does occur, oxygen reacts only slowly as indicated by the low rate of oxygen uptake and the slight influence of anaerobic conditions. One has to assume, therefore, that if the reduced pyridinium salt is formed, it is reoxidized by some component of the chloroplast fragments.

Reduction of 3-CN-Py-$CH_3I$ and, probably 3-PA-$CH_3I$, with sodium dithionite yields the 1,4-dihydropyridines (Karrer and Manz, 1946; Schenker and Druey, 1959). With sodium borohydride, 3-CN-Py-$CH_3I$ is reduced to the 1,6-dihydropyridine and to the 1,2,5,6-tetrahydropyridine in about equal amounts (Schenker and Druey, 1959). As these forms were not found to possess phosphorylation activity, they should not have been formed during ATP formation.

The two possible explanations for a redox functions of 3-CN-Py-$CH_3I$ in photophosphorylation are: (1) the 1,2-dihydropyridine component may be formed and constantly be reoxidized. At present, such a dihydro form was only prepared in pure form with *N*-(dichlorobenzyl)-3-carbamyl-4,6-dimethylpyridinium bromide (Wallenfels and Schüly, 1959b) and was found to be very unstable in solution. Chaykin *et al.* (1966) describe the lability of 1,2-dihydro-DPN. (2) The pyridinium iodides are reduced only to a radical form which is rapidly reoxidized and does not accumulate. Pyridinyls have been reported to be very sensitive to oxygen and unstable in water (Kosower and Poziomek, 1964; Itoh and Nagakura, 1965). Possible structures of pyridinium radicals are described by these authors and by Wallenfels and Gellrich (1959). Although we could not observe an epr signal during photophosphorylation, this does not rule out the possibility of radical formation; a radical may have been easily quenched and therefore escaped detection. We should note that a clear epr signal could be seen (Böger *et al.*, 1966a) with isonicotinic acid hydrazide–DPN in the photophosphorylation system. Since the responses to concentration, inhibitors, ferredoxin, and spectral changes in the phosphorylation system with the *N*-methylpyridinium salts are very similar, we assume that the redox activity of these compounds may be explained by a transient radical formation. The lack of tritium incorporation in 3-PA-$CH_3I$ and 4-$COCH_3$-Py$CH_3I$ during photophosphorylation is further evidence that electron transport is mediated by a radical form only. A mechanism involving a fully reduced (and subsequently reoxidized) form is only conceivable, provided the reduction and reoxidation exhibit the same stereospecificity (San Pietro and Lang, 1957), which seems unlikely.

The inhibition of CMU and *o*-phenanthroline indicates that the oxygen-evolving system is necessary for phosphorylation activity. It is possible that some precursor of molecular oxygen formation reacts with the pyridinyl.

Depending on the chemical environment (pH and nature of buffer) one or more reduction waves may be obtained by polarographic reduction of the same component (Schwarz *et al.*, 1961; Burnett and Underwood, 1965b). Therefore, the observed polarographic reduction waves may not necessarily represent the redox potential which is due to the biochemical reaction involved as was shown for DPN by Ke (1956) and Burnett and Underwood (1965b).

The first polarographic waves of some of the active pyridinium iodides probably could be correlated with their phosphorylation activity. In other cases, the potentials were so negative ($> -1.0$ v) that they can hardly be considered to reflect the redox potentials of the biochemical reactions under consideration.

Although ferredoxin is not necessary, it is obvious that its presence can direct reactions in which the pyridinium salts participate. In the presence of ferredoxin and 3-PA-$CH_3I$, a significant change in the spectrum during photophosphorylation was observed. A similar phenomenon has been observed with 3-pyridinealdehyde–DPN and a possible explanation was given (Böger *et al.*, 1966b). The nature of this compound remains to be investigated.

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# An Improved Method for the Partial Synthesis and Purification of 5′-Deoxyadenosylcobalamin*

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ABSTRACT: Difficulties encountered in the preparation and purification of 5′-deoxyadenosylcobalamin by published methods have led to the development of a rapid method for the preparation and purification of this cobamide in good yield.

5′-Deoxyadenosylcobalamin is prepared by reduction of cyanocobalamin (vitamin $B_{12}$) to hydridocobalamin ($B_{12s}$) and reaction of the latter with a solution of crude 5′-tosyl-2′,3′-isopropylideneadenosine. After removal of the solvent by evaporation under reduced pressure, the isopropylidene group is removed in dilute acid. Gel filtration of the products on G-15 Sephadex separates the 5′-deoxyadenosylcobalamin from impurities of lower molecular weight and partially separates it from hydroxocobalamin ($B_{12a}$) which is formed during the preparation. The 5′-deoxyadenosylcobalamin is finally crystallized from aqueous acetone.

Supplies of 5′-deoxyadenosylcobalamin are necessary for further investigation of several enzymic reactions in which the cobamide has recently been shown to participate (Weissbach and Dickerman, 1965). The substance is not commercially available so that its preparation from natural sources or by a convenient chemical synthesis is of importance. However, extraction of 5′-deoxyadenosylcobalamin from natural sources is lengthy (*e.g.*, Barker *et al.*, 1960) and, in our experience, published chemical syntheses suffer from a number of difficulties. A modified method of synthesis is described in which these difficulties are avoided.

The partial chemical synthesis of 5′-deoxyadenosylcobalamin was first reported by Johnson *et al.* (1963) and Bernhauer *et al.* (1962). They reduced hydroxocobalamin ($B_{12a}$), prepared from cyanocobalamin ($B_{12}$), with sodium borohydride (Johnson *et al.*, 1963) or zinc in 10% ammonium chloride solution (Bern-

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