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Model Reactions for Coupling Oxidation to Phosphorylation*

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ABSTRACT: The oxidation-linked phosphorylation in *N,N*-dimethylacetamide solution is further examined along with some new experimental data. Phosphorylation occurred when a ferrohemochrome solution was oxidized by air in *N,N*-dimethylacetamide solution containing imidazole, adenosine 5′-monophosphate, and/or inorganic orthophosphate. When inorganic orthophosphate was absent, P^1,P^2-diadenosyl pyrophosphate could be produced at 10% yield. Phosphorylation was also observed when a *N,N*-dimethylacetamide solution of porphyrin plus imidazole plus adenosine 5′-monophosphate and/or inorganic orthophosphate was photoreduced under nitrogen and then reoxidized by exposure to air. Controlled experiments show that phosphorylation took place in the subsequent oxidation instead of the prior photoreduction step. Kinetic measurements show the existence of an intermediate capable of phosphorylating adenosine 5′-diphosphate to adenosine 5′-triphosphate. By using ^{32}P-labeled inorganic orthophosphate and paper chromatography, it was shown that this intermediate has the same R_F value as 1-phosphoimidazole. A possible oxidation mechanism leading to the formation of 1-phosphoimidazole, which can subsequently phosphorylate adenosine 5′-monophosphate or adenosine 5′-diphosphate, is suggested.

The elucidation of the molecular mechanisms involved in photo- and oxidative phosphorylation is a very difficult problem both because of the great structural complexity of the particulate systems wherein these reactions occur and because of our very limited knowledge concerning the type of chemical mechanisms capable of coupling oxidation to phosphorylation. During the last few years, attempts have been made in our laboratory to find oxidation reactions which cause inorganic phosphate to condense with AMP[1] and ADP, respectively, to form ADP and ATP. Initially it was established that both 1-phosphoimidazole and acetylimidazole plus orthophosphate would phosphorylate AMP and ADP at respectable rates in polar organic solvents (Brinigar and Wang, 1964a;

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[1] Abbreviations used: AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates; APPA, P^1,P^2-di(adenosyl-5-) pyrophosphate; DMAC, *N,N*-dimethylacetamide; IMP, inosine monophosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, (*p*-bis[2-(5-phenyloxazolyl)]benzene; TPP, $\alpha,\beta,\gamma,\delta$-tetraphenylporphine; PP, inorganic pyrophosphate; PPP, inorganic triphosphate; AP_4, adenosine tetraphosphate.

Brinigar and Knaff, 1965). Subsequently, it was demonstrated that oxidation of chlorocruoroheme dimethyl ester in the presence of imidazole, orthophosphate, and AMP resulted in the production of appreciable amounts of ATP (Brinigar and Wang, 1964b). The results were interpreted in terms of the participation by the formyl group of the metalloporphyrin in the phosphorylation reaction, since chlorocruorohemin dimethyl ester was also observed to produce ATP under similar conditions. It has now been shown that molecular oxygen is required for these reactions and that the chlorocruorohemin works only when a small fraction of the metalloporphyrin is in the Fe(II) state. Furthermore, the infrared spectrum of chlorocruorohemin dimethyl ester reisolated from a reaction mixture appears identical with that of the starting material, the carbonyl stretching frequency of the formyl group remaining unchanged at 1660 cm^{-1}. Consequently, it must be concluded that the formyl group is neither oxidized nor hydrated in the oxidation processes. In the present work, this oxidative phosphorylation reaction is further examined along with some new experimental data.

Another reaction which in many respects resembles hemochrome oxidation is the aerobic oxidation of photoreduced porphyrins. It has been found that upon illumination, ATP is produced in a dimethylacetamide solution containing imidazole, orthophosphate, AMP, porphyrin, and a limited amount of molecular oxygen. This reaction has also been studied in the present work because of its intriguing resemblance to photosynthetic phosphorylation.

Materials

Reagents. The $\alpha,\beta,\gamma,\delta$-tetraphenylporphine was a generous gift of Dr. Henry Rosenberg. It was prepared from zinc tetraphenylporphine (Ball *et al.*, 1946) and purified by chromatography on magnesium trisilicate. Chlorocruorohemin dimethyl ester, protohemin dimethyl ester, hematohemin dimethyl ester, hematohemin, and Fe-$\alpha,\beta,\gamma,\delta$-tetraphenylporphine were prepared from the corresponding porphyrins by the ferrous acetate–acetic acid method of Warburg and Negelein (1932) with the modification that the metalloporphyrins were isolated as the acetate rather than the chloride salts. Although the chloroform extract was washed continuously with water for 1–2 hr, the isolated hemin contained in all cases from 5 to 15% Fe(II) heme as estimated by the visible absorption spectra of a N_2-swept pyridine solution before and after reduction with $Na_2S_2O_4$. That acetate was the accompanying anion was shown by the presence of a carbonyl-stretching absorption band at 1600 cm^{-1} in the infrared spectrum of Fe-$\alpha,\beta,\gamma,\delta$-tetraphenylporphine. Protohematin dimethyl ester was prepared from commercial hemin chloride as previously described (Wang *et al.*, 1958), and was entirely in the oxidized form.

APPA was prepared by mixing equimolar quantities of AMP and AMP morpholidate in dimethylacetamide solution, removing the solvent *in vacuo*, and purifying the product by ion-exchange chromatography as described below. The firefly lantern extract, AMP-morpholidate, and AMP-deaminase were obtained from Sigma Chemical Co.

Carrier-free [^{32}P]phosphoric acid was obtained from New England Nuclear Corp. The sources of other reagents used have already been described (Brinigar and Wang, 1964b; Brinigar and Knaff, 1965).

Solutions. DMAC was obtained from Matheson Coleman and Bell, passed through an alumina column to remove possible traces of acetic anhydride, and vacuum distilled before use. The imidazole plus P_i plus AMP solution was prepared by dissolving equimolar amounts of adenosine 5′-phosphoric acid and imidazole along with 2.5 equiv of diimidazolium hydrogen phosphate in DMAC. The corresponding ADP solution was prepared from the barium salt by treating it with 1 equiv of diimidazolium sulfate in water, removal of the insoluble $BaSO_4$, evaporating the supernatant to dryness under vacuum, and dissolving the residue in DMAC. The ADP concentration was determined by the absorption at 259 mμ after 1:10 dilution with water, ϵ 1.57×10^4.

The Fe(II) hemochrome solutions were prepared by dissolving 1.5 mg (2 μM) of protohemin (or hematin) dimethyl ester and 0.7 mg (10 μM) of imidazole in 1.0 ml of DMAC. After approximately 2 mg of palladium black was added to the solution, the mixture was first swept with N_2 and then with H_2 until reduction was complete (about 30 sec). The hydrogen was removed with nitrogen, and after the palladium had settled, 0.5 ml of the supernatant was withdrawn with a syringe and used immediately.

Methods

ATP Analysis. The quantitative determination of ATP was carried out by mixing diluted aliquots of the reaction mixture with firefly lantern extract in a dark chamber mounted on top of a photomultiplier tube as previously described (Brinigar and Wang, 1964b).

Illumination. The preliminary photophosphorylation experiments with AMP and P_i were conducted at 40°, using Westinghouse 250-w incandescent spotlights. The experiments with AMP alone as substrate were carried out at 25°, using a Sylvania 500-w quartz, iodine vapor lamp.

Paper Chromatography. Whatman No. 2 paper was used with a descending solvent of ethanol–0.1 F aqueous K_2CO_3 (65:35). The following R_F values were observed: orthophosphate, 0.15; 1-phosphoimidazole, 0.35–0.40; and imidazole, 0.8. Hanes–Isherwood molybdate reagent and diazotized sulfanilic acid were employed for the visualization of phosphate and imidazole, respectively, as described by Rathlev and Rosenberg (1956).

Column Chromatography. Separation of AMP and APPA was accomplished by the method of Cohn and Carter (1950). The sample, dissolved in 1 M NH_4-OH, was placed on an ice-jacketed Rexyln 201 column. After washing with 25 ml of deionized water, the

AMP was eluted from the column with 50 ml of 0.003 F HCl. The nucleotide in this eluate was exclusively AMP as demonstrated by its complete deamination after equilibration with 5′-AMP-deaminase (EC 3.5.4.6). The APPA, synthesized from AMP and AMP-morpholidate, was eluted with 0.01 F HCl (Khorana, 1961). This second fraction contained no AMP, since no spectral change was detectable after incubation with AMP-deaminase. However, after the material had been treated with 1 F HCl at 100° for 30 min, it was completely deaminated by the deaminase, indicating total conversion to AMP.

APPA Assay. The assay is based on the observation that AMP-deaminase converts AMP virtually completely to IMP (Lee, 1960) but is inactive toward APPA. The extent of deamination was followed spectrophotometrically by using $\epsilon_{AMP} - \epsilon_{IMP} = (8.0 \pm 0.4) \times 10^3\ M^{-1}\ cm^{-1}$ at 265 mμ, the wavelength at which the maximum difference between the two spectra occurs, as determined by the deamination of standard AMP solutions. The concentration of adenine groups which remain unchanged after treatment with AMP-deaminase is equal to twice the molar concentration of APPA.

Spectroscopic Measurements. Visible and ultraviolet spectra were determined with a Cary Model 11 recording spectrophotometer. Infrared spectra were taken with a Perkin-Elmer Model 421 spectrophotometer. The electron spin resonance spectra were taken with a Varian Model V4502-12 spectrometer.

Measurement of Radioactivity. The radioactive samples were counted in a Packard Model 3002 liquid scintillation spectrometer. The liquid scintillator employed was 20 g of PPO and 1.2 g of dimethyl-POPOP in 3.8 l. of toluene.

Results

Hemochrome Oxidation Coupled to ATP Synthesis. Reaction mixtures were degassed under vacuum, refilled with scrubbed nitrogen, and sealed in glass ampules. Some experiments were carried out under air in sealed vessels. The results are summarized in Table I. Listed also in Table I are the ATP yields obtained in the air oxidation of other iron porphyrins prepared similarly by the ferrous acetate–acetic acid method. It is of interest to note that hematohemin itself, in contrast to its dimethyl ester, was completely oxidized during purification and produced no detectable ATP. Data in Table I show clearly that ATP was formed only in the mixtures where the oxidation of Fe(II) porphyrin by O_2 took place. Although the oxidation of chlorocruoroheme dimethyl ester seemed to produce more ATP than the oxidation of the other iron porphyrins of lower reduction potential, the difference is not great and hence subsequent oxidation studies were carried out with the hemochrome prepared from the more readily available protohemin dimethyl ester.

Phosphorylation in the Absence of P_i. It may be noticed from Table I that the ratio of ATP produced to Fe(II) hemochrome is usually below 1%. The low yield is partly due to the large number of products formed in addition to ATP, *e.g.*, ADP, APPA, PP, PPP, AP_4, etc. Thus, the ATP represents only a small portion of the total yield of phosphorylated products.

On the other hand, in the oxidation of Fe(II) hemochrome in a DMAC solution containing only imidazole and AMP, only a single phosphorylated product, APPA, would result, and consequently the expected yield would be considerably higher. The experimentally determined ratio [APPA]:[Fe(II) porphyrin] was 10% for the oxidation of chlorocruorohemochrome and 7% for protohemochrome.

Photoreduction of Porphyrins. When a dilute solution of porphyrin in DMAC solution containing imidazole, AMP, and P_i is rigorously freed from O_2 and illuminated with a tungsten lamp, the color of the mixture changes slowly but distinctly from purple to brownish-green. The spectra of the original as well as the illuminated mixture depend of course on the particular porphyrin used. For example, the original spectrum of TPP in such a solution shows absorption peaks at 515, 547, 589, and 645 mμ in addition to the Soret peak. Upon illumination the absorption peaks at 515 and 547 mμ decrease in height, those at 589 and 645 mμ increase, and an intense new absorption peak at about 684 mμ appears. The new absorption peak at 684 mμ disappears completely in a few hours after the illuminated mixture has been exposed to air. The other absorption peaks in the visible region also return to their original height if the mixture is exposed to air at the earlier stages of illumination, but do not recover completely after prolonged illumination. It is concluded that the observed spectral change was due to photoreduction of the porphyrin by the solvent (or trace amount of unknown impurities in the solvent which survived the purification process), since similar spectral change was observed even in the absence of imidazole, AMP, and P_i.

Photophosphorylation. When a porphyrin solution in DMAC containing imidazole, AMP, and P_i was photoreduced under N_2 and subsequently oxidized by air, ATP was gradually formed in the mixture. In order to determine whether phosphorylation took place during the photoreduction or during the subsequent oxidation step, the reaction was allowed to take place in a sealed H-shaped tube which contained the following DMAC solutions in its separate arms: solution A, TPP (1 mM) plus imidazole (1 mM); solution B, imidazole (1 mM) plus diimidazolium hydrogen phosphate (2.5 mM) plus imidazolium hydrogen-AMP. In one experiment, solutions A and B in separate arms of the sealed tube were mixed, then illuminated for 60 hr at 25°. The tube was then opened to air and samples were taken from the mixture from time to time for ATP assay by the firefly method. The results are given below: [ATP] < 0.05 (0–2 hr after exposure to air), 0.1 (5 hr), 0.3 (19 hr), and 0.3 μM (24 hr). The rate-determining step in the observed ATP production is the slow transphosphoryla-

TABLE I: ATP Synthesis through Oxidation of Iron Porphyrins[a] in DMAC Solutions.[b]

Type of Heme or Hemin[c]	Initial % of Iron in the Fe(II) Form	Concn of Total Iron Porphyrin (mM)	Concn of ATP after 4 Days (μM)	
			Under Air	Under N_2
Chlorocruoro-	Undetermined	1.0	10	
Chlorocruoro-	10	1.4	7	
Chlorocruoro-	10	1.4		<0.1[d]
Proto-	10	2.8	2	
Hemato-	15	1.4	5	
Hematohemin	0	1.4	<0.1	
Iron tetraphenylporphine	Undetermined	1.4	1	
Iron tetraphenylporphine	Undetermined	1.4		<0.1

[a] Prepared by the ferrous acetate–acetic acid method. [b] The initial concentration of other solutes are: AMP (0.8 mM) and imidazolium$_2$-HPO_4 (2.4 mM). [c] All in the form of dimethyl ester except iron tetraphenylporphine and hematohemin. [d] The concentrations of ATP produced when 1-ml samples of this solution were exposed to N_2 containing 2, 0.2, and 0.02 μmoles of O_2 are 7, 0.7, and 0.4 μM, respectively.

tion from the intermediate to AMP to form ADP. The subsequent transphosphorylation from the intermediate to ADP to form ATP is 350 times faster than the phosphorylation of AMP (Brinigar and Wang, 1964b). A parallel experiment was carried out with solutions A and B kept in separate arms during illumination. After illumination the system was exposed to air for 2 hr for complete oxidation of the photoreduced TPP, then the solutions in separate arms were mixed and assayed for ATP at regular time intervals. It was found that the concentration of ATP in this mixture remained <0.05 μM after 22 hr which is indistinguishable from the [ATP] in the control mixture kept in the dark. These results show that phosphorylation took place during the oxidation of photoreduced TPP by air.

If the porphyrin solution in DMAC is illuminated under nitrogen atmosphere containing only a trace amount of oxygen, photoreduction and its reoxidation by oxygen can take place simultaneously. Since under such conditions the steady-state concentration of reduced porphyrin is relatively very small, only slight permanent change in the porphyrin spectrum was observed after the experiment. However, under air or nitrogen containing appreciable amount of oxygen, irreversible photooxidation of the porphyrin was found to take place. Consequently, all the photophosphorylation experiments summarized in Tables II and III are carried out under nitrogen atmosphere containing only a trace amount of air. Some of the results on photophosphorylation experiments in DMAC solution of porphyrin plus imidazole plus AMP but without P_i are given in Table II.

The Role of Imidazole. The initial choice of the imidazolium salts of AMP and P_i was made because of their suitable solubility in polar organic solvents such as DMAC. However, when other salts (1-methylimidazolium, pyridinium, tetrabutylammonium, morpholinium, and 1,4-di-(1-imidazolyl)butane)were used to replace imidazole, either no or very little phosphorylation occurred. Imidazole was therefore implicated as an important component of the present reaction system. Some of the results are given in Table III.

TABLE II: Photophosphorylation with Porphyrin, AMP, and Imidazole in DMAC Solution.[a]

Pigment	Illumination	APPA Formed (μM)
Tetraphenylporphine	Light	180
Tetraphenylporphine	Dark	<2
Hematoporphyrin	Light	425
Hematoporphyrin	Dark	<2

[a] Composition of the DMAC solution: porphyrin (5×10^{-4} M), imidazole (1×10^{-2} M), and diimidazolium hydrogen-AMP (5×10^{-3}). The illumination was carried out for 2 weeks at 25°. The change in optical density of the porphyrin solution at its Soret peak before and after the experiment was always less than 5%.

Evidence for the Formation of an Intermediate. In order to explore the possible existence of an intermediate capable of undergoing transphosphorylation reactions leading to ATP formation, protohemochrome dimethyl ester was oxidized by air in two separate experiments. (a) The diimidazole-ferrohemochrome solution in DMAC was added to a DMAC solution of diimidazolium salts of HPO_4^{2-} and ADP which was preequilibrated with air. (b) The ferrohemochrome was added to an air-equilibrated DMAC solution containing only diimidazolium hydrogen phosphate, then ADP was added after 1.5 hr when the oxidation was already complete. ATP production was followed as a function of time after the iron porphyrin solution

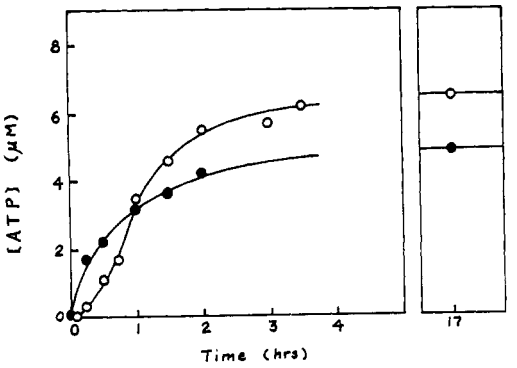

FIGURE 1: Phosphorylation of ADP caused by the oxidation of ferrohemochrome. ○, ADP present prior to the addition of ferrohemochrome. ●, ADP added 1.5 hr after the ferrohemochrome. Final concentrations: ADP (0.13 mM), diimidazolium hydrogen phosphate (1.3 mM), and diimidazole ferrohemochrome (0.4 mM). The experiments were carried out in DMAC solution at 25°.

TABLE III: Effect of the Base or Cation on Photophosphorylation.[a]

Base or Cation	Illumination Period (days)	ATP Formed (μM)
Morpholine	7	<0.10
Pyridine	14	<0.22
1,4-Di-(1-imidazolyl)butane	6	<0.024
Tetrabutylammonium	14	0.14
1-Methylimidazole	10	<0.020
Imidazole	7	49.0

[a] The pigment used in each experiment listed in Table III was hematoporphyrin at a concentration of 5×10^{-4} M. The solutions were all made 5×10^{-3} M in AMP, 1×10^{-2} M in the base in question, and 1×10^{-2} M in the appropriate phosphate salt (dibasic hydrogen phosphates except for the pyridinium salt which was pyridinium dihydrogen phosphate).

and the ADP solution were mixed. The results are illustrated in Figure 1. The data clearly demonstrate that when ferrohemochrome is oxidized by air in a DMAC solution containing imidazole and inorganic orthophosphate, an intermediate was formed which can subsequently react with ADP to form ATP.

Evidence for 1-Phosphoimidazole as the Intermediate. From the concentration of the intermediate produced in the above experiment (5–10 mM), it seemed that identification of the intermediate by using ^{32}P-labeled phosphate and chromatography may be possible. The first experiments were carried out by using the same concentrations of ferrohemochrome, diimidazolium hydrogen phosphate, now ^{32}P labeled, as before. The reaction mixture was allowed to stand for 2 hr at room temperature and was evaporated to dryness at room temperature under high vacuum. The residue was taken up in 1 F aqueous K_2CO_3 solution and the hemin ester was removed by chloroform extraction. An aliquot was then chromatographed by the procedure described in the Experimental Section. The developed chromatograms was cut into sections 3 × 2 cm, and each piece was counted separately in a liquid scintillation spectrometer. In addition to orthophosphate at R_F 0.15, another spot containing ^{32}P was evident at R_F 0.35. For identification, a suitable amount of unlabeled, synthetic 1-phosphoimidazole was then added to another aliquot of the mixture and chromatographed. After counting, the papers were washed free of the scintillator with benzene, sprayed with Hanes–Isherwood molybdate reagent, and the phosphate-positive areas were circled with an ink pen. Then they were sprayed with K_2CO_3 solution which causes the phosphate color to disappear, and finally sprayed with diazotized sulfanilic acid which developed the red color in imidazole-containing areas. A similar chromatogram was developed with the original ^{32}P-labeled diimidazolium hydrogen phosphate solution as a blank. The papers were reassembled as they had been in the original chromatogram. A photograph of a typical set of these chromatograms is shown in Figure 2, in which the number of counts per minute is also indicated for each section. The correlation between the locations of ^{32}P radioactivity, phosphate, and imidazole is highly suggestive that the intermediate formed as a result of ferrohemochrome oxidation is 1-phosphoimidazole. Shown also in Figure 2 are the chromatograms of two other solutions subjected to the same treatment. One solution contained an equivalent amount of protohematin dimethyl ester which was completely in the Fe(III) form, and the other 1 equiv of protohemin dimethyl ester which was approximately 5% reduced. No ^{32}P radioactivity above the background was observed in the 1-phosphoimidazole spot from the former solution, and only a small amount in that from the latter. The data lend further support to the conclusion that the observed phosphorylation was indeed coupled to hemochrome oxidation.

It was found that acidification of the ferrohemochrome oxidation mixture to pH ~3 for 15 min prior to spotting on the chromatographic paper resulted in a 50% decrease in the radioactivity of the 1-phosphoimidazole spot. This result is consistent with the known susceptibility of 1-phosphoimidazole to acid hydrolysis, but inconsistent with the behavior of phosphate esters and pyrophosphates. Also the intermediate was found to be stable in 1 F aqueous K_2CO_3 solution for an observed period up to 2 weeks. This last observation is again consistent with 1-phosphoimidazole but inconsistent with the known properties of acylphosphates. Similar results were obtained for the photophosphorylation system.

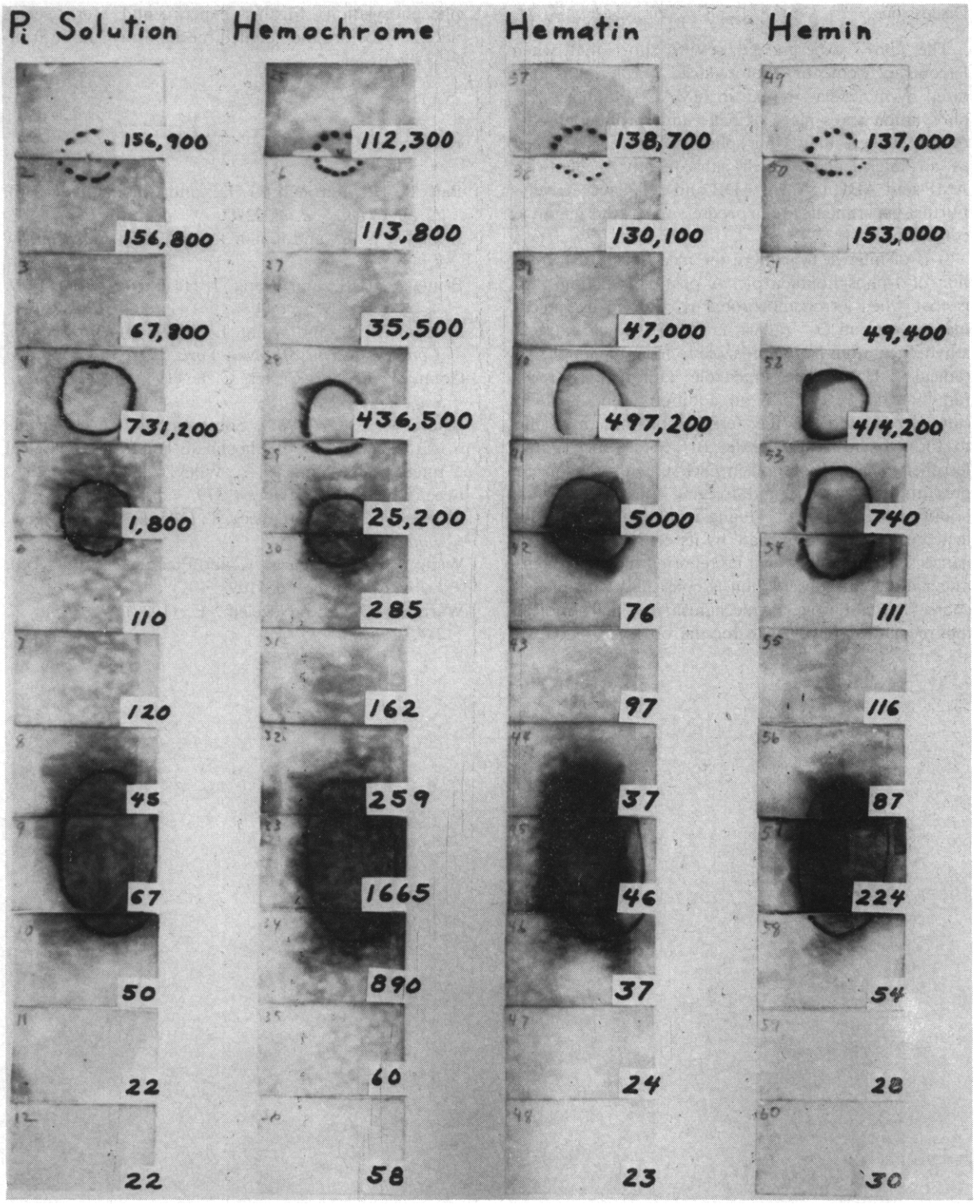

FIGURE 2: Reassembled chromatograms of reaction mixtures containing [^{32}P]phosphate. The four mixtures were prepared in each case by adding to 1.0 ml of 3.6 mM ^{32}P-labeled diimidazolium hydrogen phosphate solution in DMAC the following. P_i solution, none; hemochrome, 0.5 ml of 1 mM protoferrohemochrome dimethyl ester solution in DMAC; hematin, 0.5 ml of 1 mM protohematin dimethyl ester solution in DMAC; and hemin, 0.5 ml of 1 mM protohemin dimethyl ester (containing 5% in Fe(II) form) solution in DMAC. The phosphate-containing spots are circled and the dark areas are due to imidazole. The upper spot (R_F 0.15) is orthophosphate. The middle spot is due to an impurity in the synthetic 1-phosphoimidazole added as a carrier and identified by Ratlev and Rosenberg (1956) as 1,3-diphosphoimidazole. Further purification of the 1-phosphoimidazole eliminated this spot. The lower spot (R_F 0.35) is 1-phosphoimidazole. The number at the lower right corner of each section represent counts per minute determined for that section of the chromatogram.

Discussion

The above experimental results show that when ferrohemochrome or photoreduced porphyrin is oxidized by molecular oxygen in DMAC solution, phosphorylation takes place. Kinetic and chromatographic results suggest that 1-phosphoimidazole is produced as an intermediate which subsequently reacts with AMP and ADP to form ADP and ATP, respectively. Further experiments are in progress to test this tentative conclusion.

It is of interest to explore the mechanism of formation of 1-phosphoimidazole. A possible reaction path is that when ferrohemochrome is oxidized by molecular oxygen, an O_2^- radical is formed which extracts a hydrogen atom from imidazole to form the imidazole radical $C_3H_3N_2$. The imidazole radical may react rapidly with P_i to form an addition product which can be reduced by another ferrohemochrome molecule to form 1-phosphoimidazole. By rapidly mixing the ferrohemochrome solution in DMAC with the solvent presaturated with air and freezing the mixture with liquid nitrogen, Dr. K. Huang in our laboratory detected a radical intermediate by its electron spin resonance spectrum which will be reported in a later communication. The observation is consistent with but of course does not prove the mechanism in which a radical intermediate is responsible for the observed oxidative phosphorylation. Further experimental work seems necessary before the detailed mechanisms can be fruitfully discussed.

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