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Mitochondrial Phosphoriodohistidine. A Possible High Energy Intermediate of Oxidative Phosphorylation*

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ABSTRACT: The possibility that phosphoriodohistidine (PIH) participates in the phosphorylation of adenosine diphosphate (ADP) linked to the oxidation of the reduced mitochondrial electron carriers was investigated. Monoiodohistidine (MIH) and PIH were extracted from beef heart mitochondria and identified by paper chromatography and the P:I ratio. The amount of MIH found was of the same order as the concentration of electron carriers in mitochondria. The rate of phosphorylation of MIH to form PIH was of the same order as the rate of adenosine triphosphate (ATP) production. The phosphorylation of MIH required respiration and was inhibited by arsenate. Arsenate inhibition could be reversed by increasing the P_i concentration. The transphosphorylation reaction of PIH with nucleoside diphosphates was specific for ADP, rather than CDP, UDP, or GDP, and was oligomycin and dinitrophenol sensitive. [^{32}P]PIH in an alcohol extract of mitochondria reacted, nonenzymatically, with ADP to form [^{32}P]ATP. Addition of synthetic MIH to partially uncoupled mitochondria resulted in a decrease in respiration and an increase in phosphorylation, *i.e.*, in an increase in the P:O ratio. A possible mechanism for the reaction of MIH with P_i in the mitochondrion is proposed, and the role of the iodine group is discussed.

The nature of the intermediates responsible for the transfer of energy from the oxidation of the reduced electron carriers to the phosphorylation of adenosine diphosphate (ADP)[1] remains obscure. The most definitive evidence that an intermediate is formed from inorganic phosphate rather than from ADP is contained in the report by Boyer (1958) that the oxygen between the two terminal phosphorus atoms of adenosine triphosphate (ATP) is furnished largely or entirely by ADP. The discovery by Boyer and his associates (Boyer *et al.*, 1962; Boyer, 1963) of a protein-bound phosphohistidine, formed in mitochondria during phosphate participation in oxidative phosphorylation, pointed to the possible participation of histidine in these reactions, even though more recently phosphohistidine has been implicated with succinate thiokinase (Kreil and Boyer, 1964; Mitchell *et al.*, 1964).

The discovery of phosphohistidine led us to consider other phosphorylated imidazole compounds as possible high-energy intermediates. Of the derivatives of histidine known in biological systems, monoiodohistidine (MIH) theoretically seemed to have the biological and chemical requisites. Roche *et al.* (1952) identified MIH as a biological compound in hydrolysates of thyroglobulin extracted from rat thyroid gland. It has been found that thyroxine is rapidly deiodinated when incubated

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[1] The following abbreviations will be used throughout: monoiodohistidine, MIH; monophosphoromonoiodohistidine or phosphoriodohistidine, PIH; adenosine triphosphate, ATP; adenosine diphosphate, ADP.

with histidine (Moreale de Escobar *et al.*, 1963). Chemically, iodohistidine should be more easily phosphorylated than other imidazole compounds, including histidine. A proposed mechanism for this reaction appears later in this report.

Experimental Section

Phosphorylating beef heart mitochondria were prepared by a modification of the procedure of Schneider (1948) according to Gladte and Liss (1958). Glycerol was added to the medium as recommended by Greiff *et al.* (1961), and a nicotinamide wash was done according to Roodyn and Suttie (1961). The preparation was frozen at −76° and stored in solid CO_2 (Roodyn and Suttie, 1961). No loss of phosphorylating activity was observed even after 6 months of storage.

Monoiodohistidine was prepared according to the method of Brunnings (1947). Analysis of the product with a Beckman Model 120 B amino acid analyser produced one symmetrical peak which appeared before the histidine standard. Paper chromatography also produced one iodine-containing spot, with a different R_F value from the KI standard. We therefore concluded that only one of the two possible isomers of MIH was formed.

Monophosphoromonoiodohistidine[2] (PIH) was synthesized by treating monoiodohistidine with $POCl_3$ in a strong KOH solution at 0° (Gustafson, 1954). Paper chromatography of the product produced only one spot containing organic iodide, plus a faint spot of inorganic iodide. Mild acid hydrolysis produced only MIH. Ascending paper chromatography with Whatman No. 1 filter paper was used throughout this investigation. The time required for the development of the papers was 16–18 hr.

Iodoamino acids were detected on paper by the FFCA method of Postmes (1963) after irradiating the papers with ultraviolet light (Mineralight Model R51, 0.9 amp, Ultraviolet Products, Inc., San Gabriel, Calif.) for 30 min. This method was sensitive enough to detect and measure 3×10^{-11} mole of monoiodohistidine. Quantitative measurements were made with a Spinco Analytrol densitometer, using a 600-mμ filter, using synthetic MIH as a standard. Postmes (1963) states that the FFCA method will also detect other reducing substances, such as thioamino acids, thiouracil, and ascorbic acid; however, the difference in the R_F values in the different solvents serves to eliminate them from consideration.

The radioactivity of the paper chromatograms was detected and counted with a Nuclear-Chicago Actigraph II. The specific activity of $^{32}P_i$ was determined by comparing the radioactivity of $^{32}P_i$ to the total P_i determined by the method of Fiske and Subbarow (1925, 1929).

[2] Phosphoriodohistidine, $(HO)_2P(O)$-*N*-iodohistidine, is named according to the proposed rules for naming organic compounds containing phosphorus as published in *Chem. Eng. News 30*, 4515 (1952). Boyer *et al.* (1962) calls $(HO)_2P(O)$-*N*-histidine phosphohistidine.

Results

Identification of Mitochondrial Monoiodohistidine and Phosphoriodohistidine. Intact beef heart mitochondria were found here to incorporate inorganic ^{32}P during the oxidation of 2-oxoglutarate with malonate in the presence of a minimal amount of ADP. The mitochondria were removed from the incubation mixture, washed, and extracted with alkaline 95% alcohol (pH 13). After being concentrated, one-half of the extract was acidified with HCl. Both the acidified and alkaline aliquots were then chromatographed on paper. The acidified extract produced an iodine-containing compound and inorganic ^{32}P. The unknown iodine-containing compound had the same R_F values as synthetic monoiodohistidine in five different solvent systems (Table I).

The chromatograms of the alkaline extract showed an unknown ^{32}P-iodine compound with R_F values that were clearly different from those of synthetic MIH: 0.74, 0.78, 0.86, and 0.36 in *n*-BuOH–NH_3OH, *n*-BuOH–HOAc, 2-BuOH–NH_3, and $CHCl_3$–MeOH–NH_3, respectively. Except for the last solvent system, this

TABLE I: Correspondence of R_F Values of an Acid-Liberated Iodine Compound with Synthetic MIH.[a]

Solvent System	R_F Values: Unknown Iodide Peak	MIH Standard	KI Standard
2BuOH–NH_3 (4:1)	0.17–0.21	0.16–0.21	0.40
95% EtOH–H_2O (63:37)	0.52–0.57	0.56	0.82
$CHCl_3$–MeOH–17% NH_3 (2:2:1)	0.79–0.83	0.81	0.88
n-BuOH–HOAc–H_2O (78:5:17)	0.079	0.079	0.35
n-BuOH sat. with 2 N NH_4OH	0.06–0.14	0.078–0.155	0.24

[a] Twenty milliliters of a mitochondrial suspension (0.6 mg of N/ml) in 0.25 M sucrose, 0.002 M EDTA (pH 7.4) was incubated in 20 ml of the following solution: 30 mM 2-oxoglutarate, 30 mM malonate, 15 mM $MgCl_2$, 6 mM P_i, 1 mc ^{32}P, trace of ATP (pH 7.4), plus 20 ml 0.01 M Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) buffer, pH 7.4, for 0.5 hr with occasional shaking. The mitochondria were removed by centrifugation and extracted with 20 ml 95% ethanol, pH 12.7–13.3. After evaporation to a small volume and acidification with HCl, the extract was chromatographed on paper. Iodinated compounds were detected and measured as described under Experimental Procedure.

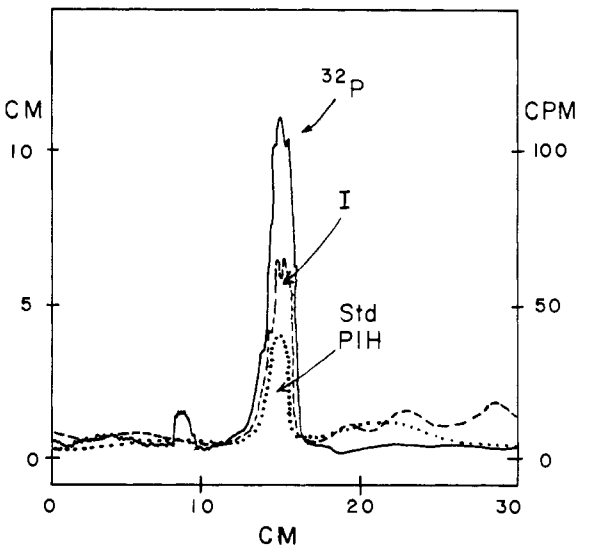


FIGURE 1: Chromatographic identification of PIH. Incubation conditions were as described in Table I, except that only 1 mM inorganic phosphate was added. One drop of 6 N KOH was added to the concentrated alcohol extract, which was then warmed gently until the solution once more became clear. The solvent system was: 95% ethanol–water (63:37). Curve labeled ^{32}P is the radioactive peak. Curve labeled I is the densitometer tracing of the FFCA–iodine peak of the unknown iodine-containing compound. Curve labeled Std PIH is the densitometer tracing of the FFCA–iodine peak of synthetic phosphoriodohistidine.

unknown ^{32}P-labeled iodine compound appeared in conjunction with a ninhydrin-positive material which was identified as consisting largely or entirely of phosphatidylethanolamine. This phospholipid protected the unknown phosphate from hydrolysis by acetic acid, but did not protect it from hydrochloric acid. Addition of disodium EDTA or KOH to the alcohol solution freed the unknown phosphate–iodine compound from the lipid. The free unknown produced a ^{32}P peak and an iodide peak in three different solvent systems (Table II) that were identical with the peaks of synthetic monophosphoromonoiodohistidine (PIH) (Figure 1). Labeling of PIH with ^{32}P by the mitochondria was independent of the substrate used: 2-oxoglutarate, malonate produced 6000 counts/min, β-hydroxybutyrate produced 8000 counts/min, and succinate produced 5260 counts/min under the same conditions. Identification of the unknown phosphate was confirmed by determining its phosphate to iodine ratio. The ratio was found to be 1:1.10 and 1:1.26 using alcohol extracts of two mitochondrial preparations. This ratio is close to that expected for monophosphoromonoiodohistidine. Acid hydrolysis of the unknown produced monoiodohistidine and inorganic ^{32}P.

The free unknown and synthetic PIH also exhibited identical behavior when they were chromatographed with a solvent system containing acetic acid. FFCA-iodide peaks with the same R_F values were produced from each; but these were different from those from MIH; however, the iodine compound did not contain any ^{32}P activity. From this we conclude that the [^{32}P]PIH reacted with the acetic acid, and that the compound we had detected in this system was, perhaps, the acetylated iodohistidine. Paper chromatography of the following iodinated compounds, diiodohistidine, monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine, produced R_F values that were substantially different from the R_F values of both MIH and PIH.

TABLE II: R_F Values of the Unknown ^{32}P-Iodine Compound Compared to Synthetic Monophosphoriodohistidine (PIH).[a]

Solvents	R_F Values: Synthetic PIH	Unknown ^{32}P Compound	Unknown I Compound
EtOH–H_2O (63:37)	0.47	0.47	0.48
$CHCl_3$–MeOH–17% NH_3 (2:2:1)	0.53	0.53	0.51
sec-BuOH–*t*-BuOH–H_2O (43:8.6:48.4)	0.47–0.51	0.51	0.49–0.56

[a] The conditions are described in Table I and Figure 1.

Quantity of Mitochondrial Monoiodohistidine. The quantity of monoiodohistidine isolated from mitochondria as measured on paper chromatograms ranged from 0.24×10^{-7} to 6.9×10^{-7} mole/g of mitochondrial protein, depending on the chromatographic solvent system. Due to the losses in the extraction procedure and the lability of iodinated compounds on paper (Taurog, 1963), it seems logical to take the highest value of MIH found, *i.e.*, 6.9×10^{-6} mole/g of protein as being closest to the actual quantity present. This figure compares well with the published figures for the quantities of electron carriers present in the mito-

TABLE III: Stoichiometry of Mitochondrial Components as Compared to Monoiodohistidine (MIH).

Component	Moles/g Protein	Molecular Ratio	Ref
Cyt *b*	6.8×10^{-7}	2	Green (1962)
Flavin (f_s)	3.2×10^{-7}	1	Green (1962)
Cyt *a*	13.0×10^{-7}	4	Green (1962)
"ATP jump"	6.1×10^{-7}	2	Eisenhardt and Rosenthal (1964)
MIH	6.9×10^{-7}	2	

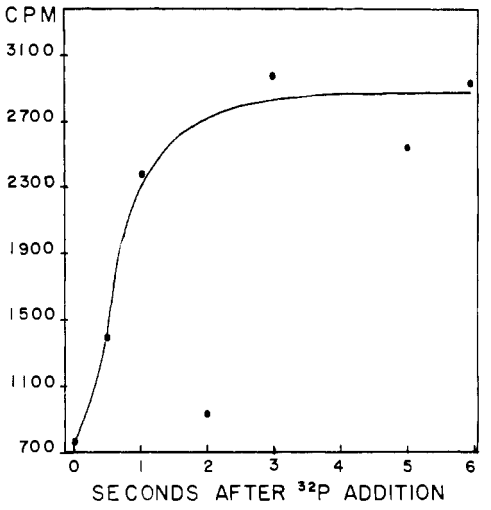


FIGURE 2: Time course of ^{32}P labeling of PIH. Each reaction vessel contained 1 ml of mitochondrial suspension (1 mg of N) in 0.25 M sucrose, 0.002 M EDTA; and 1 ml of a solution containing 30 mM 2-oxoglutarate, 30 mM malonate, and 15 mM $MgCl_2$, at room temperature. The reaction was started by addition of 0.4 ml of the following solution: 50 μcuries of $^{32}P_i$, 12 μM P_i, and 6 μM ADP. The reaction was stopped, after the time interval indicated, by quickly adding 5 ml of 0.5 M EDTA, pH to 8.8 with NH_4OH. The value for zero time was obtained by mixing the starter solution with the killer solution and pouring both into the reaction vessel. Extraction and separation of PIH was accomplished as previously described (see Figure 1).

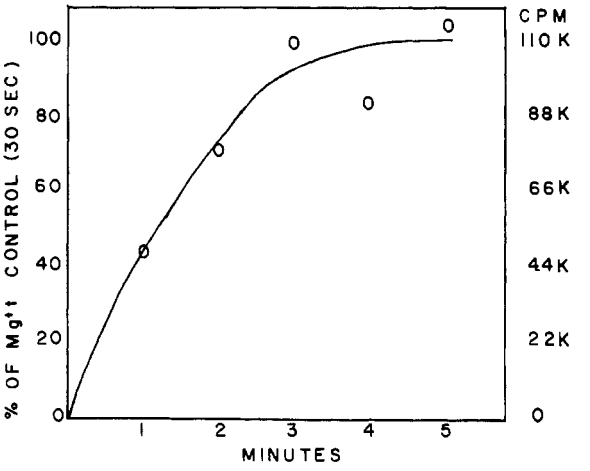


FIGURE 3: ^{32}-P labeling of PIH, without added Mg^{2+}. The conditions were as described previously (Figure 1), except that the reaction mixture contained no added $MgCl_2$. The final extracts were chromatographed on paper in *sec*-BuOH–*t*-BuOH–H_2O (43:8.6:48.4) and the [^{32}-P]PIH peaks were counted. The referance control contained 5 mM $MgCl_2$ and was killed after 30 sec.

chondrion (Green, 1962), as well as with the magnitude of the "ATP jump" (Eisenhardt and Rosenthal, 1964) which is presumably a measure of the amount of high-energy intermediate present (Table III).

Published values for the iodine content of extrathyroidal tissue are not consistant, and may vary by factors as great as 10^2 or 10^3, depending on the method and tissue used.

We were interested in determining whether the alkali-labile iodine content of the beef heart mitochondrial preparation was large enough to account for the amount of iodohistidine detected. The mitochondria were resuspended in distilled water, and one KOH pellet was added for each milliliter of suspension. The mixture was then refluxed for 3 hr, neutralized with ammonium acetate, and diluted with distilled water. Measured quantities of $^{125}I^-$ were added to the unknown and to the KI standard and different measured amounts of each were chromatographed on paper. By comparison of the radioactivity and densitometer peaks for iodide (FFCA method) of the unknown and the standard, after subtracting the reagent blank, the quantity of iodine could be calculated. The quantities of alkali-labile iodine found were: 2.26×10^{-6} mole/g protein and 2.03×10^{-6} mole/g protein, or more than enough to account for the iodohistidine detected.

Dependence of ^{32}P Labeling of Phosphoriodohistidine on Metabolism. Labeling Rate. The rapid rate of labeling of PIH with $^{32}P_i$ is shown in Figure 2. Under our experimental conditions, at 25°, maximum labeling occurred in less than 3 sec. (Preliminary experiments had indicated that there was no change in $^{32}P_i$ uptake by PIH between 6 sec and 5 min.) A better approximation of the time of 100% labeling may be made by plotting for each point of the rising portion of the curve the counts/min/sec (ordinate value/abscissa value) against time and extrapolating to zero time. If the maximum counts/min are divided by this initial rate (counts/min/sec), the result is the time required for maximum labeling at the initial rate. When this is done with the data in Figure 2, a time of 2.8 sec is obtained for maximum labeling. Manometrically, we found the phosphate uptake to be 0.435 μmole/g of protein/sec, or 1.2 μmole of P_i/g of protein/2.8 sec. This value, although higher, is of the same order as the quantity of MIH found, *i.e.*, 0.69 μmole/g of protein. Since the quantity of iodohistidine that we measured is a minimal value due to the losses mentioned previously, we feel that the two values are in good agreement. The condition suggested by Slater and Kemp (1964) that the rate of ^{32}P labeling of an intermediate of oxidative phosphorylation must be of the same order as the rate of production of ATP is thus satisfied.

Phosphoriodohistidine Labeling in the Absence of Added Mg^{2+}. A preliminary experiment had indicated that the presence of added Mg^{2+} was necessary for maximum labeling of PIH within 1 min (Perlgut and Wainio, 1965). However, we found that the absence of added Mg^{2+} had little effect on the P:O ratio or on the rate of phosphorylation, when measured manometrically for 30–40 min. If PIH is a possible inter-

TABLE IV: Inhibition of PIH Labeling by Oligomycin and Dinitrophenol.[a]

Expt	Conditions	Inhibitor	Concn	Counts/min	% Inhibition
I	P_i added to reaction mixture with ^{32}P	None	...	1100	..
		Oligomycin	1.72 μg/ml	1060	3
		DNP	1.72×10^{-4} M	1100	0
II	P_i added to reaction mixture before ^{32}P	None	...	2350	..
		Oligomycin	1.72 μg/ml	1240	48
		DNP	1.72×10^{-4} M	562	76

[a] In both experiments, the reaction mixture contained: 10 mM 2-oxoglutarate, 10 mM malonate, 5 mM $MgCl_2$, and 1 ml mitochondrial suspension (0.4 mg of N) in 0.25 M sucrose, 0.002 M EDTA; pH 7.4. For expt I, the inhibitor was added and then the starter solution consisting of 55 μc of ^{32}P, 0.165 μmole of P_i, and 0.128 μmole of ADP. Total volume was 2.9 ml. After 30 sec, 5 ml of the killer solution (Figure 2) was added. For expt II, the reaction mixture was made 0.04 mM with respect to P_i, the inhibitor was added, and then the starter solution consisting of 55 μcuries of ^{32}P plus 0.128 μmole of ADP. Total volume, time interval, and killer solution for expt II was the same as expt I. PIH was extracted and separated as described in Figure 1.

mediate of phosphorylation, an explanation of these diverse results was necessary.

Figure 3 shows the time course of PIH labeling with ^{32}P in the absence of added Mg^{2+}, compared to the radioactivity of the control, which contained added Mg^{2+} and was allowed to react for 30 sec. PIH (100% labeling) in the absence of added Mg^{2+} required 3 min, while maximum labeling occurs in less than 3 sec in the presence of added Mg^{2+} (see Figure 2).

Inhibition by Oligomycin and 2,4-Dinitrophenol. The presence of oligomycin (Lardy *et al.*, 1958) or 2,4-dinitrophenol prevents the phosphorylation of ADP. It is not known whether these inhibitors prevent the formation of the phosphorylated intermediate (X~P), or whether they prevent the transphosphorylation reaction of the intermediate with ADP, or both. If formation of the phosphorylated intermediate is inhibited, addition of the inhibitor before the addition of $^{32}P_i$ should result in a small amount of radioactive intermediate, compared to the uninhibited control. On the other hand, if only the transphosphorylation reaction is inhibited, addition of the inhibitor before addition of $^{32}P_i$ should not result in a decrease in labeling since X~P could still be formed. In this second case, it should be possible to show the inhibition if the intermediate were allowed to react with P_i in the absence of ADP, thus becoming saturated with cold phosphate. Subsequent addition of $^{32}P_i$ and ADP should result in a labeled intermediate, since the cold P_i removed by ADP will be replaced by ^{32}P. Under these conditions, addition of an inhibitor after P_i loading, but before the addition of $^{32}P_i$ and ADP, should result in a lower level of labeled intermediate.

The activity of oligomycin and dinitrophenol was tested using both of the above methods (Table IV). Our data indicate that oligomycin and dinitrophenol, at the concentrations used, inhibit the transphosphorylation reaction only. In a similar experiment where lower concentrations of oligomycin (0.83 μg/ml) and DNP (dinitrophenyl) (8.3×10^{-5} M) were used, the inhibition was 33 and 30%, respectively, with the same mitochondrial preparation, illustrating that the decrease in PIH labeling was dependent on the concentration of the inhibitor. The effect of DNP varied somewhat with the mitochondrial preparation used. The data in Table V were obtained under similar conditions to those in part II of Table IV, but with a different mitochondrial preparation. The inhibition by DNP of PIH labeling with ^{32}P in this experiment was even more marked.

Arsenate Inhibition and Its Reversal by P_i. Ter Welle and Slater (1964) have demonstrated uncoupling of oxidative phosphorylation by arsenate and its reversal by P_i by measuring the release of respiration induced by arsenate. They concluded that this competition between arsenate and P_i offers a method of distinguishing the intermediate of oxidative phosphorylation from intermediates of substrate-linked phosphorylation. The effects on ^{32}P labeling of PIH in the presence of a constant concentration of arsenate (5 mM) and of increasing concentrations of P_i are shown in Figure 5. When the P_i concentration was very low, *i.e.*, ratio of P_i to arsenate of 1:200, there was almost complete inhibition of [^{32}P]PIH formation. As the P:As ratio increased, inhibition decreased until, at a ratio of 1:5, no inhibition was detected. A similar curve was obtained from the densitometer peaks of the FFCA-iodide sprayed papers, which was a measure of total PIH formed.

Dependence of PIH Labeling on Respiration. Addition of Amytal to the reaction mixture of Table V resulted in a decrease of labeled PIH from 1344 counts/min to 402 counts/min, *i.e.*, a 70% inhibition of labeling. Amytal inhibits mitochondrial respiration 65–75%.

Reaction of [^{32}P]PIH with ADP. We had previously reported that the addition of ADP to the alkaline–alcoholic extract containing [^{32}P]PIH resulted in

TABLE V: Inhibition of PIH Labeling by DNP.

Expt	DNP Concn (M)	Counts/min	% Inhibition
III	0	4835	..
	3×10^{-5}	2160	55
IV	0	9202	..
	1×10^{-4}	346	96

the formation of [^{32}P]ATP (Perlgut and Wainio, 1964). The radioactive ATP was separated from inorganic ^{32}P on a paper chromatogram in 2-propanol–H_2O–concentrated HCl (65:18.4:16.6). Roughly 80% of the activity of the labeled PIH was transferred to ATP. The above solvent system did not separate ADP from ATP, and the possibility still existed that the radioactivity detected was due to an isotope exchange labeling of ADP. The experiment was, therefore, repeated using isobutyric acid–2 N NH_4OH (2:1) to develop the chromatograms (Figure 4). Addition of ADP to the acidified extract resulted in the appearance of the $^{32}P_i$ peak only. Addition of ADP to the alkaline, unhydrolyzed extract before acidification resulted in a [^{32}P]ATP peak, a much smaller $^{32}P_i$ peak, and no radioactivity due to ADP. About 85% of the activity of the [^{32}P]PIH was transferred to ATP by this nonenzymatic reaction, confirming the results of the previous experiment.

TABLE VI: Reaction of PIH with ADP, CDP, UDP, and GDP.[a]

Addition	Counts/min	%
ADP	2350	100
CDP	740	31
UDP	1195	51
GDP	815	35

[a] The mitochondria (4 mg of N), suspended in 4 ml of 0.25 M sucrose, 0.002 M EDTA, were mixed with 4 ml of the following solution: 30 mM 2-oxoglutarate, 30 mM malonate, 15 mM $MgCl_2$, 0.1 mM P_i, and 0.1 mM ATP. A solution (0.4 ml) containing 50 μcuries of $^{32}P_i$ and 0.09 μmole of the indicated nucleoside diphosphate was added to 2-ml aliquots of the above mixture. The reaction was stopped after 30 sec with 1 M EDTA, pH to 9.0 with NH_4OH. Extraction and separation of PIH was done as described under Figure 1, except that the 95% EtOH extract was evaporated to dryness and the residue taken up in 1 ml of 33% EtOH, pH to 13 with KOH, and warmed. This resulted in a clear homogeneous solution. The alcoholic solution was neutralized by addition of 6 N ammonium acetate and 100 μl was used for paper chromatography. The paper chromatograms were developed in EtOH–H_2O (63:37).

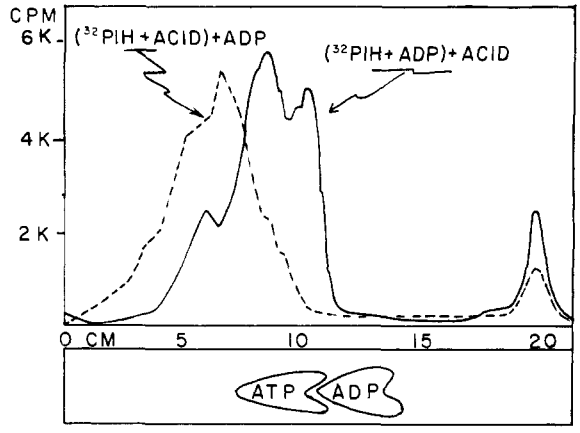


FIGURE 4: Reaction of [^{32}P]PIH with ADP. Mitochondrial PIH was labeled with ^{32}P and extracted as described previously (Figure 1). One-third of the extract was acidified with HCl before the addition of 1 μmole of ADP (dashed line). ADP was added to one-third of the extract before acidification (solid line). The extracts were then chromatographed on paper in the following solvent: isobutyric acid–2 N NH_4OH (2:1). The remaining third of the extract was treated with KOH and chromatographed on paper in $CHCl_3$–MeOH–17% NH_3 (2:2:1). The quantitative results were as follows: control (no ADP): PIH = 90,000 counts/min, $^{32}P_i$ = 46,000 counts/min, total = 136,000 counts/min. HCl before ADP: $^{32}P_i$ = 145,000 counts/min, ATP = 0 counts/min, total = 145,000 counts/min. ADP before HCl: $^{32}P_i$ = 65,000 counts/min, ATP = 80,000 counts/min, total = 145,000 counts/min.

Specificity of PIH-Nucleotide Transphosphorylation Reaction. In order to determine whether the transphosphorylation specifically requires ADP, mitochondria that had been loaded with cold P_i were allowed to react with ADP, GDP, CDP, or UDP to which the same amount of $^{32}P_i$ had been added. The extent of the transphosphorylation reaction was determined by measuring the radioactivity of the PIH, since the cold phosphate removed by the diphosphates would be replaced by radioactive phosphate. The data in Table VI show that even after 30 sec, when compared to ADP, none of the nucleoside diphosphates tested could produce more than a 50% turnover of P_i. Since a single turnover of phosphate requires 3 sec, we may assume that the ADP-treated sample turned over up to ten times in the 30 sec.

The possibility of a $^{32}P_i$–PIH isotope exchange reaction being responsible for some of the labeled PIH in this system was investigated by adding $^{32}P_i$ to the same phosphate-loaded mitochondria, in the absence of ADP. After 30 sec, the reaction was stopped, the PIH extracted, and its radioactivity counted. We found PIH with 52% of the radioactivity of the ADP-treated control. From this we concluded that the isotope exchange reaction took place, although at a significantly slower rate than the transphosphorylation reaction with ADP.

TABLE VII: Effect of Monoiodohistidine on Oxidation and Phosphorylation.[a]

MIH Concn (M)	Prepn	O_2 Uptake/mg of N (μatom/min)	O_2 Uptake/mg of N % Control	P_i Uptake/mg of N (μM/min)	P_i Uptake/mg of N % Control	P:O Ratio Actual	P:O Ratio % Control
1×10^{-3}	19	0.445	93	0.560	108	1.26	117
1×10^{-4}	19	0.510	105	0.680	131	1.33	123
1×10^{-5}	19	0.525	109	0.640	123	1.22	113
5×10^{-6}	19	0.380	93	0.575	100	1.51	107
1×10^{-6}	14	0.248	111	0.318	133	1.28	121
1×10^{-6}	18	0.506	116	1.27	119	2.60	110
1×10^{-6}	19	0.445	93	0.530	104	1.18	107
1×10^{-6}	19	0.390	96	0.600	104	1.54	109
5×10^{-7}	14	0.248	111	0.282	119	1.14	108
5×10^{-7}	18	0.417	97	1.16	109	2.78	113
5×10^{-7}	18	0.412	95	1.17	110	2.72	111
5×10^{-7}	19	0.364	89	0.640	111	1.77	125
1×10^{-7}	18	0.409	94	1.10	103	2.69	110
1×10^{-7}	19	0.387	95	0.675	117	1.74	123
5×10^{-8}	14	0.222	100	0.261	110	1.16	110
Av			100 ± 2.2		113 ± 2.6		113 ± 2.2

[a] Oxidative phosphorylation was measured by the method of Lardy and Wellman (1952) as modified by Gladte and Liss (1958) using the glucose–hexokinase–ATP trap. Each reaction vessel contained: 1 ml of mitochondrial suspension (0.6 mg of N) in 0.25 M sucrose and 0.002 M EDTA, 10 mM 2-oxoglutarate, 10 mM malonate, 5 mM $MgCl_2$, 2 mM P_i, 2 mM ATP, 0.3 ml of hexokinase–glucose solution, and MIH as indicated; the total volume was adjusted to 3 ml with 0.01 M Tris buffer, pH 7.4.

When this isotope exchange reaction was taken into account, none of the nucleoside diphosphates, except ADP, could participate in the transphosphorylation reaction. In fact, CDP and GDP seemed to slow the exchange reaction, possibly by increasing the integrity of the mitochondria. The inhibition by oligomycin of the transphosphorylation reaction (Table IV) thus becomes 100% when allowance is made for the exchange reaction, and DNP seems to inhibit the $^{32}P_i$–PIH exchange, as well as the transphosphorylation reaction, perhaps by inhibiting the dephosphorylation of PIH. The extent of the exchange reaction varied with the mitochondrial preparation, as can be seen by comparing the data in Table V with that in Table IV. The mitochondria used for the experiments in Table V evidently had a much slower rate of $^{32}P_i$–PIH exchange.

Activity of Synthetic Monoiodohistidine. The effect of synthetic monoiodohistidine on the activity of mitochondria was investigated by two different methods. MIH was added to the mixture in the reaction vessel and its effect on oxidative and phosphorylative activity of three different mitochondrial preparations was measured (Table VII). At all concentrations tested, MIH addition caused an increase in phosphorylation and in the P:O ratio, and had no effect on respiration. This increase in phosphorylative activity was blocked by the addition of 2,4-dinitrophenol.

The second series of experiments was designed to test only the effect of bound MIH. Mitochondria were preincubated in the sucrose–EDTA medium with MIH, in the presence of Mg^{2+} and inorganic phosphate. The mitochondria were then removed from the preincubation medium and resuspended in fresh MIH-free medium for use in the oxidative phosphorylation assay. At all concentrations of MIH tested, respiration was reduced and phosphorylation was increased, resulting in an increase in the P:O ratio (Table VIII). The presence of both Mg^{2+} and inorganic phosphate in the preincubation medium was required for this increased coupling of phosphorylation to oxidation. When inorganic phosphate was left out of the preincubation mixture, oxidative phosphorylation was partially uncoupled by MIH at concentrations below 2.5×10^{-7} M and above 5×10^{-7} M. This effect was the result of a sharp increase in respiration. MIH concentrations of 2.5×10^{-7} M to 5×10^{-7} M did not change phosphorylation, but depressed respiration below the control value, resulting in an increase in the P:O ratio.

Addition of 10^{-5} M 2,4-dinitrophenol to the reaction vessel uncoupled oxidation from phosphorylation equally in the MIH-treated mitochondria and in the untreated controls, resulting in P:O ratios for both that were 33% of normal. Addition of monoiodohistidine to a substrate mixture that was lacking a phosphate acceptor increased the respiratory control of an aged mitochondrial preparation by 100% (Table IX).

TABLE VIII: Activity of Mitochondria Preincubated with MIH.[a]

MIH Concn (M)	O_2 Uptake/mg of N (μatom/min)	% Control	P_i Uptake/mg of N (μM/min)	% Control	P:O Ratio Actual	% Control
5×10^{-7}	0.420	93	0.910	99	2.16	107
5×10^{-7}	0.405	89	0.945	103	2.34	116
5×10^{-7}	0.378	75	0.955	105	2.55	141
3.6×10^{-7}	0.415	83	0.972	106	2.29	127
2.5×10^{-7}	0.427	85	1.04	115	2.45	136
2.5×10^{-7}	0.475	95	0.975	107	2.06	114
1×10^{-7}	0.395	79	0.910	100	2.30	128
1×10^{-7}	0.434	87	1.09	120	2.50	139
5×10^{-8}	0.435	79	1.13	101	2.60	128
1×10^{-8}	0.439	79	1.13	101	2.59	128
Av		85 ± 2.1		106 ± 2.2		126 ± 3.5

[a] The mitochondrial preparation (0.6 mg of N) suspended in 0.25 M sucrose, 0.002 M EDTA, was preincubated in 5 mM $MgCl_2$, 2 mM P_i, and MIH as indicated, for 15 min. The mitochondria were then removed from the mixture and tested for oxidative phosphorylation as described under Table VII.

Increased respiratory control is indicative of increased dependence of respiration on phosphorylation (Chance and Williams, 1956).

The activities of iodine, potassium iodide, and histidine were tested under the conditions described in Table VII. Iodine at 10^{-7} M had no effect on oxidation, phosphorylation, and the P:O ratio. At higher concentrations, iodine had little effect on phosphorylation while respiration increased with increasing concentration, resulting in lower P:O ratios. KI (10^{-6} M) had no effect on oxidation, phosphorylation and the P:O ratio. Histidine (10^{-5}–10^{-7} M) had a slightly inhibitory effect on phosphorylation.

TABLE IX: Effect of Monoiodohistidine (MIH) Addition on the Respiratory Control of Isolated Mitochondria.[a]

MIH Concn (M)	O_2 Uptake/mg of N (μatom/min)	Respiratory Control Ratio
0	0.176	2.45
1×10^{-6}	0.195	2.20
5×10^{-5}	0.084	5.12
3×10^{-4}	0.105	4.10

[a] The mitochondrial preparation was aged for 3 days at 5° in the sucrose–EDTA medium. Respiration was measured manometrically for 30 min. The reaction vessels contained the mixture indicated in Table VII, except that the ATP and the hexokinase–glucose solution were omitted. Respiratory control ratio = (respiration in the presence of ADP)/(respiration in the absence of ADP).

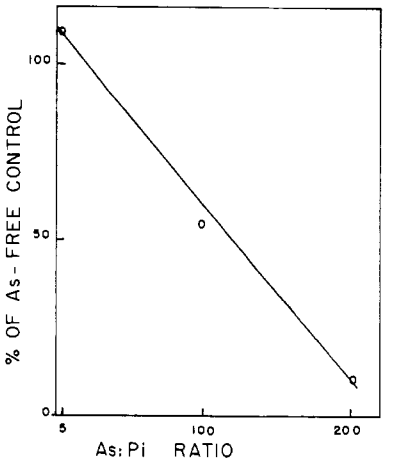
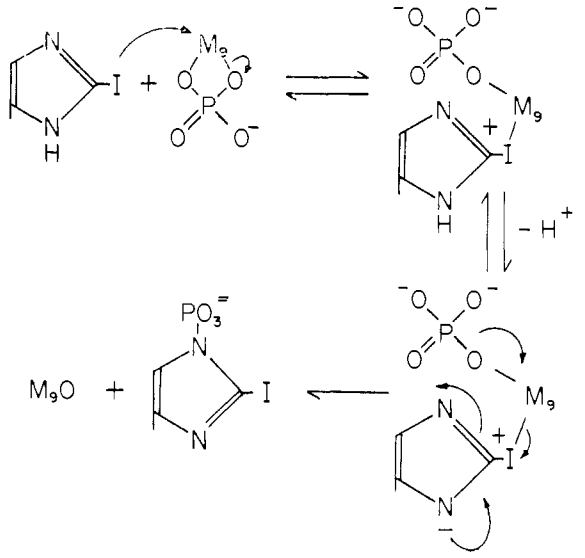


FIGURE 5: Reversal of arsenate inhibition of PIH formation by P_i. The experiment was carried out as described under Table VI. The starter solution for the controls contained only $^{32}P_i$ and the indicated concentration of P_i; for the experimental samples the starter was also made 5 mM with respect to Na_2AsO_4. Each arsenate-containing sample was, therefore, compared to an identical control without arsenate.

Discussion

The identification of mitochondrial iodohistidine and phosphoriodohistidine is based on their chromatographic and chemical identities with the synthetic compounds. Mitochondrial iodohistidine was chromatographically identical with synthetic MIH in five solvent systems, one acid, one neutral, and three alkaline. The correspondence of the R_F values in each of these systems eliminates the possibility that we are detecting

SCHEME 1: The Reaction of Iodohistidine with Inorganic Phosphate.



substances such as thioamino acids, thiouracil, and ascorbic acid, which are also sensitive to the FFCA reagent. Treatment of both the biological and the synthetic compounds with strong alkali and heat resulted in the appearance of free iodide ion. We have also measured the appearance of ^{131}I activity associated with mitochondrial MIH after injection of rats with ^{131}I-thyroxine. The R_F values of thyroxine and MIH are entirely different in our system, and the radioactivity of both can be counted separately.

Similarly, mitochondrial phosphoriodohistidine was found to be chromatographically identical with synthetic PIH in three solvent systems, both alkaline and neutral. Mild acid hydrolysis of both the biological and synthetic compounds resulted in the liberation of free MIH, and the P:MIH ratio was close to one, as expected of monophosphoromonoiodohistidine. Both synthetic and biological PIH reacted with acetic acid to produce chromatographically identical iodine compounds that were not the same as MIH, PIH, or iodide. Finally, in the arsenate inhibition experiment, we found that the FFCA-iodine values for PIH fell off in the same manner as the [^{32}P]PIH values, with increased As:Pi ratios (Figure 5). Arsenate interfered with the formation of PIH and both methods of measurement of PIH agreed.

Positive identification of mitochondrial MIH by column chromatography has not been completely successful to date. Our Beckman amino acid analyzer produced a double peak in the histidine region with the unknown peak appearing about 20 ml before and running into the added histidine standard. Synthetic MIH appears about 30 ml before the standard histidine peak, so that perfect correspondence was not achieved. This may be due to the presence of a large amount of lipid in the mitochondrial extract, which slowed the movement of all the amino acids through the column.

We found that incubation of the mitochondria with oxidizable substrate and P_i was necessary in order to extract PIH, which in turn was easily hydrolyzed to MIH. If the incubation was omitted, an unknown iodine-containing compound was obtained that was different from MIH or PIH, and stable to acid and base. Preliminary experiments indicate that the unknown may be MIH bound to a 262 mμ absorbing material.

Two isomeric forms of MIH are possible: 2-iodohistidine or 4-iodohistidine. Synthetic MIH appeared as a single compound, and we made no attempt to determine which isomer it was. The most probable isomer, chemically, is 2-iodohistidine, since monoiodination of imidazole yields the 2-iodoimidazole (Schipper and Day, 1957; Ridd, 1955). There are four possible isomers of PIH: 1-phosphoro-2-iodohistidine, 3-phosphoro-2-iodohistidine, 1-phosphoro-4-iodohistidine, and 3-phosphoro-4-iodohistidine. Since only one compound was detected upon paper chromatography of synthetic PIH, we believe that only one isomer was synthesized from the 2- (or 4-) iodohistidine. We believe that 3-phosphoromonoiodohistidine is the compound most likely to be formed, since Brunnings (1947) concludes from the pK_3 shift of iodohistidine that the 1 position is hydrogen bonded to the amino group of the side chain. If this is so, the 1 position of the histidine ring would not be readily available for the phosphorylation reaction. We were indeed fortunate that the biological compounds were identical with the synthetic ones. This will simplify the eventual determination of the structure of MIH and PIH. The fact that our identification of iodohistidine in the mitochondrion is not absolute does not detract from the metabolic relationships that we have established.

The data presented here for phosphoriodohistidine indicate that it behaves as expected of an high-energy phosphorylated intermediate of oxidative phosphorylation. Griffiths (1963) concluded that the concentration of the high-energy intermediate should be very low and of the same order as that of the respiratory carriers and that it is likely to be a highly labile compound with a rapid turnover rate. The concentration of PIH and its acid lability fit this criteria. In addition, the ^{32}P-labeling rate of PIH is of the same order as the rate of ATP production, as required by Slater and Kemp (1964). We have also shown that this reaction is respiration dependent. The transphosphorylation reaction of PIH with ADP is oligomycin and 2,4-dinitrophenol sensitive, and is specific for ADP rather than for CDP, UDP, or GDP. The fact that oligomycin only inhibits the transphosphorylation reaction in our system agrees with the data of Lardy and Connelly (1964). Our data on the inhibition by arsenate of PIH formation and its reversal by P_i distinguish PIH from intermediates of substrate-linked phosphorylation, according to the findings of Ter Welle and Slater (1964). That [^{32}P]PIH is a high-energy compound is shown by the nonenzymatic reaction with ADP to form [^{32}P]ATP. From this, we may conclude that PIH has a larger negative ΔF of hydrolysis than ATP. Our findings that one of the functions of exogenous Mg^{2+} is to facilitate the transport of inorganic phosphate to the reaction site may

explain the metabolic reason for mitochondrial accumulation of Mg^{2+} and P_i (Brierley *et al.*, 1962).

Very low concentrations of synthetic monoiodohistidine, when added to mitochondria, behaved in many ways as a coupling agent of oxidative phosphorylation. This activity would be expected of an intermediate of phosphorylation, provided it could be bound in the proper manner to the mitochondrion. We found that the presence of both Mg^{2+} and P_i were necessary for binding of MIH to occur. The proposed mechanism for the reaction of iodohistidine with inorganic phosphate (Scheme I) in the lipid matrix of the mitochondrion is compatible with current concepts in theoretical organic chemistry. The first reaction results in the formation of a $MgPO_4^-$ complex with iodohistidine, the iodine group acting as the bridge atom. This complex formation could take place in a nonaqueous medium, and does not rule out the possibility of ligand formation between Mg^{2+} and phospholipid. Ionization of the ring hydrogen to form the imidazolide ion is next. This ionization is aided by the presence of the I^+ group in the 2 (or 4) position. Electron transport may be involved in this step, since with an increased π electron density of the ring complete ionization would not be necessary for the final reaction to occur. The latter reaction should take place as the rotation of the PO_4 group around the Mg–I bridge brings it close enough to the un-ionized nitrogen of the ring. The possibility of the phosphate group being positioned by an enzyme is also very likely. The reaction presumably would go through a six-membered transition state during which the dipolar ion is discharged.

This mechanism agrees with Ernster's proposal that a metal–phosphate and/or a metal–adenine nucleotide complex are concerned with the energy coupling mechanism of the respiratory chain (Ernster, 1963). In the above mechanism, it can be seen that the functions of the iodine group of iodohistidine are: (1) to make possible the histidine–I–Mg–PO_4 complex, (2) to aid in the ionization of the ring hydrogen, and (3) to form the positive half of a zwitterion, whose discharge results in the formation of phosphoriodohistidine.

The other halides, bromine and chlorine, should function as well as iodine as electron-withdrawing groups, but would form the +bridge atom with increasing difficulty with decreasing size. I^+ is formed more easily than is Br^+, which is formed more easily than Cl^+. An OH group can form the $MgPO_4$ complex, but in so doing, the tendency would be for it to lose a proton and remain uncharged.

$$\text{R–OH} + \text{MgPO}_4^- \rightleftharpoons \underset{\text{H}}{\overset{+}{\text{R–O}}}\text{–MgPO}_4^{2-} \overset{-\text{H}^+}{\rightleftharpoons} \text{R–O–MgPO}_4^{2-}$$

This stable complex would not aid in the formation of the imidazolide ion by withdrawing electrons from the ring; it may even increase the difficulty of ionization by increasing the electron density of the ring.

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Aliphatic Diether Analogs of Glyceride-Derived Lipids. III. Synthesis of Dialkenyl and Mixed Alkylalkenylglycerol Ethers*

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ABSTRACT: L-2,3-Di-*O*-*cis*-9′-octadecenylglycerol (L-2,3-dioleylglycerol, III) was synthesized by alkylation of D-3-*O*-triphenylmethylglycerol with 1-bromo-*cis*-9-octadecene (oleyl bromide) and potassium hydroxide in boiling benzene, followed by acid hydrolysis of the triphenylmethyl group. L-2-*O*-Octadecyl-3-*O*-*cis*-9′-octadecenylglycerol (VII) was synthesized by blocking the 1 position of L-3-*O*-*cis*-9′-octadecenylglycerol (L-selachyl alcohol, IV) with the triphenylmethyl group, alkylation of the resulting triphenylmethyl derivative (V) with 1-bromooctadecane, and finally removing the blocking group by acid hydrolysis. The positional isomer of VII, namely, L-2-*O*-*cis*-9′-octadecenyl-3-*O*-octadecylglycerol (XI), was synthesized in an analogous way, starting with L-3-*O*-octadecylglycerol (L-batyl alcohol, VIII).

The oleyl bromide used was prepared by reduction of pure (>99%) oleic acid with lithium aluminum hydride to oleyl alcohol, formation of the *p*-toluenesulfonate, and conversion to the bromide by reaction with lithium bromide in boiling acetone. L-2-*O*-Hexadecanoyl-3-*O*-octadecylglycerol (XIII) was synthesized by acylation of L-3-*O*-octadecyl-1-*O*-triphenylmethylglycerol (IX) with palmitoyl chloride in pyridine followed by removal of the triphenylmethyl group by hydrogenolysis with palladium catalyst. The infrared spectra and physical properties of these compounds are described.

Previous communications in this series have dealt with the synthesis of 2,3-dialkyl ethers of glycerol (Kates *et al.*, 1963), and of the D and L isomers of 2,3-dihydrophytylglycerol (Kates *et al.*, 1965a). The latter compound was found to be identical with the glycerol diether isolated from hydrolysates of the lipids of *Halobacterium cutirubrum* (Kates *et al.*, 1965b).

These glycerol diethers have proved to be useful as starting materials for the synthesis of saturated dialkyl ether analogs of phosphatides, such as the diether analogs of phosphatidylcholine (Stanacev *et al.*, 1964) and of phosphonocephalin (Baer and Stanacev, 1965). In order to prepare diether phosphatides containing unsaturated hydrocarbon chains the corresponding unsaturated glycerol diethers were required. The latter were also required for metabolic studies and for identification of natural glycerol diethers. Synthesis of this class of diethers, however, required a modification of our previous procedure (Kates *et al.*, 1963) to avoid the use of catalytic hydrogenolysis for removal of the benzyl blocking group. This has been achieved by using the triphenylmethyl (trityl) group as a blocking agent, since it is readily removed by acid hydrolysis without affecting the double bond.

The present report describes the synthesis of a representative diether containing two unsaturated long-chain groups, namely, 2,3-di-*O*-*cis*-9′-octadecenylglycerol (dioleylglycerol), as the DL and L isomers, and two representative diethers containing one saturated and one unsaturated hydrocarbon group, namely, L-2-*O*-octadecyl-3-*O*-*cis*-9′-octadecenylglycerol and its positional isomer, L-2-*O*-*cis*-9′-octadecenyl-3-*O*-octadecylglycerol. The L stereoisomers were synthesized here since the only natural glycerol diether isolated to date proved to have the L configuration (Kates *et al.*, 1965a).

To ensure that the final unsaturated diethers contained only the *cis*-9-octadecenyl group without contamination with the *trans* isomer it was found necessary to start with highly purified oleic acid uncontaminated with the *trans* isomer and to convert it to oleyl bromide by a procedure which did not produce any *cis–trans* isomerization. This was accomplished by reducing pure oleic acid with lithium aluminium hydride and converting the alcohol to the bromide by treating the *p*-toluenesulfonate with lithium bromide. In contrast to

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