PYRIDINE NUCLEOTIDE STIMULATION OF LIPID PHOSPHORYLATION*

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Attempts to demonstrate high level incorporation of ^{32}Pi into the common phosphatides such as LEC and PE in cell free mammalian systems have met with little success (Friedkin and Lehninger, 1949, Kennedy, 1953, Marinetti et. al., 1957, Conover et. al., 1960, Garbus et. al., 1963, Galliard and Hawthorne, 1963). However, in these systems (homogenates, microsomes, mitochondria) one can show high level incorporation of ^{14}C-glycerol into these common phosphatides (Marinetti et. al, 1962, Marinetti et. al, 1964, Erbland et. al, 1967). This anomaly led us to investigate further the nature of lipid phosphorylation. The major objectives of this study were to determine the cellular localization of lipid phosphorylation, the donor high energy phosphate compound, and the dependency of high energy phosphate production on substrate phosphorylation versus oxidative phosphorylation.

After a long search with studies on rat liver homogenates and cell particles we have found that a critical factor necessary for lipid phosphorylation is the coenzyme NADH (or NAD^+). In these systems $NADP^+$ or NADPH cannot substitute for NAD^+ or NADH. The pattern of lipid labeling with ^{32}Pi is dependent on other cofactors such as Mg^{++}, FDP, CMP and is also dependent on the time of incubation and on the cell particles which are present.

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Abbreviations: LEC, lecithin, PE, phosphatidylethanolamine, PA, phosphatidic acid, MPI, monophosphoinositide, GPX, a glycerolphosphatide tentatively identified as phosphatidylglycerolphosphate, ^{32}Pi, radioactive orthophosphate, DNP, dinitrophenol, FDP, fructose diphosphate.

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Materials and Methods

ATP, ADP, CTP, CMP, NAD^+, NADH, $NADP^+$, NADPH were obtained from P-L Biochemicals Inc., FDP from Schwartz Biochem., DNP from Eastman Kodak, antimycin A from Nutritional Biochemical, ^{32}Pi from Nuclear Science and Engineering Corp., γ-^{32}P-ATP from International Chemical and Nuclear Corp., oligomycin from Mann Research Lab.

Liver homogenates were prepared as described previously (Erbland et.al, 1967). Incubations were carried out in a shaking water bath at 37° C. Air was the gas phase. The lipids were separated by two dimensional chromatography on Whatman SG 81 silica gel paper. 20 µl aliquots of each incubation system was spotted on the silica gel paper (20 x 20 cm). Ascending chromatography was employed. The first solvent was chloroform-methanol-water 65:25:4 (v/v). The second solvent was diisobutyl ketone-acetic acid-water 40:25:5 (v/v). Autoradiograms were made on Kodak rapid process X-ray film. The exposure time was one week. The radioactive lipid spots were carefully delineated, cut out, and the radioactivity determined in a Packard Liquid Scintillation counter. The counts given in the tables are for each lipid component in the 20 µl aliquot. Since the total incubation medium volume was 1.0 ml, the cpm must be multiplied by 50 to get the total cpm in the lipid per 200 mg of wet weight liver.

Results and Discussion

Table 1 demonstrates the effect of a number of agents on the incorporation of ^{32}Pi into lipids of rat liver homogenates. Mg^{++} ions alone stimulate labeling of PE and MPI to a small extent. However, the marked stimulation of lipid labeling requires NADH (or NAD^+). Labeling of GPX occurs with NADH alone but high level labeling of PE and MPI requires both NADH and Mg^{++}. The labeling of LEC and PE is stimulated by FDP when NADH and Mg^{++} are present. Higher labeling of LEC is dependent on CMP as shown in Table 2. CMP also stimulates the labeling of PE and MPI but not that of GPX. CTP is not as effective as CMP in this system. Labeling of GPX, PE and MPI is rapid whereas labeling of LEC is slower and requires 1-2 hours incubation.

Table 1

Incorporation of ^{32}Pi Into Lipids of Rat Liver Homogenates*

Additions (all 10 mM)	cpm - 120 min. incubation GPX	PE	LEC	MPI
None	190	68	61	0
Mg^{++}	111	378	58	63
FDP	174	24	24	0
NADH	5293	169	63	0
NADH + Mg^{++}	7218	1664	36	1388
FDP + Mg^{++}	68	72	0	0
NADH + Mg^{++} FDP	1753	2695	187	399

* (100 mg wet weight liver, 125 mM sucrose, 45 μC ^{32}Pi)

Table 2

Effect of CMP on the Incorporation of ^{32}Pi Into Lipids of Rat Liver Homogenates*

System	cpm - 60 min. incubation GPX	PE	LEC	MPI
Control	385	1550	80	110
CMP 1 mM	370	2200	280	160
CMP 10 mM	325	1450	195	180

* (100 mg wet weight liver, 125 mM sucrose, 10 mM NADH, 10 mM $MgCl_2$, 10 mM FDP, 44 μC ^{32}Pi)

Table 3

Effect of NADH Concentration on the Incorporation of ^{32}Pi Into Lipids of Rat Liver Homogenates*

Concentration of NADH (mM)	cpm - 30 min. incubation			
	GPX	PE	LEC	MPI
0	158	225	55	43
5	2931	800	65	613
10	4742	962	112	1010
15	4160	541	72	706
20	4160	470	94	660

* (100 mg wet weight liver, 125 mM sucrose, 10 mM $MgCl_2$, 50 µC ^{32}Pi)

Table 3 shows the effect of NADH concentration on lipid phosphorylation in a liver homogenate. The optimal concentration of NADH is 10 mM. The optimal concentration of Mg^{++} and FDP was also found to be 10 mM.

In order to elucidate the stimulatory action of NADH the effect of inhibitors of oxidative phosphorylation and of uncouplers of oxidation phosphorylation was examined. Since NAD^+ has essentially the same action as NADH in these systems one can assume that this coenzyme was undergoing oxidation-reduction. This was supported by the data in Table 4. The marked inhibition of labeling of GPX, PE, and MPI by DNP and cyanide is evident. The inhibition of labeling of GPX by antimycin A is also apparent.

These data give evidence that the NADH effect is in part mediated through the electron transfer chain and that high energy phosphate is involved. This

Table 4

Effect of Inhibitors on the Incorporation of ^{32}Pi Into Lipids of Rat Liver Homogenates*

			cpm - 30 min. incubation		
	Inhibitor		GPX	PE	MPI
Exp. 1	none		1312	160	138
	DNP	0.1 mM	77	74	0
	CN^-	1.0 mM	59	90	0
Exp. 2	none		3010	**	**
	DNP	0.1 mM	194	**	**
	CN^-	1.0 mM	27	**	**
	Antimycin A	10 µg	127	**	**

* (100 mg wet weight liver, 125 mM sucrose, 10 mM NADH; 10 mM $MgCl_2$, 50 µC ^{32}Pi)

** (These lipids were not analyzed in this experiment.)

was supported by our finding that oligomycin also gave a strong inhibition of lipid phosphorylation.

The data for lecithin are not given in Table 4 since lecithin labeling is very low in these systems if FDP is not added. However, we later found that the inhibition of lipid labeling by DNP, cyanide and antimycin in homogenates is greatly reduced, or entirely eliminated by addition of 10 mM FDP. This action of FDP may be mediated by FDP enhancing substrate phosphorylation during glycolysis in the cytoplasm.

It was found that γ-^{32}P-ATP can replace NADH + ^{32}Pi for labeling of GPX either in homogenates or in isolated mitochondria. Hence ATP appears to be the primary donor of phosphate for phosphorylation of this lipid. However, γ-^{32}P-ATP did not replace NADH + ^{32}Pi for labeling of PE in a microsomal-cytoplasm system (Chatterjee, Marinetti and Pettit).

The following observations have also been made: (a) ADP inhibits lipid phosphorylation from ^{32}Pi in homogenates. (b) Lipid phosphorylation in a microsomal-cytoplasm system requires NADH, Mg^{++} and either pyruvate or FDP. (c) The labeling of lipids in homogenates from ^{14}C-glycerol requires either ATP + Mg^{++} or NADH + Mg^{++}. However, whereas the major phosphatide labeled with ^{14}C-glycerol is LEC, the major lipids labeled with ^{32}Pi are PE and GPX. (d) When pyruvate and NADH are added to a homogenate or microsomal-cytoplasm system a yellow fluorescent NADH-pyruvate complex is rapidly formed and is then metabolized (Chatterjee, Marinetti and Pettit).

Chemical and enzymatic studies on GPX showed that it is an acidic glycerophosphatide which is labeled by both ^{32}Pi and ^{14}C-glycerol. There are several forms of GPX which we believe differ in the number and type of fatty acids esterfied to the glycerol moieties. Mild alkaline hydrolysis of GPX yields a water soluble phosphate ester of glycerol.

This compound does not react with hydroxylamine or 2,4-dinitrophenyl hydrazine but is cleaved by periodic acid. Hydrolysis of GPX with phospholipase A yields at least two lyso-derivatives. Our evidence to date indicates that GPX represents phosphatidylglycerol phosphate which has one or more fatty acids esterfied to the two glycerol units.

These experiments point to a major role for NAD^{+}-NADH in lipid phosphorylation. High energy phosphate required for this process is produced both by mitochondrial and non-mitochondrial systems. The non-mitochondrial system (glycolysis) can predominate when FDP is added to homogenates.

The significance of the rapid and high level labeling of lipid GPX is not clear at present, especially since we do not observe a similar high labeling of

PA in these systems. It is of interest that mitochondria can synthesize GPX in our systems and can produce acyl dihydroxyacetonephosphate under other conditions (Hajra et. al. 1968).

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