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Biological Oxidation of Phospholipids by Rat Liver Homogenates.*

PAUL W. O'CONNELL AND ELMER STOTZ. (Introduced by A. L. Dounce.)

From the Department of Biochemistry, School of Medicine and Dentistry, The University of Rochester, Rochester, N. Y.

The normal saturated C_4 to C_{18} fatty acids have been shown to be oxidized by homogenized rat liver preparations in the presence of adenosine triphosphate (ATP) and cytochrome c,[1] with acetoacetate as a product of the reaction.[2] Presumably this reaction represents fatty acid oxidation *in vivo*, but the concentration of free fatty acids in animal tissues is actually very low.[3] Hence other lipids may also serve as the source of fatty acid for oxidation, and among these the phospholipids have been strongly implicated as intermediates in fat metabolism.[4] Consequently, in the work reported here, measurements were made of the oxygen consumption and acetoacetate production from phospholipids, utilizing the type of enzyme preparation which oxidizes free fatty acids.

Experimental. Substrates. The petroleum ether-soluble, acetone-insoluble fraction of beef brain lipid was prepared and found to contain 3.3% phosphorus and to have an iodine number of 85.5. It was stored in petroleum ether, under nitrogen, at 5°. The beef lung hydrolecithin was generously supplied by Dr. S. J. Thannhauser. Emulsions of these compounds were prepared by suspending 80 mg of the phospholipid in 10 ml of 0.1 M phosphate buffer, pH 8.0, and homogenizing the mixture in a Potter-Elvehjem glass homogenizer. A 0.01 M sodium octanoate solution, used to test the activity of the enzyme preparations for fatty acid oxidation, was prepared by neutralizing redistilled *n*-octanoic acid with the required amount of NaOH and adjusting the pH to 7.0-7.5.

Reagents. Cytochrome c was prepared from beef heart according to the method of Keilin and Hartree.[5] The stock solutions used varied from 1.3 to 2.0 $\times$ 10^{-4} M. Adenosine triphosphate was prepared from rabbit muscle according to the method of Dounce *et al.*[6]

Analytical methods. For the measurement of oxygen consumption, Warburg flasks of about 20 ml total volume were used. The flasks were constructed with two side arms. All experiments were run at 30° and in all cases the gas phase was air. The center well of each flask was equipped with a filter paper roll and 0.2 ml of 20 per cent KOH. Acetoacetic acid was determined by distilling a metaphosphoric acid filtrate of the incubated samples and determining the acetone by the vanillin method of Alyea and Backström.[7] The detailed procedure has been described by Witter and Stotz.[8]

Liver Homogenate. White rats, which had been starved overnight, were decapitated and bled. The liver was removed and homogenized in the manner described by Lehninger,[1] using an all-glass homogenizer which has been described by Dounce.[9]

The Oxidation of Beef Brain Phospholipid.

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TABLE I.
Oxidation of Beef Brain Phospholipid.

Substrate	ATP*	Oxygen consumption, cmm	Acetoacetic acid, μM
Endogenous†	+	24	1.95
Octanoate	+	85	4.50
Phospholipid	+	75	1.80
		104	1.80
Phospholipid	—	0	1.00
Endogenous	+	13.6	1.50
Octanoate	+	110	3.60
Phospholipid	+	109	1.55
”	—	3.2	0.75
Endogenous	+	8	1.40
Octanoate	+	51	2.25
Phospholipid	+	73.1	1.45
		103	1.50
”	—	13.5	1.05

* When absent, replaced by equal volume of water in side arm.
† Substrate replaced by equal volume of water.

TABLE II.
Oxidation of Beef Lung Hydrolecithin.

Substrate	ATP	Oxygen consumption, cmm	Acetoacetic acid, μM
Endogenous	+	114	—*
Hydrolecithin	+	182	—
”	—	27.0	—
Endogenous	+	41.0	1.35
Octanoate	+	165	6.70
Hydrolecithin	+	167	1.40

* Not determined.

The main compartment of each Warburg flask contained 0.4 ml of 0.1 M phosphate buffer, pH 7.7, 0.2 ml of cytochrome c, and 0.1 ml of 0.1 M magnesium chloride. One side arm of each flask contained 0.4 ml of 0.01 M ATP solution, adjusted to pH 7.5. The other side arm contained 0.4 ml of substrate solution. Just before equilibration, 0.5 ml of liver homogenate were added to the main compartment of each flask. The equilibration period was 5 minutes, at which time the taps were closed and the contents of the side arms tipped. The oxygen uptake was nearly linear through a 30 minute period of measurement and the results reported are expressed in terms of a total 30 minute oxygen consumption. After the measurements of the oxygen consumption, the contents of the flasks were analyzed for acetoacetic acid. The results of typical experiments are recorded in Table I.

The homogenized rat liver preparation oxidized both free fatty acids and phospholipid in the presence of ATP. Only the oxidation of the free acid resulted in the production of acetoacetic acid in amounts significantly greater than was produced by preparations to which no substrate was added. The necessity of ATP for phospholipid oxidation and the rate of the oxygen consumption suggested that the oxidation was of a true biological nature, rather than an auto-oxidation.

The Oxidation of Beef Lung Hydrolecithin. The above conclusion was supported by results obtained through the use of beef lung hydrolecithin as a phospholipid substrate. Both the fatty acids of the molecule are palmitic acid.[10] Since auto-oxidation is believed to involve the reaction of oxygen with the unsaturated acids

[10] Thannhauser, S. J., Benotti, J., and Boncoddo, N., *J. Biol. Chem.*, 1946, **166**, 669.

of the phospholipid molecule, hydrolecithin should not be susceptible to auto-oxidation.

The hydrolecithin was used as a substrate with the enzyme preparation previously described. The results of typical experiments are recorded in Table II.

Discussion. Since acetoacetic acid is not metabolized by the homogenized rat liver preparation, and there is no inhibition of acetoacetic acid formation when octanoate and phospholipid are added to the same preparation, the failure of the oxidizing phospholipid to produce acetoacetic acid indicates that free fatty acids are not liberated from the phospholipid in the preparation employed.

It was found that the rat liver homogenate was able to dehydrogenate phospholipid in the presence of ATP, as measured by the Thunberg technic. The aerobic reaction, however, did not result in an increased phospholipid iodine number, and it was found impossible to determine what changes might have occurred in the fatty acids of the phospholipid as a result of the oxidation. Nevertheless, the fact that both free fatty acids and phospholipid are oxidized by the same enzyme preparation prompts the suggestion that this new oxidative reaction of phospholipid is somehow related to intermediary fatty acid metabolism.

Summary. Beef brain phospholipid and beef lung hydrolecithin are oxidized by rat liver homogenates in the presence of adenosine triphosphate. Although this preparation oxidizes free fatty acids with the production of acetoacetic acid, the phospholipid oxidation does not result in such a product.

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Effects of Glucose Fermentation Products on Determination of Mannitol by Periodate-Titrametric Method.

A. B. KENDRICK, W. P. SWISHER, AND R. A. FORREST. (Introduced by R. W. Keeton.)

From the Department of Medicine, College of Medicine, University of Illinois, Chicago.

When mannitol clearance and maximal glucose reabsorptive capacity are measured simultaneously in studies of renal function,[1,2] it becomes necessary to determine mannitol in the presence of high concentrations of glucose in both plasma and urine. Satisfactory recoveries of mannitol under these conditions have been reported by others,[2] but certain discrepancies have been noted by us. This report deals with a) the extent of interference of glucose fermentation products with the determination of mannitol, b) a correction factor, and c) the nature of the interfering substance.

Glucose was added to water in varying concentrations from 0 to 1250 mg %. The increases were made in steps of 50 mg %. The solutions were then analyzed for mannitol by the method of Smith, Finkelstein, and Smith[3] except that $CdSO_4$-NaOH[4] was used instead of $ZnSO_4$-NaOH as the precipitating reagent, and the time of fermentation was extended to 2 hours. The method entails fermentation of glucose from the samples by yeast, removal of yeast by centrifugation, precipitation of the proteins, oxidation of the mannitol in the filtrate with periodic acid, and the determination of the excess periodic acid, together with the iodic acid formed, by titration with sodium thiosulfate. All of the filtrates used in our determinations were analyzed quantitatively for glucose[5] to make cer-

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