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Effect of Inhibition of Oxidative Phosphorylation on Incorporation of Inorganic Phosphate (P^{32}) into Liver Mitochondrial ATP and Phospholipids.* (30512)

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A number of enzymes found in mitochondria involved in oxidative phosphorylation contain phospholipids(1,2). However, if the phospholipids are removed there is a decrease in enzymatic activity and activity is restored with the addition of phospholipids(3). Compounds such as bilirubin(4), dinitrophenol (5), arsenate(6,7), and oligomycin(8,9) have been shown to inhibit oxidative phosphorylation. All of these compounds inhibit the incorporation of inorganic P (P_i^{32}) into the individual liver mitochondrial phospholipids (10). Heart mitochondria isolated from rats fed a magnesium deficient diet for 4 to 8 days exhibit uncoupling of oxidative phosphorylation with a decrease in P/O ratios (11) and result in decrease synthesis of the individual phospholipids of heart mitochondria(12). However, in none of the above conditions was the synthesis of ATP investigated. With these observations in mind, experiments were undertaken to determine the effect of inhibition of oxidative phosphorylation on incorporation of P_i^{32} into liver mitochondrial ATP and phospholipids.

Methods. Male albino rats of Sprague-Dawley strain, weighing 110-150 g, were divided into groups. The animals in Group 1 were injected intraperitoneally daily with dinitrophenol (30 mg/kg for 3 days), in Group 2, rats were injected with a single dose of arsenate (14 mg/kg). Control rats for each group received intraperitoneal injections of saline. Intraperitoneal injections of 200 μc of P^{32} as NaH_2PO_4 were administered one hour following injection of the above compounds. One hour after P_i^{32} administration the animals were killed by decapitation. This time interval corresponds to the ascending part of the specific activity time curve, where synthesis of ATP occurs to a degree greater than breakdown(13).

The liver was removed, rapidly weighed, homogenized in a Potter-Elvehjem tissue grinder with teflon pestle in ice-cold 0.25 M sucrose containing 0.00018 M $CaCl_2$. The homogenate was divided into 2 fractions. From one fraction acid-soluble phosphorus was removed by extracting with 10% TCA, and the radioactivity(14) and acid soluble phosphorus(15) were determined. The other homogenate fraction was separated into cellular fractions by the method of Hogeboom *et al*(16). The homogenate was centrifuged at 600 $\times$ *g* for 15 minutes to remove the nuclei. The mitochondria were separated from the supernatant by centrifugation at 10,000 $\times$ *g* for 15 minutes. The mitochondria were washed twice with 0.25 M sucrose and recentrifuged at 10,000 $\times$ *g* for 15 minutes

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TABLE I. Incorporation of P_i^{32} into Mitochondria ATP Nucleotides Following Inhibition of Oxidative Phosphorylation.

Tissue	Materials administered	No. of animals	Specific activity*	Relative specific activity†
Liver	Control	5	103.1 ± 10.5	.124 ± .008
	Dinitrophenol	5	78.8 ± 15.5§	.083 ± .010‡
	Control	5	87.6 ± 5.3	.130 ± .007
	Arsenate	5	72.9 ± 2.8	.096 ± .006§
Heart	Control	8	73.3 ± 8.3	.314 ± .013
	Magnesium deficient	8	58.6 ± 7.6‡	.274 ± .010‖

* c/m/μg of phosphorus.
† Specific activity of ATP/specific activity of acid soluble P.
The P probability for chance occurrence of this difference was: ‡ <.01, § <.05, and ‖ <.02.
Numbers preceded by ± are standard deviations. Test of significance was applied to difference between mean of control and experimental values.

and the washings discarded. This procedure removed over 99% of the microsomes as determined by assaying for microsomal glucose 6-phosphatase(17). To the mitochondrial pellet was added 5 ml of cold 0.6 N perchloric acid, mitochondria were resuspended, centrifuged, and supernatant collected. The mitochondrial pellet was washed with 5 ml of 0.2 N perchloric acid and the supernatants combined. To the combined perchloric acid extract, phenol-red indicator and 5 N KOH were added one drop at a time until a pink color momentarily appeared. The perchloric acid extract was further neutralized with 1 N KOH until the pink color persisted. The volume was then adjusted to 16 ml. The ATP was separated on a Dowex 1-XB column, (200-400 mesh) using the formic acid gradient eluting system described by Hurlbert *et al* (18). The ATP fraction from the column was evaporated to dryness, and phosphorus (15) and radioactivity(14) determined. From the phosphorus and radioactivity analysis the specific activity (c/m/μg of P) and relative specific activity (ratio of specific activity of ATP to specific activity of inorganic P_i) were calculated(19). The relative specific activity expresses the relationship between the isotopic concentration in a compound and in its precursor(20). Under the conditions of our experiments, a decrease in formation of ATP may be reasonably assumed when lower values for specific and relative specific activities are found. An additional group of animals, 45-50 g, was fed a diet similar to that reported by Vitale *et al*(11) for 12 days in which magnesium deficiency was shown to uncouple oxidative phosphorylation. Animals were injected with P_i^{32} and sacrificed one hour later, the heart removed and ATP extracted as previously described. Mitochondria were prepared from similar dinitrophenol treated and magnesium deficient animals. The animals were injected intraperitoneally with 200 μc of P_i^{32} and sacrificed by decapitation 4 hours later. This time interval corresponds to the ascending part of the specific activity time uptake curve, where incorporation of the isotope into phospholipids occurs to a greater degree than does their breakdown(21, 22). The organs were immediately weighed and homogenized in cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with a teflon pestle. Aliquots were taken for inorganic phosphorus by extracting with 10% trichloroacetic acid and radioactivity(14) and acid soluble phosphorus(15) determined. The mitochondria were prepared by differential centrifugation and lipids extracted and purified(21). Radioactivity(14) and total lipid phosphorus(22) were determined. From these results the relative specific activities of the total phospholipids were calculated.

Oxidative phosphorylation efficiency was determined in mitochondria, prepared from dinitrophenol treated and magnesium deficient animals. The Warburg vessels contained the following in main compartment: 0.5 ml of mitochondrial suspension, 0.042 μmole of cytochrome c, 22.5 μmoles of $MgSO_4$, 6 μmoles of adenosine triphosphate (ATP), 40 μmoles of potassium phosphate buffer, pH 7.3, 20 μmoles of α-ketoglutarate, and 4 μmoles of NaF. The side arm contained 5.0

TABLE II. Relative Specific Activity of Mitochondrial Phospholipids and P/O Ratio.

Tissue	Materials administered	No. of animals	Relative specific activity of total phospholipids*	No. of animals	P/O ratio
Liver	Control	5	.062 ± .007	9	4.60 ± .50
	Dinitrophenol	5	.033 ± .004†	5	3.67 ± .18‡
Heart	Control	5	.028 ± .001	6	5.30 ± .20
	Magnesium deficient	5	.011 ± .001†	7	3.41 ± .74†

* Specific activity of phospholipids/specific activity of acid soluble P.
The P probability for chance occurrence of this difference was: † <.01, ‡ <.05.
Numbers preceded by ± are standard deviations. Test of significance was applied to difference between mean of control and experimental values.

mg of crystalline hexokinase and 50 μmoles of glucose. The final volume was made up to 3.0 ml with 0.154 M KCl. The center well contained 0.2 ml of 20% KOH. Equilibration was set for 5 minutes in an air phase and the reaction was run at 37° for 10 minutes. O_2 uptake was measured, the reaction was stopped by addition of 2 ml of 5% TCA. Phosphorus determinations were carried out by the Fiske and Subbarow method(15). Average oxygen uptake ($Qo_2^{(N)}$ μl taken up per mg tissue nitrogen per hour) for the dinitrophenol-treated animals was 258±25 and 351±31 for the controls. The volume uptake for the magnesium deficient animals was 208±33 with their respective control 288±58.

Results. Table I shows the specific activities and relative specific activities of mitochondria ATP following administration of dinitrophenol, arsenate, and in magnesium deficiency. To evaluate the significance of results, the t test of significance(23) was applied to the difference between the mean value of the control and experimental groups, where t =

$$\frac{(\bar{X}_1 - \bar{X}_2)\sqrt{1/N}}{\sqrt{\frac{S(X_1^2) - (S(X_1))^2}{N_1} + \frac{S(X_2^2) - (S(X_2))^2}{N_2}}}$$

There is a statistically significant decrease in the incorporation of P_i^{32} into mitochondrial ATP following administration of dinitrophenol, arsenate and in magnesium deficiency. Administration of dinitrophenol(5), arsenate (6,7) and dietary magnesium deficiency(11) have been shown to inhibit oxidative phosphorylation as measured by a reduction in the P/O ratio. This decreased incorporation of P_i^{32} into ATP is in agreement with this observed inhibition of oxidative phosphorylation. Table II shows the effect of dinitrophenol and dietary magnesium deficiency on the incorporation of P_i^{32} into the total mitochondrial phospholipids of the liver. These results agree with the inhibition of the synthesis of the individual mitochondrial phospholipids following administation of the same compounds(10,12). Kennedy(24) and Garbus *et al*(25) have demonstrated that synthesis of phospholipids takes place in isolated mitochondria and requires the maintenance of oxidative phosphorylation or addition of ATP. It would appear from these results (Table I and II) that inhibition of oxidative phosphorylation results in a decrease synthesis of ATP as measured by decreased incorporation of P_i^{32} into the ATP, and is a direct reflection of lipid phosphorylation since ATP is needed for phospholipid synthesis.

Summary. 1. The effect of single dose of dinitrophenol, arsenate, and dietary magnesium deficiency on incorporation of inorganic P into mitochondrial ATP of the liver of rat was studied. 2. A statistically significant decrease occurred in the incorporation of P_i^{32} into mitochondial ATP and total phospholipids.

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Hydrolases of Rabbit Macrophages. III. Effect of BCG Vaccination, Tissue Culture, and Ingested Tubercle Bacilli.* (30513)

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Rabbit macrophages contain active proteases, esterases, lipase, lysozyme and acid phosphatase(1,2,3). To provide information on the *rate* at which immune and normal MN‡ might increase these hydrolases in the presence of specific antigen, the effects of (a) BCG vaccination, (b) a 2-day residence in cell culture, and (c) ingestion of heat-killed tubercle bacilli were investigated. The hydrolase levels of peritoneal and alveolar macrophages were also compared. These studies represent an effort to gain further insight into the mechanisms of cellular immunity.

Materials and methods. Thirty-two New Zealand white rabbits weighing about 2 kg were vaccinated with 2.0 ml of live BCG (Phipps) (1 mg per ml of Sauton's medium) by means of 20 intradermal injections. Six unvaccinated rabbits served as controls. Of the 32 rabbits, 12 received a booster injection of 1 ml of live BCG subcutaneously into *each* flank 7 weeks after their initial vaccina-

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‡ Abbreviations used: MN: mononuclear(s) or specifically oil-induced mononuclear peritoneal exudate cells; AM: pulmonary alveolar macrophages; PMN: polymorphonuclears; BPN: N-benzoyl-D,L-phenylalanine-β-naphthol ester; BPNase: the enzyme hydrolyzing BPN; BCG: Bacillus of Calmette and Guérin, an attenuated strain of *Mycobacterium tuberculosis* employed world-wide as a vaccine.