Oxidative Phosphorylation of Heart Tissue from Vitamin E. Deficient Rabbits.* (18691)

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With regard to the yet unexplained function of vit. E, the suggestion has been made that vit. E may be required in some manner for phosphorylation(1) and that this requirement may be the basis for the increased oxygen uptake of tissues observed in vit. E deficiency(2-4). This suggestion has received some support from observations that minces(5) and water homogenates(6) of dystrophic muscles have a reduced capacity to couple oxidation of substrates with the phosphorylation of creatine. In those investigations, however, the composition of the assay systems apparently limited the efficiency of oxidative phosphorylation as demonstrated by the low ratio of moles of phosphate esterified to atoms of oxygen consumed (P/O ratio) obtained with normal as well as dystrophic tissues. An investigation of the effect of vit. E deficiency on phosphorylations in an assay system normally operating at a high level of phosphorylating efficiency thus appeared desirable. The experiments reported here are the results of such assays on heart tissue preparations from normal and vit. E-deficient rabbits. Rabbit heart was chosen for these experiments since it had been found that high P/O ratios can be obtained with heart preparations(7). In addition, in the rabbit, lack of vit. E adversely affects the heart(8,9) and leads to death from heart failure(10).

Methods. Preparation of animals. Ten young female rabbits weighing 400 to 685 g were divided into 2 groups by equally distributing littermates. They were fed a dystrophy-producing ration designed after that of Basinski and Hummel(11). Half the animals were supplemented orally with 9 mg of synthetic d,l-α-tocopherol (Merck) in 0.15 ml of olive oil each day, 6 days a week. The food was removed 3 hours before supplementation and returned 3 hours afterward, to decrease the destruction of the vitamin in the intestinal tract(12). After 3 to 4 weeks, when the unsupplemented animals were unable to get up by themselves if placed on their sides or back, they were considered ready for the experiment.

Determination of the P/O ratio. Each rabbit was sacrificed by a blow on the head, and the heart was quickly removed and placed in a beaker of ground frozen distilled water. The heart was then cut into 4 or 5 portions, which were blotted and rapidly weighed. After rough mincing, the heart tissue was homogenized in cold buffer(13) in an ice-jacketed Potter-Elvehjem homogenizer. The homogenate was then diluted to 30 ml with cold buffer, and centrifuged at 8500 $\times$ gravity for 10 minutes in a room at —5°C. The sediment was resuspended in 30 ml buffer by homogenization, and centrifuged again at 8500 $\times$ gravity for 10 minutes. This washing was repeated a

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third time. Finally the sediment was homogenized in a volume of buffer in milliliters corresponding to twice the weight of the whole heart in g. Assays were started immediately after preparation of the final homogenate. The following additions‡ were made in the order given to the main compartments of Warburg flasks: Water to make a final volume of 3.0 ml, 0.20 ml of .50 *M* potassium chloride, 0.30 ml of 0.10 *M* magnesium chloride, 0.20 ml of .01 *M* adenosine triphosphate (ATP) sodium salt, pH 7.2, .300 ml of .10 *M* potassium phosphate buffer, pH 7.2, .10 ml of 3.5×10^{-4} *M* cytochrome C, .30 ml of .050 *M* sodium pyruvate pH 7.2, .10 ml of .050 *M* sodium fumarate, pH 7.2, .10 ml of .50 *M* glucose, .25 ml of yeast hexokinase solution, .10 ml of .50 *M* tris (hydroxymethyl) aminomethane buffer, pH 7.2(17), .30 ml of 0.20 *M* potassium fluoride, and .30 or .50 ml heart homogenate. The center wells contained .20 ml of 20% potassium hydroxide and a strip of filter paper. The flasks were chilled in crushed ice before addition of the homogenate.

For a single determination of a P/O ratio 2 Warburg flasks with identical contents were incubated at 20°C for 10 minutes. One flask was then rapidly removed from the manometer and treated with 2.0 ml of 17.5% trichloroacetic acid. Oxygen uptake was measured in the other flask for 15 to 30 minutes, and then the reactions were stopped with trichloroacetic acid as described above. After sedimentation of the proteins, the inorganic phosphate of aliquots was determined by the method of Sumner(18). The difference in inorganic phosphate between the first and second flasks gave a direct measure of the phosphate which had been esterified during the oxidation. No correction was applied for possible hydrolysis of phosphate esters during the incubation period. It was necessary to establish that the observed phosphorylation resulted from oxygen uptake and not from glycolysis. The absence of phosphorylation due to glycolysis was shown by the lack of disappearance of inorganic phosphate in flasks in which fumarate was omitted and the system incubated under nitrogen with 15 micromoles of fructose-1,6-diphosphate.

TABLE I. Oxidative Phosphorylation of Heart Preparations from Normal and Dystrophic Rabbits.

Assay periods of 15-30 min. were adjusted so that the total oxygen uptakes in all trials were roughly comparable. The observed rate of oxygen uptake was proportional to tissue concentration.

		Volume of homogenate			
		0.30 ml		0.50 ml	
Rabbit		O_2 uptake ml	P/O	O_2 uptake ml	P/O
Normal	1	79 78	2.4, 2.4		
	2	68	2.6	68	2.3
	3	59	2.6	65	2.6
	4	71	2.6	66	2.4
	5	68	2.4	78	2.2
Dystrophic	1	64 65	2.7, 2.7		
	2	61	2.6	64	2.7
	3	51	2.6	65	2.8
	4	66	2.5	62	2.4
	5	73	2.4	79	2.4

Results. The results of determinations of the P/O ratios of heart preparations from normal and dystrophic rabbits are given in Table I.

These results clearly show that there is no decrease in the ability of dystrophic rabbit heart to couple oxidation and phosphorylation. As stated by Hummel(16), the decreased ability of dystrophic muscle homogenates to

‡ The ATP solution was prepared from Sigma dibarium salt. The cytochrome C (Sigma) was standardized spectrophotometrically(14). The pyruvate was prepared and recrystallized according to Robertson(15). The fumaric acid was recrystallized from water and alcohol, and tris (hydroxymethyl) aminomethane from alcohol. The yeast hexokinase preparation was fraction 3a of Berger, *et al.*(16) in 2% glucose. The bakers' yeast used for hexokinase preparation was kindly supplied by Anheuser-Busch, St. Paul. All inorganic salts were reagent grade, and all solutions were made with glass redistilled water. The solutions of ATP, cytochrome C, pyruvate, fumarate, glucose and hexokinase were kept frozen when not in use.

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phosphorylate creatine is probably a secondary phenomenon related to the marked pathological condition of that tissue in advanced dystrophy. However, the possibility cannot be excluded that vit. E plays some role in these enzymatic processes *in vivo*, which were not detected by our analytical procedures.

It is of interest that with test system used the rate of pyruvate oxidation was increased 60 to 80% by addition of either glucose or hexokinase to the otherwise complete system. This demonstrates that in such preparations substrate oxidation may be controlled by the presence of phosphate acceptors.

Summary. 1. An assay system is described for the direct measurement of the ability of heart preparations to couple esterification of phosphate with oxygen uptake. With this system a high efficiency of phosphorylation is obtained with normal rabbit heart tissue. 2. Heart tissue preparations from rabbits made dystrophic by lack of vit. E showed no decrease in the ability to carry out oxidative phosphorylation.

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Effect of Nitrogen Mustard on Nephrotoxic Nephritis in Rats. (18692)

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The hypothesis that glomerulonephritis is a manifestation of an abnormal immune response(1,2) has led to attempts to alter its characteristics by pharmacological means (3,4). Nitrogen mustard protects rabbits against bovine gamma globulin nephritis and has been reported of value in treatment of the nephrotic stage of human glomerulonephritis (5-7). Since nephrotoxic nephritis in rats is believed to be the consequence of an antigen-antibody reaction(8-10), the effect of nitrogen mustard, which inhibits antibody production(4,11), in preventing the development of this type of nephritis has been studied.

Methods. Male Wistar rats, weighing 60-80 g were used. They were fed dog food cubes and given tap water *ad lib*. Three lots of nephrotoxic anti-rat kidney rabbit serum were prepared by Smadel's method(12), which produced "mild," "moderate," and "severe" nephritis as judged by proteinuria, edema, and survival when 0.1-0.2 ml per 100 g of rat was given intravenously on 3 successive days. Methyl-bis (beta-chloroethyl) amine hydrochloride (HN_2) 0.3-0.4 mg/kg was given intravenously for 4 or 5 successive days beginning 2 or 3 days before the first injection of nephrotoxic serum. The minimal leucocyte depression averaged 67% and usually coincided with the time of administration of the nephrotoxic serum. Urines were collected for 16-hour periods for quantitative protein determinations by the Shevky and Stafford method and for microscopic examination of the sediment. The animals were weighed and examined for evidence of edema

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