istered a single dose of 150 r x-irradiation on the 10th or 14th day of pregnancy showed a significant increase in severity of caries over that of the offspring of non-irradiated rats. Severity of caries was not increased in the offspring of rats administered a similar dose of x-irradiation on the 18th day of pregnancy.

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Creatine-Phosphate Utilization by Muscle Extracts of Rabbits on Vit. E-Deficient Diets.* (23691)

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The main pathway for creatine metabolism is presumed to be reaction I which is linked to glycolysis by reaction II. The overall reaction III was studied by following stabilization of phosphate from creatine-phosphate.

$$\text{C-P} + \text{ADP} \rightleftharpoons \text{ATP} + \text{Creatine} \quad \text{(I)}$$

$$\text{ATP} + \text{HMP} \rightarrow \text{ADP} + \text{HDP} \quad \text{(II)}$$

$$\text{C-P} + \text{HMP} \rightarrow \text{Creatine} + \text{HDP} \quad \text{(III)}$$

Since phosphate of creatine-phosphate also may be transferred to HMP with production of creatinine as measured by the Jaffe reaction(1), creatinine production was determined. It was necessary to ascertain that the effects observed in utilization of creatine-phosphate by muscle from Vit. E-deficient animals were specific for the system by which C-P is used. To observe whether generalized and similar changes occurred in other metabolic pathways in muscle, activity of another soluble enzyme, phosphoglucomutase, and rate of glycolysis by muscle strips were also investigated.

Materials and methods. Animals and diets. New Zealand male rabbits weighing 500-600 g were used. One group of 26 animals was fed Mason and Harris diet(2), and to half of them a supplement of cod liver oil (4 ml/week) was given while the other half received 75 mg of α-tocopheryl acetate (Distillation Products Industries)/week. A second group of 60 rabbits received the diet shown in Table I. Creatinuria (Cr/Cn values varied from 1 to about 5) occurred after 18 days on Mason-Harris diet in animals not receiving tocopherol. Neither of these diets produced a severe dystrophy, but instead a relatively mild effect on muscle in terms of weakness and wasting, similar to that reported with diets R-14 and R-20, by Hove and Copeland(3). The animals on Diet II survived for 100 days or more and, although they became weak,

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† The following abbreviations will be used: C-P creatine-phosphate, ADP adenosine diphosphate, ATP adenosine triphosphate, HMP hexosemonophosphate, HDP hexosediphosphate, G-1-P glucose-1-phosphate, G-6-P glucose-6-phosphate, F-6-P fructose-6-phosphate, TCA trichloracetic acid, Cn creatinine, Cr creatine, PGM phosphoglucomutase, G-1-6-P glucose-1-6-diphosphate.

TABLE I. Composition of Diet II.

10 kg	Casein	(Vit. extracted)
10	Lard	(Molecularly distilled)
28	Sucrose	(Commercial)
2	Salt mixture*	
523 g	Vitamins†	
5 kg	Alphacel	
	Vit. E orally, 50 mg twice weekly to controls	

* Hubbell, *et al.*(10).

† Vitamin Fortification Mix, Nutritional Biochemicals Corp.

they never reached the "state 3" described by Mackenzie and McCollum(4). Muscles had the waxy appearance of dystrophic muscle, with fibers varying greatly in size; but did not show complete wasting of severe muscular dystrophy. The explanation for the mild effects obtained with these diets is not clear to us, but it is not due to small amounts of Vit. E in the dietary ingredients, since the same materials used in a combination indicated by Young and Dinning(5), produce a very serious muscular dystrophy with deaths of animals after 3 to 5 weeks on the diet. Animals were sacrificed in pairs, one deficient in and one supplemented with Vit. E. Those on Mason-Harris diet were sacrificed at intervals between 7 and 42 days. The rabbits on Diet II were studied at about 60 days after starting on diet. *Enzyme preparations.* Animals were stunned by blow on head and exsanguinated. Back muscles were excised immediately; muscle of one side was used to prepare muscle strips, while the other was used to prepare an extract. A sample usually weighing a gram, was ground in a cooled mortar and extracted with 3 volumes of cold salt solution of 0.1 M KCl, 0.25 M $NaHCO_3$, and 0.01 M $MgSO_4$. The suspension was centrifuged at 0°C and the supernatant used as enzyme source. For some experiments it was desirable to dialyze the enzyme preparation. Dialysis was performed in the cold for 1½ to 3 hours in the salt solution to which 16 mg % of cysteine was added and then diluted with 1 vol. of distilled water. The dialysis medium was changed each half hour. *Enzyme determinations: PO_4 transfer from C-P to HMP.* Preliminary determinations showed that transference of phosphate to HMP was faster when G-1-P was used as co-substrate rather than G-6-P or F-6-P. Consequently, G-1-P was used as standard acceptor. The reaction system consisted of 1.3 μM of C-P (Sigma Ca-C-P converted to Na-C-P), 3 μM of Na-G-1-P (Schwarz), 2 μM cysteine, and 0.01 or 0.02 ml of enzyme preparation. Total volume was 0.25 ml, pH 7.5, and incubated 10 minutes at 30°C. Reaction was stopped with 0.3 ml of 10% TCA and precipitated protein removed by centrifugation. Phosphate was determined by the Fiske and Subbarow method. Control experiments were identical except that C-P was added after enzyme precipitation by TCA. Results were calculated from the difference in acid-labile phosphate between experimental and control. Between 0.01 and 0.07 ml of 1:3 extract, the transference of P is a linear function of amount of muscle extract. In all experiments the effect of added ATP (Na_2ATP Pabst Laboratories) at a concentration of 0.1 μM was also determined. *Creatinine production.* The same system employed in determination of phosphate transfer was used except that the reaction was incubated 15 minutes at 30°C, stopped by addition of 1 ml of alkaline picrate (15 ml of 1% picric acid plus 1 ml of 4N NaOH), diluted with distilled water to 3 ml, and measured for absorption in Beckman D. U. spectrophotometer at 520 mμ. C-P was added to incubated control immediately after denaturation of protein by alkaline picrate. Readings were taken at 20 and 30 minutes and extrapolated to zero time to correct for conversion of creatine to creatinine in alkaline medium. *Phosphoglucomutase reaction.* A modification of the method of Cardini, *et al.*(9), was used. The reaction mixture consisted of 3 μM of G-1-P, 1 μM of $MgSO_4$, 1 μM cysteine, and 0.02 ml of muscle extract diluted 1:20 or 1:30 with cold distilled water. Final volume was 0.25 ml, pH 7.5. Incubation was 15 minutes at 30°C. Formation of G-6-P was measured by reducing power according to the Somogyi-Nelson method(6). *Glycolysis.* Rate of glycolysis was determined manometrically at 30°C. Strips of muscle fibers were dissected in the cold, from rabbit back muscle without cross-cutting of fibers. Strips were selected for uniformity in diameter (approximately 0.5

TABLE III. $HPO_4^=$ Transferred and Sugar Esters Formed in Different Conditions.

Conditions for PGM reaction: 1 μM mg, 3 μM G-1-P, 0.02 ml dialyzed muscle extract; incubated 10 min., 35°C. *Conditions for measurement of fructose formed and $HPO_4^=$ transferred:* 3 μM substrate, 1.5 μM C-P, 0.02 ml dialyzed muscle extract; incubated 10 min., 25°C.

Substrate	μM ATP added	μM $HPO_4^=$ transferred	μM G-1-6-P* formed	μM G-6-P+ F-6-P formed	μM fructose formed
G-1-P	.0	.69		2.0	
	.025	.58		1.09	.849
	.050	.37		.72	
	.100	.04		.41	.216
G-6-P	.0	.59			.503
	.100	1.07			.716
G-1-P	.0	.923			.690
	.100	.290			.235
G-1-P	.0	.51	.015		
	.0	.88	.022		

* Determined by enzymatic method of Cardini, *et al.*(9).

mm) of fibers. Incubation was performed in 7 ml flasks containing 1 g of muscle strips, 1.8 ml of Krebs-Ringer bicarbonate buffer, 0.2 ml of 0.2 M glucose in side bulb, and 0.2 ml of 20% KOH in center well. Vessels were gassed with 5% CO_2 - 95% N_2 for 10 minutes. After 15 minute equilibration period, glucose solution was tipped into the main chamber. CO_2 production was measured 30 minutes.

Results. Method to measure phosphate transfer: a) *Selection of acceptor of phosphate.* F-6-P is presumed to be the acceptor of phosphate in reaction (II), however, (Table II) our experience has been that the reaction is consistently faster in presence of G-1-P. It is not known whether the preparation of F-6-P has an inhibitory impurity, or if the F-6-P itself is an inhibitor at rather high concentration that must be used for accurate determination. To corroborate that in reaction II the HMP directly concerned is F-6-P, fructose esters found as end products of reaction were determined (Table III) by the method of Roe(7). Micromoles of fructose formed, when compared to micromoles of phosphate transferred, were sometimes less than would be expected if the reaction is terminated at fructose-1-6-diphosphate. Loss of fructose esters may have resulted from further steps in glycolysis. The known mechanisms which could mediate an increase in total phosphate transferred via glucose esters are the reactions by which the phosphate of C-P is transferred to G-1-P to form G-1-6-P(1,8). Formation of G-1-6-P can be measured specifically by its coenzymatic property(9), and since only about 3% of total phosphate transferred appears to be this compound (Table III), it was concluded that the role of G-1-P as a direct phosphate acceptor is a very minor one. b) *Participation of the adenylic system in the reaction.* An adenylic derivative is not added to the reaction in the system for measurement of phosphate transfer from C-P. Preliminary experiments showed that addition of ATP in concentration of 0.1 μM/0.25 ml resulted in slight activation, no effect, or sometimes a small inhibition. At higher concentrations of ATP, inhibition resulted uniformly. This variability in effect of ATP may be a function of quantity of endogenous ATP present in individual enzyme extracts. To study the effect more precisely, it was necessary to dialyze the enzyme extract. Phosphate transfer was stimulated by ATP at concentrations up to 0.1 μM when dialyzed extracts were used (Table IV). c) *Inhibition of PGM by*

TABLE II. Comparison of Rate of $HPO_4^=$ Transfer from C-P to HMPs by Non-dialyzed Muscle Extract. Conditions: As stated under *Methods.*

Phosphate acceptor, 3 μM	μM $HPO_4^=$ transferred Time in min. 3	6	9	12
G-1-P	.408	.914	1.078	1.112
G-6-P	.182	.482	.816	.854
F-6-P	.246	.408	.638	.768

TABLE IV. Effect of ATP on $HPO_4^=$ Transfer from C-P to HMP. Conditions: As stated under *Methods.*

Phosphate acceptor, 3 μM	μM ATP added	μM $HPO_4^=$ transferred: Dialyzed enzyme preparation	μM $HPO_4^=$ transferred: Non-dialyzed enzyme preparation
G-1-P	.0		
	.001	.07	
	.01	.379	
	.1	.842	
	1.0	.600	
G-1-P	.0	.154	.558
	.2	.485	.218
G-6-P	.0		.489
	.2		.479
F-6-P	.0	.285	.445
	.2	.520	.445

ATP. The inhibitory effect of ATP observed above was probably due to inhibition of the phosphoglucomutase reaction. Table III shows that with G-1-P as acceptor, there is a decrease in formation of G-6-P, fructose phosphates, and in activity of phosphate transfer when the concentration of ATP is increased. When G-6-P is the acceptor both production of fructose phosphate (as determined by the resorcinol reaction of Roe) and transference of phosphate from creatine-phosphate are enhanced in presence of 0.1 μM of ATP as compared to no ATP. With G-1-P as co-substrate, this level of ATP inhibits both fructose and G-6-P formation and also phosphate transfer. However, G-1-P still appears to be the best acceptor of phosphate from C-P in our reaction system (Tables III and IV).

Effect of tocopherol deficiency on enzyme activities of muscle extracts. The first change in enzyme activity was observed in rabbits fed the Mason-Harris diet for 25 days (Fig. 1). At this time, rate of glycolysis of glucose by muscle strips of Vit. E-deficient animals (Mean 4.30 μM/CO_2/g/30″) begins to increase as compared to that of tocopherol-supplemented controls (3.02 μM/CO_2/g/30″). Glycolysis remains elevated to about 40 days on the diet, after which there is a trend to return to normal. Concurrent with changes in glycolysis, transfer of phosphate from C-P to HMP by Vit. E-deficient animals decreases (Mean 0.33 μM $HPO_4^=$) as compared to that of controls (Mean 0.45 μM $HPO_4^=$) and shows no tendency to return to normal value. Production of creatinine is also diminished in Vit. E-deficient animals (Means: +E = 0.185 μM, −E = 0.132 μM). Differences observed in the above systems are significant at the 1% level when submitted to the Fisher F test. Activity of the phosphoglucomutase reaction did not differ significantly among experimental and control animals (Means: μM G-6-P, +E .457 μM, −E .421 μM).

Animals on Diet II were studied between 60 and 70 days on the diet. Data from deficient animals compared with supplemented animals (Table V) show no significant differences in glycolysis between experimental and control animals, confirming the apparent return to normal of values observed with the Mason-Harris diet. The transfer of phosphate, however, remains significantly impaired in experimental animals and the decrease from normal (27%) is essentially as observed with the Mason-Harris diet (28%). Creatinine production is significantly diminished. Although the mean for phosphoglucomutase activity for Vit. E-deficient animals is lower it does not represent a significant difference from the controls.

Effect of ATP on transfer of $HPO_4^=$ from C-P by muscle extracts. In conjunction with phosphate transfer measurements, parallel experiments were done in which the effect of addition of 0.1 μM of ATP was tested (Table IV). Addition of ATP decreases transfer of $HPO_4^=$ of animals on the Mason-Harris diet. The decrease is significant for both supplemented and deficient animals. In the same reaction system but using muscle extracts from animals fed Diet II, ATP does not lower the mean for phosphate transfer significantly.

Discussion. The results presented show that there is impairment of the phosphate transfer from C-P to the HMP chain. Since neither glycolysis in general, nor phosphoglucomutase reaction are decreased, this seems to represent a rather specific effect on the system that normally phosphorylates and dephosphorylates creatine phosphate. This indicates that reaction II is also functioning

TABLE V. Enzyme Activities of Muscle Extracts from Rabbits on Diet II.*

Diet	No. of animals	Glycolysis, μM CO_2/g /30 min.	$HPO_4^=$ transfer from C-P to HMP	Cn formed from C-P	PGM reaction G-6-P formed
			μM		
Deficient in vit. E	12	8.15 (None)	.411 (Between 1-5%)	.119 (1%)	.264 (None)
Complete	11	8.00	.576	.168	.327

* Numbers in parentheses represent significance level of value obtained for F when activities of deficient animals are compared with those on a complete diet.

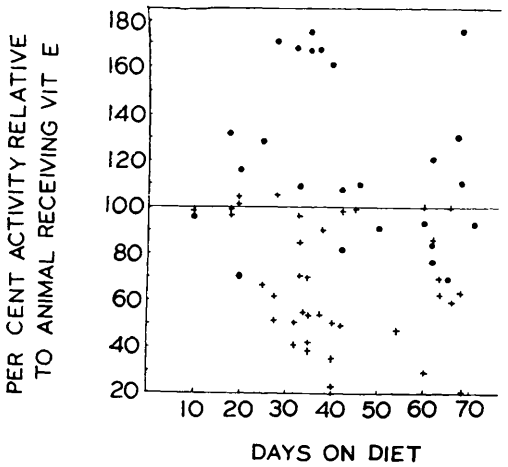

FIG. 1. Phosphate transfer by muscle extracts (+) and glycolysis by muscle strips (○) of rabbits fed the Mason and Harris diet for various lengths of time. Values are expressed as percent of the same activities of control animals.

normally, since a substantial decrease in activity here would diminish glycolysis. It is known that creatine is stored in muscle mainly as C-P and impairment of reaction I should result in decreased uptake of creatine formed by other organs. To maintain steady concentration of total creatine, this uptake has to be in equilibrium with rate of decay of creatine to creatinine. A decrease in rate of reaction I presents a possible explanation for creatinuria which is produced in tocopherol-deficient animals before other symptoms of muscle wasting and weakness. The role of tocopherol, or perhaps a derivative in reaction III, for which known co-factors are ATP and SH, is probably not that of co-factor. Also the possibility can be discarded that decrease of ATP accounts for decreased rate of phosphate transfer, since addition of ATP to the reaction system was not found to stimulate activity. Therefore, the enzyme itself remains the most likely decreased component.

That there is a specific impairment of this enzymatic system relative to others studied in this work, makes it plausible to suggest that the severity of Vit. E deficiency on the muscle could be related to the requirement of muscle for unusually high concentrations of C-P as compared to other tissues.

Summary. A method for determination of the transfer of phosphate from creatine-phosphate to the hexosemonophosphates is described. It has been found that this transference is decreased in the muscle of rabbits fed tocopherol-deficient diets at a time when phosphoglucomutase activity and glycolysis are normal. The decrease in transference is not corrected by addition of ATP. ATP added at concentrations above 0.004 M inhibits phosphoglucomutase.

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