spread of typical slow potential changes in nearby cortex. 2. Where previously absent, typical slow potential changes and spreading depressions were produced in the cingular area by a) prolonged exposure of the cortex, b) intravenous infusion of concentrated sucrose solution, c) cooling of the cortex, d) treatment of the cortex with isotonic sucrose solution, and e) with Ringer's solution containing 10 times the normal amount of potassium. 3. Asphyxial potentials were recorded both from the cingular area and from the cortex on the convexity of the hemisphere. However, the latent period of this potential from the cingular area was markedly longer than that recorded from the convexity.

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Received December 20, 1955. P.S.E.B.M., 1956, v91.

Enzyme Studies in Muscular Dystrophy. II. Muscle Dipeptidase Activity and Vitamin E-Deficiency.* (22244)

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Vit. E-deficiency in the rabbit and other species results in characteristic muscular wasting and weakness. In previous investigations, on a number of tissues of the deficient rabbit, we found the cathepsin activity, *in vitro*, to be markedly increased in muscle, but unchanged in the other tissues(1). To investigate further the metabolism of protein in this form of muscular dystrophy, we have studied the activity of two previously characterized peptidase systems of muscle, glycyl-l-leucine and glycylglycine dipeptidase(2-4).

Methods. Vit. E-deficiency was induced in young rabbits by maintaining them on Diet 11 of Goettsch and Pappenheimer(5) treated with 1% ethereal $FeCl_3$. Control animals received the untreated diet supplemented with 25 mg % dl-α-tocopherol. After the appearance of symptoms of Stage II(6) of muscular

* Aided by Grants from Muscular Dystrophy Associations of America, and National Institutes of Health, U. S. Public Health Service.

TABLE I. Effect of Vit. E-Deficiency on Dipeptidase Activity of Muscle Extracts.

Substrate	Extract	Buffer	0.001M activator	Control: No. animals	Control: No. determ.	Control: Specific activity†	Vit. E-deficient: No. animals	Vit. E-deficient: No. determ.	Vit. E-deficient: Specific activity	t	P*
						60 min.			60 min.		
Glycyl-l-leucine	KCl	$PO_4^{=}$	—	7	14	100 ± 38‡	6	10	410 ± 235	5.2	<.01
			$+Mn^{++}$	4	8	85 ± 29	5	8	300 ± 213	3.0	"
	H_2O	"	—	3	12	155 ±100	7	17	540 ± 250	5.3	"
			$+Mn^{++}$	3	6	75 ± 32	4	8	390 ± 237	5.3	"
						120 min.			120 min.		
Glycylglycine	KCl	"	—	5	10	65 ± 36	7	14	100 ± 59	1.8	<.1
			$+Co^{++}$	5	10	85 ± 32	7	14	410 ± 243	4.5	<.01
		Veronal	—	4	8	60 ± 10	7	14	80 ± 48	1.1	<.3
			$+Co^{++}$	4	8	80 ± 26	7	14	205 ± 99	3.8	<.01
	H_2O	$PO_4^{=}$	—	4	8	110 ± 46	6	12	94 ± 37	1.0	<.4
			$+Co^{++}$	4	8	125 ± 53	6	12	405 ± 200	4.2	<.01
						mg protein N/ml			mg protein N/ml		
Protein nitrogen content of extracts	KCl			12		3.05 (2.19-3.72)§	12		2.45 (1.68-3.33)		
	H_2O			15		1.84 (1.41-2.52)	18		1.86 (.89-2.69)		

* P = Level of significance. † Specific activity = % hydrolysis/mg of muscle extract protein nitrogen/ml of incubation mixture. ‡ Stand. dev. § Figures in parentheses are range of values.

TABLE II. Effect of Vit. E-Deficiency on Dipeptidase Activity of Muscle Homogenates.*

Substrate	.001M activator	Control: No. of animals	Control: mg PN,† g wet wt	Control: Activity,‡ g wet wt	Control: Specific activity	Vit. E-deficient: No. of animals	Vit. E-deficient: mg PN, g wet wt	Vit. E-deficient: Activity, g wet wt	Vit. E-deficient: Specific activity
			a	b	b/a		a	b	b/a
Glycyl-l-leucine	—	4	23.5 (21.3–25.2)§	810 (710–1080)	34 (29–44)	3	19.2 (16.4–23.0)	5910 (2200–10800)	310 (95–660)
	Mn^{++}	3	24.2 (23.6–25.2)	700 (495–840)	29 (20–36)	3	19.2 (16.4–23.0)	3410 (1470–5950)	175 (65–365)
Glycylglycine	—	4	23.5 (21.3–25.2)	160 (0–480)	7 (0–20)	4	20.7 (16.4–25.0)	184 (90–280)	9 (5–16)
	$+Co^{++}$	4	23.5 (21.3–25.2)	350 (200–500)	15 (8–21)	4	20.7 (16.4–25.0)	2350 (500–4000)	110 (22–250)

* All muscle homogenates were prepared in H_2O. † PN = Protein nitrogen. ‡ Activity = % hydrolysis of glycyl-l-leucine at end of 60 min. incubation period and of glycylglycine at end of 120 min. of incubation. § Figures in parentheses are range of values.

dystrophy, the animals were sacrificed. Mixed portions of the muscles of the leg, thigh and back were homogenized with 2 volumes of either water or 2% KCl. The enzyme source was either a muscle extract obtained by centrifuging at 1400 x g, or the whole muscle homogenate. The extracts were diluted to 5-15 times their original volumes, and activity was determined at 2 or more different dilutions. The homogenates were diluted to 5-10 times their original volumes and strained through gauze. The observed values of activities of duplicate aliquots were averaged. Protein nitrogen content of all of the preparations was determined by a colorimetric nesslerization procedure. The assay system consisted of 0.15 ml buffer (1 M phosphate, pH 7.4-7.6 or 0.015 M veronal, pH 7.7-7.9), 0.15 ml 0.01 M metal ion activator when used, 0.5 ml 0.1 M substrate, 0.5 ml of the enzyme source, and sufficient water to make the final volume 1.5 ml. The mixtures were incubated at 37.5 $\pm$ .5°C for periods of one and 2 hours. Hydrolysis of the dipeptides was determined by the colorimetric ninhydrin procedure of Moore and Stein(7) as adapted by Schwartz and Engel(8). Preparations containing the enzyme source showed no measurable change in ninhydrin reacting material in the absence of substrate.

Results. Dipeptidase activities of the muscle extracts are shown in Table I and those of the whole homogenates are given in Table II. The incubation periods used were relatively short as compared to previous studies on these systems. Although the rates of hydrolysis of both substrates decreased somewhat during the second hour of incubation, as previously reported for rat muscle(9), the hydrolysis of glycylglycine had to be determined over a 2-hour period in order to obtain measurable values in the control group. Hydrolysis of glycyl-l-leucine is reported for one hour periods of incubation although in many experiments activity during the second hour also was determined. The addition of Mn^{++} did not increase glycyl-l-leucine hydrolysis by either the normal or E-deficient tissues, and, in fact, was somewhat inhibitory. We do not know why the Mn^{++} activation of glycyl-l-leucine dipeptidase activity reported by Smith(2,4) was not observed in these experiments; however, it is noteworthy that in at least one instance, this investigator also failed to note any stimulation by Mn^{++}(2). The addition of Co^{++} caused a slight increase in glycylglycine hydrolysis by the control group and a considerable increase by the E-deficient group. During vit. E-deficiency there was a marked increase in dipeptidase activity of the rabbit muscle extracts and whole homogenates. The increase in glycyl-l-leucine dipeptidase activity was not dependent upon the addition of Mn^{++}, but the increase in glycylglycine dipeptidase activity during vit. E-deficiency required the addition of Co^{++} activator. Elevation in dipeptidase activity was observed during both the first and the second hours of incubation, although the absolute rates of hydrolysis by both the control and deficient groups decreased slightly during the second hour. In one experiment, hydrolysis of glycyl-l-leucine by a mixture of extracts from control and vit. E-deficient rabbit muscle was equal to the sum of hydrolysis by the individual extracts. This result suggests that the difference between the activities of normal and E-deficient muscle was not due to the presence of either an inhibitor in the control group or of an activator in the deficient preparations. Although there was usually some decrease in protein nitrogen content of the muscle during the deficiency, which could give slightly higher values for the specific activity, this relatively small difference in nitrogen content cannot account for the large increases in dipeptidase activity we have observed.

An increased incorporation of glycine into muscle protein, *in vivo*, occurs, at least over short periods of time, in deficiency of vit. E (10). Therefore, the decrease in muscle mass that is observed in this condition appears to be due to an even greater increase of protein catabolism. Both dipeptidase and catheptic (1) activity of muscle is increased *in vitro* during the deficiency. It has been demonstrated that purified cathepsins have the ability, under proper conditions, to synthesize polypeptides *in vitro* by transpeptidization (11,12). While the functioning of these en-

zymes *in vivo* is yet to be demonstrated and other and more important enzyme systems are probably involved in protein metabolism, it is plausible to speculate that the net breakdown of muscle protein, observed *in vivo* during vit. E-deficiency, may be due to increase in rate, and a shift in the equilibrium of a reversible enzyme system(s) catalyzing peptide bond formation and breakdown.

Summary. The effect of vit. E-deficiency on hydrolysis of glycylglycine and glycyl-l-leucine by skeletal muscle extracts and homogenates was investigated. During this deficiency there was a marked increase in dipeptidase activity of both muscle extracts and homogenates. The increase in glycylglycine dipeptidase activity could be demonstrated only after the addition of Co^{++}.

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Received January 23, 1956. P.S.E.B.M., 1956, v91.

Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin-Ciocalteau). (22245)

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In tissue culture studies, the determination of the amount of growth under varying environmental conditions is often of central importance. In this laboratory, the actual enumeration of the cells(1) proved a useful but laborious procedure(2-4); and studies were therefore undertaken to devise a rapid yet reliable method of measuring growth. Consideration was given to the direct turbidimetric measurement of the resuspended cells, the determination of total nitrogen by nesslerization, or of cell protein by precipitation procedures; but the method to be here described proved superior to any of these in its simplicity and reproducibility. Essentially, the technic is a modification of the colorimetric method of Lowry, *et al.*(5) for measuring protein, using a phenol reagent (Folin-Ciocalteau) for the development of color. As here described, the method is designed for use in cultures adherent to a glass surface, and overlaid with a fluid medium.

Materials. 1. Earle's salt solution(6) (or any similar balanced salt solution) for washing the cell layer in the culture flask. 2. Alkaline copper solution to dissolve the cells. This is kept as 2 stock solutions, A and B: A, Na_2CO_3, 200 g; NaOH, 40 g; NaK tartrate, 2 g; q.s. to 10 liters; B, $CuSO_4 \cdot 5H_2O$, 5 g; q.s. to 1 liter. (The tartrate was incorporated with the alkali, rather than the $CuSO_4$, because of the slow development of a precipitate in the copper-tartrate mixture.) Solutions A and B are mixed in the proportion of 50 parts of A to 1 part of B to form Lowry's solution C, which is prepared fresh daily.

* Public Health Service, U. S. Department of Health, Education and Welfare.