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^{++}.

1. Weinstock, I. M., Goldrich, A. D., and Milhorat, A. T., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 257.

2. Smith, E. L., *J. Biol. Chem.*, 1948, v173, 553.

3. ———, *ibid.*, 1948, v173, 571.

4. ———, *ibid.*, 1948, v176, 9.

5. Goettsch, M., and Pappenheimer, A. M., *J. Exp. Med.*, 1931, v54, 145.

6. Mackenzie, C. G., and McCollum, E. V., *J. Nutrition*, 1940, v19, 345.

7. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1948, v176, 367.

8. Schwartz, T. B., and Engel, F. L., *ibid.*, 1950, v184, 197.

9. Engel, F. L., and Schwartz, T. B., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 615.

10. Dinning, J. S., Sime, J. T., and Day, P. L., *J. Biol. Chem.*, 1955, v217, 205.

11. Jones, M. E., Hearn, W. R., Fried, M., and Fruton, J. S., *ibid.*, 1952, v195, 645.

12. Fruton, J. S., Hearn, W. R., Ingram, V. M., Wiggans, D. S., and Winitz, M., *ibid.*, 1953, v204, 891.

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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.

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3. Folin-Ciocalteau reagent† (7). The reagent should be standardized by determining that concentration which gives the maximum and most stable color. This standardization need be carried out only once for each lot. Crystalline bovine albumin may be used as the reference standard. One part of a solution at 50 μg/ml is added to 5 parts of solution C. Six ml are then distributed into each of 6 cuvettes, and a parallel series is prepared using H_2O instead of the protein solution. Dilutions of the F-C reagent are prepared as indicated below:

Reagent	5	5	5	5	5	5
H_2O	4	5	6	7	8	9
Dilution	5/9	5/10	5/11	5/12	5/13	5/14

0.5 ml of each of these dilutions is rapidly blown into 1 cuvette† containing the protein solution, and 1 control cuvette containing H_2O. The color is read after 15, 30, 60, 90, 120, 180, and 240 minutes, in each case against the corresponding water control. That dilution of the F-C reagent which reaches maximum color within approximately ½ hour, and remains stable for 2 hours thereafter, is the one used in the test.

Details of procedure. Step 1. Washing of cultures. The culture flasks are drained of medium by inversion, and the adherent cell layer washed twice with Earle's salt solution (6). For the culture flasks previously described (2,8), with a surface area of approximately 15 sq cm, and a 15 ml capacity, an 8 ml wash is used. After the second wash the flasks are left inverted over clean gauze to drain for approximately 15 minutes, when clean stoppers are inserted. At this stage, the washed and dry cells may be stored in the original culture flasks for several days with no change in the protein analysis. *Step 2. Solution of cells in alkaline copper tartrate.* Ten ml of Lowry's reagent C(5) are added to the flasks with an automatic syringe and allowed to remain in contact with the cell layer for 10 minutes, after which the flasks are shaken manually to complete the dissolution of the cells. At this stage also, the flasks may be stored for several days with no loss in protein. *Step 3. Color development.* A biuret color is produced by the interaction of the alkaline copper sulfate tartrate solution C with the protein; and the intensity of this color may serve as a rough measure of the amount of cells, and thus of the amount of the solution which must be used in the final test in order to produce a color within the optimum measurable range (optical density = <0.4). When the color is not detectable, 2 ml is taken for analysis; when the color is pale violet, 0.5 ml will suffice; while with a deep violet color, the solution should be diluted with 1 or 2 volumes of solution C, and 0.5 ml taken for analysis. The appropriate amount of the cell solution is pipetted into a Coleman cuvette,† and solution C added to a total of 5 ml. One ml of H_2O is then added, and finally, 0.5 ml of the properly diluted F-C reagent is jetted in by a syringe. The rapid admixture of the color reagent with the solution is essential; and the slow addition of the reagent without such rapid admixture leads to erroneous results. The controls consist of 3 cuvettes, each containing 1 ml of crystalline bovine albumin at 50 μg/ml instead of the H_2O used in the test proper. A water blank is also run. Absorption is read after 30 minutes at 660 mμ; but the color remains stable for 2 hours.

Calculation of results. Results of the test are most simply expressed in terms of the bovine albumin equivalent of the unknown cell culture. For 4 different cell lines the ab-

† Available from the Fisher Scientific Co.

† Round 19 x 105 mm tube, 5.5 min ml capacity.

TABLE I. Conversion of "Bovine Albumin Equivalent" of 8 Tissue Culture Strains to Cell Mass, Nitrogen and Number.

Cell line	Multiplying factors to convert bovine albumin equivalent (mg) to: Cell mass (mg)	Cell N (mg)	Cell count (×10⁶)
Mouse fibroblast (Earle)	1.4	.18	.12
HeLa (Gey)	1.4	.16	.16
Intestinal epithelium (Henle)	1.5	.18	.16
Human leukemia (Osgood #111)	1.6	.17	.13
#39 "liver" (Chang)	1.6	.19	.18
KB (Eagle)	1.6	.18	.15
Human conjunctiva (Chang)	1.7	.18	.17
"Liver" (Henle)	1.8	.19	.14

TABLE II. Progressive Growth of 3 Cell Strains as Measured by Protein Determinations and by Nuclei Counts.

Cell line			Days after planting culture 1	2	3	4
Human leukemia (Osgood #111)	Pd‡	$\mu g \pm$ S.D.*	784 ± 16	1217 ± 12	1694 ± 11	2520 ± 43
		V†	2%	1%	.64%	1.7%
	Cc‡	$\times 10^4$**	97 ± 6.6	144 ± 4.5	217 ± 9.3	391 ± 42
		V	6.8%	3.1%	4.3%	11%
	Ratio of protein, μg /Cc $\times 10^4$		8.1	8.5	7.8	6.4
HeLa (Gey)	Pd	$\mu g \pm$ S.D.	746 ± 19	1025 ± 53	1498 ± 60	1988 ± 159
		V	2.5%	5.2%	4%	8.0%
	Cc	$\times 10^4$	80 ± 5.8	101 ± 16	163 ± 15	267 ± 39
		V	7.2%	16%	9.2%	14%
	Ratio of protein, μg /Cc $\times 10^4$		9.3	10.1	9.2	7.4
Intestinal epithelium (Henle)	Pd	$\mu g \pm$ S.D.	454 ± 11	743 ± 39	1005 ± 34	1607 ± 176
		V	2.4%	5.3%	3.3%	11%
	Cc	$\times 10^4$	51 ± 4.2	77 ± 8.8	101 ± 12	204 ± 18
		V	8.1%	12%	12%	8.8%
	Ratio of protein, μg /Cc $\times 10^4$		8.9	9.6	10.0	7.9

* Mean of 4 replicate flasks $\pm$ stand. dev. (σ). † Pearson's coefficient of variation $\frac{\sigma \times 100}{\text{mean}}$. ‡ Pd = Protein determination; Cc = Cell count.

sorption curve was a straight line function of the amount of cells up to an optical density $\left(\log \frac{I_o}{I}\right)$ of 0.4.† For a given cell type, the bovine albumin equivalent as measured colorimetrically may be converted to cell count, mg dry weight, total nitrogen, protein nitrogen, or any other desired criterion, by appropriate determinations on test suspensions simultaneously measured by the method here described. Some of these conversion factors are indicated for 8 cell types in Table I.

An experiment to show the reliability of the protein determination as a measure of cell growth with 3 different cell lines is summarized in Table II. A number of replicate T-15 flasks were inoculated with each strain, and their protein content and cell count measured daily. The coefficient of variation in the protein content of 4 flasks varied from 1 to 11%, averaging 3%. The coefficient of variation in the cell count procedure varied from 3 to 16%, averaging 9.5%. With each cell line the ratio of the protein content to cell count remained essentially constant until the 4th day, when there was a smaller amount of protein per cell, presumably indicative of a smaller average cell size.

Summary. An analytical method for the measurement of cell growth in tissue culture is described, based on the Lowry method for the determination of protein, and employing a phenol reagent (Folin-Ciocalteau) for color development. The results, referred to a bovine serum albumin standard, may be converted to dry weight, nitrogen or cell count by appropriate conversion factors. Those factors are here given for 7 human cell lines and 1 mouse line.

1. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., Jr., *J. Nat. Cancer Inst.*, 1951, v11, 773.

2. Eagle, H., *J. Biol. Chem.*, 1955, v214, 839.

3. ———, *J. Exp. Med.*, 1955, v102, 37.

4. ———, *ibid.*, 1955, v102, 595.

5. Lowry, O. H., Rosebrough, N. J., Fair, A. L., and Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

6. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

7. Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, 1927, v73, 627.

8. Earle, W. R., and Highouse, F., *J. Nat. Cancer Inst.*, 1954, v14, 841.

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