were cytopathic on MKTC's. MKTC's were much more sensitive (80%) than HEp-2 cells. Seventeen of 24 isolates were revealed between the 3rd and 6th days post-inoculation. Growth curves in MKTC's revealed virus by the 7th hour, with peak titers at 78 to 96 hours. Rotation yielded a 90% net increase of virus over those obtained in stationary cultures. RS virus is not stabilized by 1 M $MgCl_2$. The virus can be maintained in 50% glycerin for at least 9 months of storage at $\geq -20°C$. Even at 4°C the decay rate in glycerin is markedly delayed. Preservation in glycerin is superior to 0.5% gelatin or 20% chicken serum. Preservation was not enhanced by lyophilization.

1. Chanock, R. M., Parrott, R. H., Vargosko, A. J., Kapikian, A. Z., Knight, V., Johnson, K. M., *Am. J. Pub. Health,* 1962, v52, 918.

2. Rowe, D. S., Michaels, R. H., *Pediatrics,* 1960, v26, 623.

3. Lewis, F. A., Rae, M. L., Lehmann, N. J., Ferris, A. A., *Med. J. Australia,* 1961, v2, 932.

4. Wulff, H., Kidd, P., Wenner, H. A., 1963, *Pediatrics,* in press.

5. Wallis, C., Yang, C.-S., Melnick, J. L., *J. Immunol.,* 1962, v89, 41.

6. Hilleman, M. R., Hamparian, V. V., Ketler, A., Reilly, C. M., McClelland, L., Cornfeld, D., Stokes, J., *J.A.M.A.,* 1962, v180, 445.

7. Jordan, W. S., *J. Immunol.,* 1962, v88, 581.

8. Kisch, A. L., Johnson, K. M., Chanock, R. M., *Virology,* 1962, v16, 177.

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Peroxidation and Lysosomes in Nutritional Muscular Dystrophy of Chicks.* (28942)

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Nutritional muscular dystrophy is characterized by appearance of white striations in the pectoral and, to a lesser extent, in the leg muscles of chicks deficient in both Vit. E and sulfur amino acids(1). Various studies on similar nutritional dystrophies are reported in the literature but the biochemical mechanism of such pathology is not well understood. Oral administration of large amounts of polyunsaturated fatty acids or injection of their peroxides has been found to cause severe symptoms of encephalomalacia in chicks(2,3). Recently, it has been shown in the dystrophic tissues of Vit. E deficient rabbits(4) and in the genetic dystrophy of mice and chickens(5) that a significant increase occurs in all hydrolytic enzymes of the lysosomes. This paper describes the increases which occur in the hydrolytic enzymes of the lysosomes in the muscles of chicks suffering from nutritional muscular dystrophy produced by a dietary deficiency of Vit. E and sulfur amino acids and by feeding excess linoleic acid.

Methods. One-day-old chicks from Vit. E-depleted White Plymouth Rock × Vantress hens were used in the study. Each treatment was replicated and consisted of equal numbers of male and female chicks. They were housed in electrically-heated battery brooders with wire mesh floors. Feed and water were supplied *ad libitum.* The basal diet used in the experiment reported in Table I was the casein-torula yeast diet previously described by Scott and Calvert(6). In all other experiments the casein-gelatin diet of Calvert *et al*(7) was used with modifications as follows: the casein was methanol-extracted, the stripped lard and diphenyl-p-phenylene diamine (DPPD) were omitted and the diet was supplemented with 0.5% hydrogenated coconut oil, 0.1 ppm selenium (as Na selenite) and 0.0125% ethoxyquin.

Chicks were sacrificed at the end of the 4-week experimental period and scored for

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TABLE I. Lysosomal Enzymes in Pectoral Muscle of Nutritional Dystrophic Chick.

Enzyme	Specific activity*		
	Dystrophic	Methionine added, .4%	d-α-tocopheryl acetate added, 80 mg/kg diet
Acid phosphatase	207 ± 18.8	105 ± 4.9[a]	146 ± 7.6[c]
Cathepsin	79 ± 4.3	40 ± 1.7[a]	48 ± 3.7[a]
Aryl sulfatase	0	0	0
Ribonuclease	271 ± 18.8	57 ± 2.7[a]	71 ± 3.4[a]
β-Glucuronidase	61 ± 10.2	11 ± 1.4[a]	8 ± 1.0[a]
β-Galactosidase	16 ± 2.5	3 ± .1[a]	8 ± .4[b]

* Expressed as μmoles of substrate hydrolyzed per mg of N per min × 10^4.

[a] $p<.001$, [b] $p<.01$, [c] $p<.02$; p determined by Student's "t" test for significant difference from dystrophic group.

muscular dystrophy. Pectoral muscles were removed and frozen immediately in liquid nitrogen. For chemical analysis, a 10% homogenate was made in ice-cold, de-ionized water by blending the tissue in Servall Omnimixer for one minute.

Activity of individual lysosomal enzymes was determined by several assays as follows: acid phosphatase, β-glucuronidase and cathepsin were determined by the methods of Gianetto and de Duve(8). β-Galactosidase was assayed by the method of Sellinger *et al* (9) and ribonuclease according to de Duve *et al*(10). Aryl sulfatase was assayed by the method of Roy(11) with 2-hydroxy-5-nitrophenyl sulfate as substrate.

Tissue peroxidation was estimated in unincubated tissue using thiobarbituric acid (TBA) index as described by Tappel and Zalkin(12). Respective controls and reagent blanks were run for all enzyme assays and TBA measurements.

Results. The activities of 5 lysosomal enzymes, acid phosphatase, cathepsin, ribonuclease, β-glucuronidase and β-galactosidase were significantly increased in the pectoral muscle of the nutritionally dystrophic chicks as compared with the normal controls. These results are presented in Table I. Aryl sulfatase was absent in both dystrophic and nondystrophic tissues. Supplementation of the diet with 0.4% DL-methionine or 80 mg d-α-tocopheryl acetate per kg completely prevented dystrophy and significantly reduced the activity of lysosomal enzymes. The increase in lysosomal enzymes in the dystrophic tissues ranged from about 2 × for acid phosphatase and cathepsin to about 5 to 6 × for ribonuclease, β-glucuronidase and β-galactosidase.

The results presented in Table II show that a correlation was obtained between the levels of linoleic acid in the diet and the incidence and severity of muscular dystrophy, up to a level of about 1% linoleic acid. Correlations also existed between incidence of dystrophy, the thiobarbituric acid index and activity of the indicator lysosomal enzyme, β-glucuronidase. One percent of linoleic acid in the diet increased the values of muscle

TABLE II. Influence of Dietary Linoleic Acid Stress on Tissue Peroxidation and Lysosomal Enzymes.

Dietary linoleic acid, %	Muscular dystrophy		TBA index†	Specific activity of indicator lysosomal enzyme β-glucuronidase‡
	Incidence*	Score (0-3)		
—	0/8	0	24.1	12.1
.25	6/8	.75	55.2	21.0
.50	5/8	1.12	69.6	33.0
1.00	8/8	1.62	81.6	41.4
.50 (in form of lard)	7/8	1.31	74.8	35.0

* Denominator shows total No. of chicks per treatment and numerator denotes chicks with muscular dystrophy.

† Expressed as absorbancy at 535 mμ per mg N × 10^3.

‡ Expressed as μmole substrate hydrolyzed per mg N per min × 10^4.

TABLE III. Comparative Effects of Dietary Linoleic and Oleic Acids on Tissue Peroxidation and Lysosomal Enzymes.

Added to basal diet, %	Muscular dystrophy Incidence*	Score (0-3)	TBA index†	Specific activity of indicator lysosomal enzyme β-glucuronidase‡
—	0/8	0	24.1	12.1
Oleic, 1.95	0/8	0	30.1	12.5
Idem + .25 linoleic	3/8	.50	39.6	22.3
" + .50 "	8/8	1.12	97.3	30.3
" + 1.00 "	8/8	1.90	86.1	32.1

* Denominator shows total No. of chicks per treatment and numerator denotes chicks with muscular dystrophy.
† Expressed as absorbancy at 535 mμ per mg N × 10^5.
‡ Expressed as μmole substrate hydrolyzed per mg N per min × 10^4.

TBA index and the lysosomal enzyme, β-glucuronidase, almost 3 times as compared to the levels present in the chicks receiving no added linoleic acid. Addition of 4% lard had an effect equivalent to its content of linoleic acid (0.5%). However, inclusion of oleic acid alone in an amount equivalent to that present in lard, either alone or in combination with linoleic acid, had no effect on either muscular dystrophy or the biochemical parameters (Table III). Supplementation of the diet with 5-10 mg d-α-tocopheryl acetate per kg in the presence of linoleic acid prevented the muscular dystrophy and reduced TBA index and lysosomal enzyme activity accordingly (Table IV).

Discussion. The results presented here indicate direct relationships between peroxidation of the tissues, increases in lysosomal enzymes and occurrence of muscular dystrophy. Analyses of the dystrophic muscles of Vit. E and sulfur amino acid-deficient chicks showed significant increases in the activities of 5 characteristic lysosomal hydrolases as compared to the level of these enzymes present in non-dystrophic chickens protected from dystrophy by supplementation of the diet with methionine or Vit. E (Table I). Similar increases in the activities of these enzymes have been observed in the muscles of nutritionally dystrophic rabbits(4) and genetically dystrophic mice and chickens(5).

The primary function of lysosomes is believed to be associated with cellular digestion in pathological conditions such as cell injury, autolysis and necrosis(13). These findings of large increases in activity of the entire lysosomal complement of enzymes in a variety of nutritional and genetic muscular dystrophies may be explained on the basis of their role in cellular catabolism.

Nutritional muscular dystrophy in chicks occurs with a deficiency of Vit. E and sulfur amino acids, especially cystine(14). Dietary alterations in the level of fat, particularly unsaturated fatty acids, are known to influence the fatty acid pattern of the muscle tissues and Vit. E deficiency symptoms. The results

TABLE IV. Inhibitory Action of Dietary d-α-Tocopheryl Acetate on Tissue Peroxidation and Lysosomal Enzymes Induced by Linoleic Acid.

Added to basal diet, %	Muscular dystrophy Incidence*	Score (0-3)	TBA index†	Specific activity of indicator lysosomal enzyme β-glucuronidase‡
—	0/10	0	24.4	16.9
Linoleic acid, 1.0%	10/10	2.0	81.7	43.3
Idem + Vit. E,§ 5.0 mg/kg	0/10	0	26.7	19.9
" + " , 10.0 "	0/10	0	22.7	20.2

* Denominator shows total No. of chicks per treatment and numerator denotes chicks with muscular dystrophy.
† Expressed as absorbancy at 535 mμ per mg N × 10^3.
‡ Expressed as μmole substrate hydrolyzed per mg N per min × 10^4.
§ In form of d-α-tocopheryl acetate.

presented here (Table II) show a direct correlation between the symptoms of muscular dystrophy and the tissue peroxidizability (TBA index) as influenced by the dietary level of linoleic acid. These results are in accord with those of Century and Horwitt(15) that increased levels of unsaturated lipids in diets of Vit. E-deficient rats increased muscular dystrophy and creatine excretion.

Addition of d-α-tocopheryl acetate to the diet prevented muscular dystrophy, inhibited peroxidizability and decreased the lysosomal enzyme activities of the tissues (Table IV). This demonstrates an interrelationship between the damaging effect of peroxidizable linoleic acid in the tissue, the development of muscular dystrophy and the role of d-α-tocopheryl acetate in modifying this effect.

The evidence presented here demonstrates two events occurring during the development of the biochemical lesions and the gross dystrophy. These are induction of peroxidation as evidenced by the increased TBA values and increases in hydrolytic lysosomal enzymes. Increased peroxidizability of the tissues is accompanied by membrane susceptibility of subcellular particles, mitochondria (17,18) and lysosomes(19), to changes in permeability and lysis.

These findings of higher activities of lysosomal enzymes in nutritional muscular degeneration of chicks present biochemical evidence in accord with earlier histological observations of perivascular infiltration with "round cells"(1), since these probably are phagocytes and lymphocytes which are among the richest source of lysosomes. The accompanying tissue breakdown may be responsible for many biochemical changes, such as increased catabolism of protein and nucleic acids(20,21), increased excretion of urea and α-ketoglutarate in a variety of species(22) and increases in inorganic phosphates and phosphorus turnover of nutritionally dystrophic chicks(23). The increases in activities of the lysosomal hydrolases may be enhanced by increased phagocytic infiltration and by increased susceptibility of the lysosomes to lysis.

The results presented here support the hypothesis that nutritional muscular dystrophy in the chick is initiated by peroxidative tissue damage. It is a logical assumption that Vit. E prevents muscular dystrophy by protecting the cells from the initial peroxidative changes which allow the sequence of catabolic events to occur. The question remains, however, as to the biochemical mechanism responsible for protection of the muscle tissues from this degeneration, especially in regard to the role of the sulfur amino acids which have been shown to prevent dystrophy in the absence of Vit. E, and in respect to inorganic selenium compounds which have a marked sparing effect upon the amount of Vit. E required to prevent dystrophy. The results presented here indicate that it may be a special mechanism in which Vit. E, sulfur amino acids and selenium are interrelated in an overall system whose function is the prevention of initial peroxidation of cellular membranes such as those which contain the lysosomal enzymes.

Summary. Muscle tissues of chicks with nutritional muscular dystrophy produced by feeding a diet deficient in Vit. E and sulfur amino acids and stressed with excess dietary linoleic acid showed significant increases in peroxidation as evidenced by TBA index and also showed increased activities of the lysosomal enzymes. Addition of DL-methionine or d-α-tocopheryl acetate to the deficient diet prevented the dystrophy, reduced the peroxidation and reduced the lysosomal enzyme activities.

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1. Dam, H., Prange, I., Søndergaard, E., *Acta pathol. microbiol. scand.*, 1952, v31, 172.

2. Dam, H., Nielson, G. K., Prange, I., Søndergaard, E., *Nature,* 1958, v182, 802.

3. Nishida, T., Tsuchiyama, H., Inoue, M., Kummerow, F. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 308.

4. Zalkin, H., Tappel, A. L., Caldwell, K. A., Shibko, S., Desai, I. D., Holliday, T. A., *J. Biol. Chem.*, 1962, v237, 2678.

5. Tappel, A. L., Zalkin, H., Caldwell, K. A., Desai, I. D., Shibko, S., *Arch. Biochem. Biophys.*, 1962, v96, 340.

6. Scott, M. L., Calvert, C. C., *J. Nutrition,* 1962, v77, 105.

7. Calvert, C. C., Nesheim, M. C., Scott, M. L., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 16.

8. Gianetto, R., De Duve, C., *Biochem. J.,* 1955, v59, 433.

9. Sellinger, O. Z., Beaufay, H., Jacques, P., Doyen, A., De Duve, C., *ibid.,* 1960, v74, 450.

10. De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., Appelmans, F., *ibid.,* 1955, v60, 604.

11. Roy, A. B., *ibid.,* 1953, v53, 12.

12. Tappel, A. L., Zalkin, H., *Arch. Biochem. Biophys.,* 1959, v80, 326.

13. De Duve, C., *Symposium on Biological Approaches to Cancer Chemotherapy,* Louvain, June 1960, Academic Press, Inc., London, p101.

14. Scott, M. L., *Vitamins and Hormones,* Academic Press, Inc., New York, 1962, v20, 621.

15. Century, B., Horwitt, M. K., *J. Nutrition,* 1960, v72, 357.

16. Horwitt, M. K., *Vitamins and Hormones,* Academic Press, Inc., New York, 1962, v20, 541.

17. Hoffsten, P. E., Hunter, F. E., Gebicki, J. M., Weinstein, J., *Biochem. Biophys. Res. Comm.,* 1962, v7, 276.

18. Century, B., Horwitt, M. K., *J. Nutrition,* 1963, v80, 145.

19. Desai, I. D., Sawant, P. L., Tappel, A. L., *Biochim. et Biophys. Acta,* in press.

20. Coleman, D. L., Ashworth, M. E., *Am. J. Physiol.,* 1959, v197, 839.

21. Kruh, J., Dreyfus, J., Schapira, G., Gey, G. O., *J. Clin. Invest.,* 1960, v39, 1180.

22. McGaughy, C., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 730.

23. Calvert, C. C., Monroe, R. A., Scott, M. L., *J. Nutrition,* 1961, v73, 355.

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Carbohydrate Metabolism in *Pasteurella multocida.* I. Mechanism of Glucose Oxidation.* (28943)

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In the genus *Pasteurella,* studies on carbohydrate metabolism have been concerned mostly with *Past. pestis.* Resting cells of *Past. pestis* (strain Tjiwidej) oxidize glucose through the combined action of the Embden-Meyerhof pathway and the Krebs cycle(1,2) while proliferating cells oxidize glucose almost exclusively through the pentose pathway(3). The present study deals with the mechanism of carbohydrate oxidation in *Past. multocida.* This organism oxidizes easily hexoses, pentoses and Krebs cycle intermediates(4,5) and the operation of the pentose pathway is suggested by the presence of aldoheptose in the cellular lipopolysaccharide (6). A preliminary account has been published(7).

Materials and methods. Suspensions of *Past. multocida* (resting or proliferating) were obtained as described previously(8). *Preparations of cell-free extracts.* The acetone-dried(9) bacteria (5 g) were homogenized with 30 ml 92 mM K_2HPO_4. The suspension was left at 22-25° (room temperature) for 2 hours and then at 3° for 24 hours. The pH was maintained at 7.0 ± 0.5 by addition of 0.1 N NH_4OH. The suspension was then centrifuged at 25000 *g* and the sediment resuspended in 10 ml 90 mM pH 7.0 phosphate buffer, kept at 3° for 24 hours and centrifuged as above. Supernatants of the first and second centrifugation were joined and kept at —18°. Protein concentration was measured at 280 mμ(10).

Reagents. Xylulose and ribulose were a gift of Prof. M. Calvin; glucose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate, Polidase Type S and DL-glyceraldehyde-3-phosphate(diethylacetal) from Schwarz Bio-Research Inc.; glucose-1-phosphate, DL-

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