Proteolytic Activity During Growth of Hypertrophic and Atrophic Muscles of Genetically Dystrophic Chickens[1] (36932)

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Elevated proteolytic activity occurs in muscle in pathological conditions characterized by variable degrees of muscle wasting such as the hereditary muscular dystrophies in the mouse (1, 2) and the chicken (1, 3), in muscular dystrophy in man (4) and in muscular dystrophy of vitamin E deficiency in the rabbit (5–7).

Tappel *et al.* (1) found concomitant elevation of activities of several hydrolytic enzymes associated with lysosomes to occur in dystrophic chicken muscles and proposed that increased lysosomal enzyme activity is responsible for muscle wasting. Since lysosomes have not been morphologically demonstrated in muscle they also proposed that the increased lysosomal activity might be due to invasion of macrophages. However it has been found that phagocytic activity in dystrophic chicken muscle did not correspond to degree of muscle atrophy at 6 wk of age (8), or at 17, 45, and 140 days of age (9). Canonico and Bird (10) employing zonal centrifugation of muscle homogenates have presented some evidence for the existence of two groups of lysosomal enzymes in rat muscle and suggested that one group arises in the muscle cells and the other in macrophages. Thus it is possible that the lysosomal hydrolytic enzymes originate in the muscle itself.

The dystrophic chickens studied by Tappel *et al.* (1) were 17 mo of age and presumably in an advanced stage of dystrophy. While it appears to be generally assumed that elevated levels of proteolytic activity are associated with net loss of muscle protein there is no clear-cut evidence in favor of this idea. In both normal and dystrophic chicken muscle proteolytic activity is elevated in the early rapid stage of growth, and while the activity level decreases with age in normal muscle it remains elevated in the dystrophic muscle (3, 11). In dystrophic chicken muscle the rate of protein synthesis (amino acid incorporation) is much greater than in normal muscle (11, 12). However, in some dystrophic chickens relative gross hypertrophy of the pectoralis muscle occurs and is persistent while in others an early relative gross atrophy occurs with replacement of muscle fibers by fat (13). It is not possible to reconcile these two opposing end results with the simple statements that hydrolytic enzyme activity is increased and rate of protein synthesis is increased in dystrophic muscle unless there are differences in rates of these two processes between the two types of dystrophic muscle. Weinstock and Lukacs (3) have proposed that acid hydrolytic enzymes are involved in both anabolic and catabolic functions and that net changes in muscle constituents would be a function of the balance between the synthetic mechanisms and the catabolic effects of the acid hydrolases. In denervated rabbit muscle cathepsin activity is increased in the stage of "mild atrophy" but is decreased in "severe atrophy" compared with the contralateral control muscle (6). This is in accord with the above ideas since presumably in the denervated muscle there is a cessation or decrease in protein synthesis. It should be pointed out that even in the dystrophic chicken muscle which is grossly atrophic the muscle continues to grow, albeit at a reduced rate compared with either normal or hypertrophic muscle (see

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Fig. 1 and Table I).

A suggestion as to the effect of relative level of proteolytic activity is seen in the experiments of Iodice, Leong and Weinstock (14) in which the rate of autolysis of chicken muscle extracts was increased by addition of purified cathepsin D. The specific substrate(s) for this enzyme is unknown.

Because of the variability in dystrophic chickens of the incidence of relative gross hypertrophy and relative gross atrophy it has been possible to select a line of dystrophic chicken in which relative gross atrophy and high accumulation of fat occurs in the pectoralis muscle at an early age (line 307), and other lines in which hypertrophy occurs and persists and accumulation of fat remains low (lines 304 and 308) (13, 15–18). In the latter two lines gross atrophy may not be evident until a relatively advanced age (2 or 3 yr) and frequently is not as severe as in the 307 line (19).

Because of the availability of these lines of dystrophic chickens this study was conducted to determine whether or not a relationship exists between muscle atrophy and hypertrophy and the levels of activity of cathepsins A, B, C and D.

Materials and Methods. Animals. The chickens used in these experiments were from University of California lines of New Hampshire chickens with hereditary muscular dystrophy which are as follows: line 308 which exhibits early and persistent gross hypertrophy of the pectoralis muscle and line 307 which exhibits early relative gross atrophy of the pectoralis with fatty infiltration. The birds used in these experiments were from the 11th and 12th generations of continuous selection. Control chickens were from a normal line of New Hampshire chickens (line 200).

Preparation of samples. Birds were killed by disarticulation of the cervical vertebrae. Pectoralis muscles were dissected out, weighed, put into heat sealable polyester pouches, immediately frozen in an alcohol–dry ice bath, and stored at about −18° until used.

Muscle homogenates. The entire pectoralis muscle from one side was homogenized in a Sorvall Omnimixer which was immersed in an

TABLE I. Weights and Total Nitrogen Content of Pectoral Muscles Used in Cathepsin D Assays.[a]

Age (wk)	200 (Normal)		308 (Hypertrophic)		307 (Atrophic)	
	Av wt muscle (g)	N in muscle (g)	Av wt muscle (g)	N in muscle (g)	Av wt muscle (g)	N in muscle (g)
1/7	0.22 ± 0.01	0.0039 ± 0.0002	0.18 ± 0.01	0.0035 ± 0.0001	0.17 ± 0.01	0.0031 ± 0.0002
1	1.55 ± 0.12	0.052 ± 0.004	1.01 ± 0.11	0.026 ± 0.003	0.96 ± 0.18	0.026 ± 0.004
2	2.86 ± 0.24	0.107 ± 0.008	4.41 ± 0.41	0.139 ± 0.014	2.45 ± 0.18	0.068 ± 0.008
4	8.89 ± 0.31	0.326 ± 0.013	12.0 ± 0.7	0.404 ± 0.030	7.37 ± 1.09	0.212 ± 0.037
6	18.3 ± 1.3	0.689 ± 0.049	22.6 ± 1.5	0.715 ± 0.058	6.15 ± 0.42	0.167 ± 0.021
8	32.8 ± 0.5	1.24 ± 0.02	39.7 ± 5.3	1.27 ± 0.14	19.4 ± 2.1	0.539 ± 0.058
10	53.2 ± 3.2	2.08 ± 0.12	54.5 ± 8.4	1.75 ± 0.23	30.4 ± 5.7	0.823 ± 0.167
12	73.9 ± 3.9	2.88 ± 0.15	100.7 ± 21.6	3.15 ± 0.64	41.5 ± 5.6	1.13 ± 0.17
14	88.2 ± 3.6	3.44 ± 0.14	119.3 ± 5.5	3.89 ± 0.18	45.5 ± 9.3	1.70 ± 0.30
16	94.4 ± 3.7	3.72 ± 0.14	109.5 ± 25.0	3.70 ± 0.80	52.9 ± 13.6	1.28 ± 0.36
18	100.8 ± 6.6	4.08 ± 0.30	116.6 ± 17.1	3.98 ± 0.52	68.8 ±12.7	1.85 ±0.33

[a] Data are for means and SEM. Each weight value is a mean for 5 *left* pectoral muscles except data for 1 day which is based on 100 muscles, and 1 wk which is based on 25 muscles. Nitrogen values at 1 day are for 5 composites of 20 muscles and those for 1 wk for 5 composites of 5 muscles. All other nitrogen values are means for 5 muscles. Similar data (not included) was obtained for muscles used in other assays.

ice bath. Samples of homogenate were taken and analyzed for total nitrogen by the Kjeldahl method and for water content by drying for 48 hr in a vacuum oven at 65°.

Muscle extracts. The muscle homogenate was extracted with 2% cold KCl solution as described by Martins and Whitaker (20). It was allowed to stand overnight in the refrigerator and centrifuged at 13,000*g* in a refrigerated centrifuge for 2 hr at 4°.

Cathepsin D. Measurement of activity was by the hemoglobin method as described by Martins and Whitaker (20).

Cathepsin A. Measurement of activity was by the method used by Iodice and Weinstock (21). The substrate was *N*-carbobenzoxy-α-L-glutamyl-L-phenylalanine. The reaction mixture containing 2.5 ml substrate, 1.5 ml acetate buffer, and 1 ml muscle extract was incubated 1 hr at 37°.

Cathepsin B. Measurement of activity was by method similar to the method used by Iodice, Leong and Weinstock (22). The substrate was benzoyl-L-arginine amide. The substrate mixture was prepared by adding 20 mg cysteine, 1.3 mg penicillin G, 20 mg streptomycin sulfate (23) to 7.5 ml 0.1 *M* substrate and diluting to 10 ml with 0.16 *M* citrate buffer. The reaction mixture containing 1 ml substrate mixture and 1 ml muscle extract was incubated 24 hr (25).

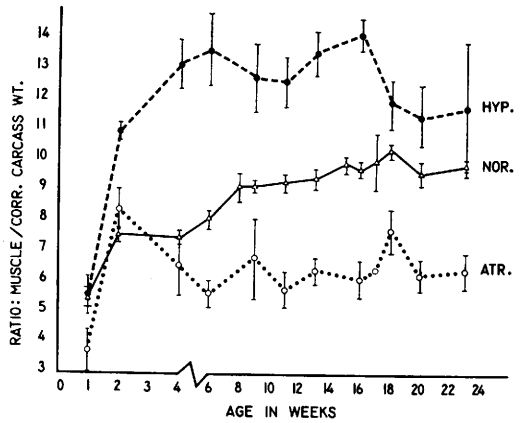

FIG. 1. Relative growth of normal, atrophic, and hypertrophic muscles. The ratio is that of the weight of 2 pectoralis muscles to the body weight minus weight of pectoralis muscles and viscera. Vertical lines represent SEM. Values are for 3 to 11 pairs of muscles except for 1 pair at 17 wk of age.

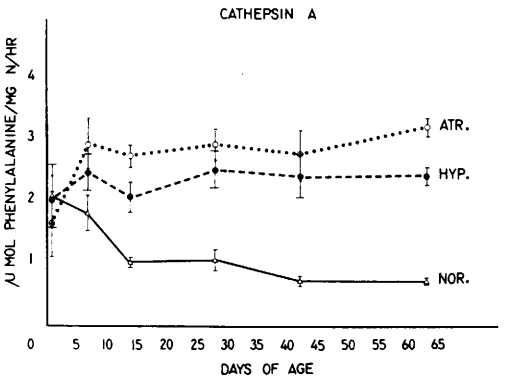

FIG. 2. Cathepsin A activity. The points are means ± SEM. The numbers of muscles in each mean is stated in footnote *a*, Table I.

Cathepsin C. The method for cathepsin C was the same as that for cathepsin B except that the substrate was glycyl-L-phenylalanine amide acetate (22).

All substrates were obtained from Schwartz/Mann, Orangeville, NY.

Units of enzyme activity. Enzyme activity is expressed per milligram of nitrogen of the whole muscle homogenate. This expression is justified since the percentage of nitrogen on a fat-free basis is constant from 31 to 140 days of age in both normal and dystrophic pectoral muscles but has different values for the 2 genotypes (13, 18). Our observations indicate that noncollagen protein nitrogen is a relatively constant proposition of total nitrogen (75–80%) in both normal and dystrophic muscles (18). However noncollagen protein of pectoral muscle increases from hatching up to about 4 wk of age in both normal (26) and dystrophic chicks (3). Therefore activities expressed here at these early ages would be somewhat higher if expressed on a protein basis.

Results. Relative growth of muscle. The relative growth of the three pectoralis muscles is shown in Fig. 1. This is shown as percentage ratio of the two pectoralis muscles to the "corrected carcass weight" (13). The latter is defined as the body weight minus the weights of both pectoralis muscles and viscera. This ratio is the same at 1 day of age for all three lines of chickens (not shown). By 2 wk of age (Fig. 1) the rate of growth of pectoralis muscles in the hypertrophic line

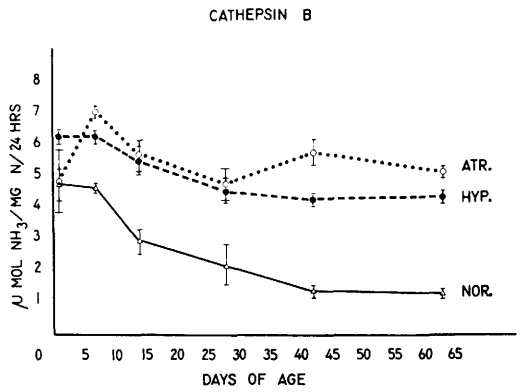

FIG. 3. Cathepsin B activity. Means ± SEM (see footnote *a*, Table I for number in mean).

of dystrophic birds (line 308) had greatly surpassed that of the other two lines. This relatively larger ratio was maintained in line 308 until 23 wk of age although by this time considerable variability had occurred.

The pectoralis muscles of the atrophic line (line 307) at first grew at the same rate as that of the normal birds but by 4 wk of age the growth rate had declined and remained below the normal thereafter. It can be seen from Table I however that these muscles continue to increase in size and total nitrogen content.

The data in Fig. 1 were obtained from muscles at autopsy collected over a period of 2 yr on birds of the three lines. Data for muscle weights and nitrogen contents of muscles actually used in the experiments reported are shown in Table I.

Cathepsin A. (Fig. 2). Activity of cathepsin A in normal pectoralis muscle declined after 1 day of age to less than half that of its initial activity by 6 wk of age. In both types of dystrophic muscle the cathepsin A activity increased and was maintained at a significantly higher level ($p < .01$) than in normal muscle from 14 to 63 days of age. While the mean values for cathepsin A activity were higher in atrophic muscle than in hypertrophic muscle from 7 days onward these differences could only be shown to be significant at 14 days ($p < .05$) and 63 days ($p < .001$). According to Iodice, Leong and Weinstock (22) these differences in cathepsin A activity represent a true difference in quantity since isolated enzymes from both normal and dystrophic muscle showed the same activity.

Cathepsin B (Fig. 3). Cathepsin B activity also declined with growth in the normal muscle but remained significantly higher in the dystrophic muscles than in the normal muscles from 7 days onward (p range $< .02$ to $< .001$). Again the trend was for activity to be higher in the atrophic than in the hypertrophic muscle but these differences were only shown to be significant at 6 wk ($p < .01$) and 9 weeks of age ($p < .005$).

Cathepsin C (Fig. 4). The cathepsin C activity of normal muscle showed the same downward trend with age as with cathepsins A and B and differed significantly from the dystrophic muscles (p range $< .05$ to $< .001$) from 7 days onward. While the mean activity of atrophic muscle was higher at all points but one, only 3 out of 5 of these showed significant differences.

Cathepsin D (Fig. 5). Because cathepsin D activity showed highly consistent trends and significant differences in muscle from the three different lines of birds this study was expanded to include muscles at two week age intervals up to 18 wk of age. Analysis of variance indicates that all three lines differed significantly from each other (Table II). In the dystrophic muscles the cathepsin D activity remained consistently higher in the atrophic muscle than in the hypertrophic muscle. In normal muscle the level fell rapidly from

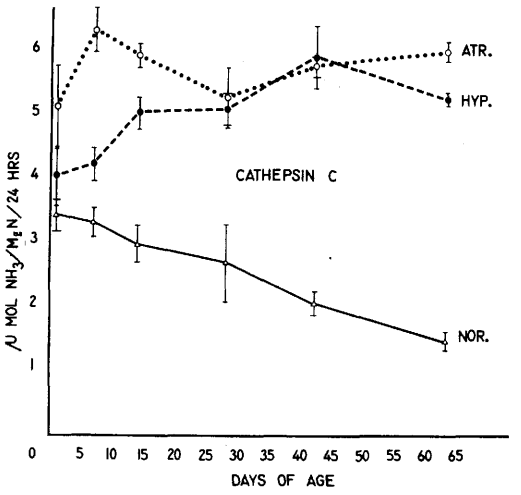

FIG. 4. Cathepsin C activity. Means ± SEM (see footnote *a*, Table I for number in mean).

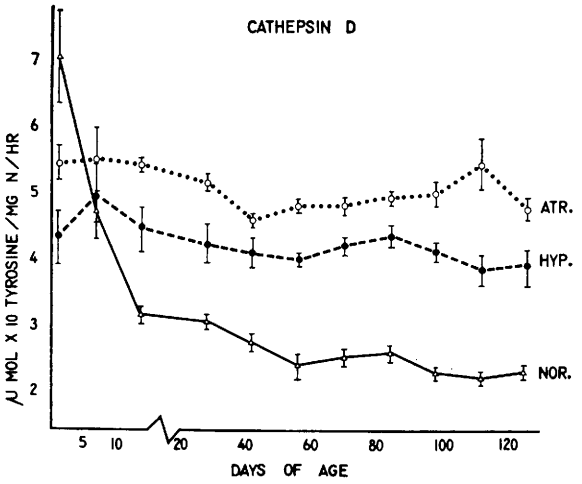

FIG. 5. Cathepsin D activity. Means ± SEM (see footnote *a*, Table I for number in mean).

1 day to 14 days of age and tended to level out thereafter.

Discussion. It is evident from the data presented here that elevated proteolytic activity in muscle cannot be related specifically to net muscle wasting or atrophy. In the normal pectoral muscle the highest levels of proteolytic activity are found in the early stage of growth when muscle weight is increasing most rapidly relative to body weight. Proteolytic activity declines as relative growth rate of the muscle slows down. The activities of the four cathepsins are also greatly elevated in the hypertrophic dystrophic muscle which is growing more rapidly than normal muscle. This would indicate that anabolic processes are proceeding at a more rapid rate than catabolic processes and that in agreement with the idea expressed by Weinstock and Lukacs (3) and Weinstock (11) proteolytic enzymes may have some function in preventing rapid changes in muscle composition. In the dystrophic muscle which shows relative gross atrophy, however, the cathepsin D activity is significantly increased over that in the hypertrophic muscle. This appears to relate to slowing down of the growth of the muscle and actual atrophy. This would seem to agree with the *in vitro* data of Iodice, Leong and Weinstock (14) which showed that the rate of autolysis of muscle extracts could be increased by adding purified muscle cathepsin D.

TABLE II. Analysis of Variance of Cathepsin D Activity.

	Mean square	*F*	*p*
Line	.66115	456	<.001
Age	.00682		
Line × age	.00258	1.78	<.05
Error	.00145		

What is here represented as cathepsin D may actually be the combined activities of cathepsin A and D since while purified cathepsin A does not attack hemoglobin it can apparently attack breakdown products of hemoglobin (22). The increased levels of activities of cathepsins A and D observed in dystrophic muscles are probably due to increased levels of enzyme since the properties of these enzymes isolated from normal muscle appear to be identical with those isolated from dystrophic muscle (22).

In agreement with statements made by Iodice, Leong and Weinstock (22) cathepsins B and C have a low order of activity as evidenced by the 24 hr incubation time necessary to demonstrate differences between normal and dystrophic muscle.

The specific substrates for the cathepsins in muscle have not been elucidated. Because the protein content and distribution of proteins in the dystrophic pectoral muscle differ from that of the normal pectoral it has been speculated that there is differential loss of

proteins from the muscle fibers (12, 27). In the dystrophic pectoral muscle the protein concentration is less than in the normal muscle (3, 25) and the ratio of sarcoplasmic to myofibrillar proteins is also less than in the normal muscle (27, 28). In this respect dystrophic pectoral muscle is very similar to immature pectoral muscle and resembles normal red chicken leg muscle (29). Two separate hypotheses have been advanced to explain the characteristics of dystrophic pectoralis muscle: (a) that red muscle fibers fail to undergo the normal maturation into white fibers (11, 12, 25, 30) and (b) using terminology suggested by Ashmore and Doerr (31) that, subsequent to transformation of α-red fibers into α-white fibers, degeneration of the α-white fibers occurs. Neither of these hypotheses adequately explains the fact that some dystrophic muscles atrophy rapidly and others do not.

Since atrophic dystrophic muscle contains quite variable amounts of fat its protein content on a percentage basis appears to be low and variable. However when atrophic and hypertrophic muscles from 19 wk old dystrophic chickens were compared on a fat-free basis the percent of noncollagen protein nitrogen was found to be identical (25). It seems probable on a compositional basis that when loss of protein occurs it is due to loss of entire fibers and not due to differential loss of specific protein fractions. This aspect of dystrophic muscle composition has been little studied and needs to be examined in detail in both types of dystrophic muscle over a wide age range.

Summary. The activities of cathepsins A, B, C, and D were assayed during growth of pectoralis muscles from normal chicks and from two lines of chicks with genetic muscular dystrophy. One dystrophic line was characterized by persistent hypertrophy of the pectoralis and the other by early atrophy of this muscle. At 1 day of age activities of all cathepsins were high in both normal and dystrophic muscle. They declined rapidly in normal muscle but remained elevated in both types of dystrophic muscles. Cathepsin D activity was significantly higher in atrophic muscle compared to hypertrophic muscle at all ages studied. Cathepsins A, B and C were not greatly different in activity between the muscles of the two dystrophic lines. These data suggest that relative level of cathepsin D activity is related to retarded growth and degeneration of the atrophic muscle.

1. Tappel, A. L., Zalkin, H., Caldwell, K. A., Desai, I. D., and Shibko, S., Arch. Biochem. Biophys. **96**, 340 (1962).

2. Weinstock, I. M., Epstein, S., and Milhorat, A., Proc. Soc. Exp. Biol. Med. **99**, 272 (1958).

3. Weinstock, I. M., and Lukacs, M., Enzymol. Biol. Clin. **5**, 103 (1965).

4. Pennington, R. J., and Robinson, J. E., Enzymol. Biol. Clin. **9**, 175 (1968).

5. Weinstock, I. M., Goldrich, A. D., and Milhorat, A. T., Proc. Soc. Exp. Biol. Med. **88**, 257 (1955).

6. Weinstock, I. M., and Lukacs, M., Enzymol. Biol. Clin. **5**, 89 (1965).

7. Zalkin, H., Tappel, A. L., Caldwell, K. A., Shibko, S., Desai, I. D., and Holliday, T. A., J. Biol. Chem. **237**, 2678 (1962).

8. McMurtry, S. L., Julian, L. M., and Asmundson, V. S., Arch. Pathol. **94**, 217 (1972).

9. Holliday, T. A., Julian, L. M., and Asmundson, V. S., unpublished data.

10. Canonico, P. G., and Bird, J. W. C., J. Cell Biol. **45**, 321 (1970).

11. Weinstock, I. M., Ann. N.Y. Acad. Sci. **138**, 1, 199 (1966).

12. Weinstock, I. M., Soh, T. S., Freedman, H. A., and Cutler, M. E., Biochem. Med. **2**, 345 (1969).

13. Holliday, T. A., Julian, L. M., and Asmundson, V. S., Anat. Rec. **160**, 207 (1968).

14. Iodice, A. A., Leong, V., and Weinstock, I. M., Enzymol. Biol. Clin. **6**, 269 (1966).

15. Asmundson, V. S., Kratzer, F. H., and Julian, L. M., Ann. N.Y. Acad. Sci. **138** (Art. 1), 49 (1966).

16. Asmundson, V. S., Julian, L. M., and Jordan, J. P., Can. J. Genet. Cytol. **3**, 313 (1961).

17. Jordan, J. P., Kratzer, F. H., and Zarghami, N. S., Proc. Soc. Exp. Biol. Med. **116**, 243 (1964).

18. Peterson, D. W., Hamilton, W. H., and Lilyblade, A. L., J. Nutr. **101**, 453 (1971).

19. Julian, L. M., and Asmundson, V. S., *in* "Muscular Dystrophy in Man and Animals" (G. H. Bourne and Ma. N. Golarz, eds.), Chap. 1, p. 1. Karger, Basel (1963).

20. Martins, C. B., and Whitaker, J. R., J. Food Sci. **33**, 59 (1968).

21. Iodice, A. A., and Weinstock, I. M., Nature (London) **207**, 1102 (1965).

22. Iodice, A. A., Leong, V., and Weinstock, I. M.,

Arch. Biochem. Biophys. **117**, 477 (1966).

23. Ali, S. Y., Biochem. J. **102**, 10C (1967).

24. Ali, S. Y., and Evans, L., Biochem. J. **112**, 427 (1969).

25. Peterson, D. W., Hamilton, W. H., and Lilyblade, A. L., Proc. Soc. Exp. Biol. Med. **127**, 300 (1968).

26. Dickerson, J. W. T., Biochem. J. **75**, 33 (1960).

27. Barany, M., Gaetjens, E., and Barany, K., Ann. N.Y. Acad. Sci. **138**, (Art. 1), 360 (1966).

28. Brown, S. J., MS thesis. University of California, Berkeley, 1967.

29. Khan, A. W., J. Food Sci. **27**, 431 (1962).

30. Cosmos, G., and Butler, J., *in* "Exploratory Concepts in Muscular Dystrophy and Related Disorders" (A. T. Milhorat, ed.), p. 197. Excerpta Medica, Amsterdam (1967).

31. Ashmore, C. R., and Doerr, L., Proc. Soc. Exp. Biol. Med. **137**, 1066 (1971).

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