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Comparison of Muscle Tissue from Normal and Dystrophic Chick at Different Stages of Development.* (30445)

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Explants of pectoralis muscle from hatched dystrophic chick show a greater deterioration of histological structure and a more pronounced spreading of cells than similar explants from normal chick muscle of the same age(1). These results, as well as biochemical analyses by Kaplan and associates(2), Cosmos(3) and Perkoff(4), indicate that in dystrophic chick muscle maturation is delayed. In the experiments reported here, it was intended to define further the beginning of this apparent developmental retardation in dystrophic muscle. Since the DNA level has been found to be higher in muscle of newly hatched dystrophic chick than in that of normal animals(5), determinations of the DNA content were carried out in the normal and dystrophic muscle at different stages of development. As a second index of the state of maturation, the rates of amino acid incorporation into muscle proteins were measured *in vitro* using muscle preparations immediately after dissection of the tissue or after explantation of muscle for several days.

Materials and methods. The origin of the normal and genetically dystrophic chick muscle tissue and the explantation technique were described previously(1). Only pectoralis muscle was used in the present tissue culture experiments. An explantation period of 5 days was chosen because only small changes occur in the measured incorporation on further *in vitro* maintenance up to 11 days. For the measurement of amino acid incorporation about 7 to 10 mg of a mince or of a single bundle of fibers (strap) were incubated in a Dubnoff shaker for 2 hours in 10 ml beakers with 1 ml of a tissue culture medium(6) from which glycine-C^{12} was omitted, adding 1 μC of glycine-1-C^{14} (Spec. Act. 5.49 mc/mM) to each sample. The freshly dissected material was suspended in the incubation medium. The tissue incubated with glycine-1-C^{14} after explantation remained attached to the coverslips on which it was explanted(1). The explants were washed before adding glycine-1-C^{14} by 2 ten-minute incubations in a Dubnoff shaker with 2 ml aliquots of the glycine-free medium in order to remove glycine present in serum. After incubation, soluble material was removed by 2 washings with cold 5% trichloracetic acid and one washing with hot alcohol ether (1:1).

The DNA was extracted with hot perchloric acid and its quantity determined according to the method of Burton(7). The residue was collected in 3 ml centrifuge tubes and 0.2 ml each of HCl and acetic acid were added. The centrifuge tubes were capped and kept at 95°. After 5 hours the acid mixture was driven off with jets of air while maintaining the tubes at about 110°C. The now easily soluble dry residue was taken up in water, plated on aluminum planchets, and

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TABLE I. Contents of DNA and Protein and Incorporation of Glycine-1-C^{14} into Protein in Mince of Pectoralis Muscle from Normal and Dystrophic Chick at Different Stages of Development.

Day		DNA, μg/mg	PN, μg/mg	PN/DNA	Cts/μg DNA	Cts/μg PN	Cts/mg FW
11 E	N (5)	2.1 ± .1	3.7 ± .1	1.8 ± .1	419.3 ± 49.7	228.8 ± 22.6	842.3 ± 87.8
16 E	N (6)	2.8 ± .04	5.7 ± .2	2.0 ± .1	131.9 ± 13.1	87.8 ± 19.7	368.4 ± 34.4
	D (6)	2.8 ± .1	5.8 ± .2	2.1 ± .1	134.5 ± 20.3	79.6 ± 22.4	374.1 ± 50.5
18 E	N (11)	3.1 ± .1	7.6 ± .3	2.5 ± .1	76.8 ± 5.8	29.9 ± 3.2	237.3 ± 15.1
	D (7)	2.7 ± .1	7.1 ± .5	2.6 ± .1	92.6 ± 11.0	32.8 ± 5.2	250.7 ± 31.6
21 E	N (5)	3.0 ± .2	8.9 ± .5	3.0 ± .1	66.1 ± 5.8	22.0 ± 1.3	194.5 ± 14.9
	D (5)	3.4 ± .1	10.0 ± .4	3.0 ± .1	65.7 ± 10.4	22.0 ± 3.1	216.6 ± 27.0
2 H	N (10)	2.4 ± .1	8.6 ± .4	3.6 ± .2	94.6 ± 4.1	27.3 ± 1.6	230.0 ± 13.3
	D (10)	3.4 ± .2*	8.8 ± .4	2.6 ± .1*	62.1 ± 7.1*	23.4 ± 2.3	203.2 ± 16.1
4 H	N (5)	2.6 ± .1	9.7 ± .4	3.8 ± .3	83.1 ± 11.1	21.4 ± 1.1	210.4 ± 16.9
	D (5)	3.8 ± .4*	10.6 ± .8	3.0 ± .4	66.2 ± 13.3	25.5 ± 2.9	268.4 ± 33.3
7 H	N (8)	2.0 ± .1	14.1 ± .8	7.5 ± .8	49.2 ± 6.1	7.3 ± 1.3	97.6 ± 13.8
	D (9)	2.7 ± .2*	12.2 ± .8	4.8 ± .5*	64.2 ± 7.1	15.1 ± 2.3*	175.4 ± 23.1*
11 H	N (34)	1.2 ± .1	17.8 ± .5	16.6 ± .9	31.5 ± 2.7	2.1 ± .2	35.9 ± 2.8
	D (28)	1.8 ± .1*	14.7 ± .4*	8.6 ± .5*	60.0 ± 4.4*	9.1 ± 1.5*	105.5 ± 7.7*

Numbers in parentheses indicate triplicate analysis on tissue from individual chick for 18 (E) to 11 (H). At 11 (E) tissue from 3 embryos and at 16 (E) tissue from 2 embryos were pooled per sample.

* Indicates significant differences ($p < .05$) between normal and dystrophic muscle.

E: days of embryonic development; H: days of post-hatching development; N: normal muscle; D: dystrophic muscle; PN: protein nitrogen; FW: freshweight; Cts: counts/min.

All tabulated values shown are means ± standard errors in Table I and II.

counted in a Nuclear Chicago counter (D47) with micromil window. PN was determined by Nesslerization.

Results. The DNA contents were similar in muscle from normal and dystrophic chicks (Table I), both increasing slightly from the 16th to the 21st day of development (E).‡ Immediately after hatching (21 days of development) the DNA content of the normal muscle declined while the DNA level of the dystrophic muscle stayed at a high level until 4 days (H). After the 4th day (H) the DNA content of the dystrophic muscle began to decline but a difference of about 1 μg of DNA per mg of muscle was maintained up to 11 days (H) of development.

The protein content of the normal muscle tissue increased sharply during 11-18 days (E) and 4-11 days (H) of development. Values for the dystrophic muscle were similar to those of normal muscle from the 16th day (E) to 4 days (H), becoming slightly but significantly lower than normal muscle by the 11 day (H).

‡ (E) and (H) indicate days of pre-hatching and post-hatching development respectively.

Incorporation rates were calculated as counts per mg freshweight, per μg protein nitrogen and per μg DNA. Incorporation of the labeled glycine followed a linear course during the 2-hour period of incubation. In a tissue-like developing muscle in which the proportion of cell constituents changes, an interpretation of incorporation data is facilitated by the calculation for all 3 parameters. From Table I it can be seen that in the normal muscle the incorporation rate declined sharply from the 11th to the 18th day (E) for each form of calculation. From the 18th day (E) until 4 days (H) there was not much change in the rates of incorporation calculated for freshweight and protein nitrogen. From the second to the fourth day (H), significantly higher values were observed for the counts per DNA only in normal muscle in which a drop of the DNA level occurred. Incorporation rates per freshweight or per PN did not follow this drop in DNA content and were at this phase independent of the DNA content per freshweight. After the fourth day post-hatching incorporation rates dropped in both muscle types when calcu-

TABLE II. DNA and Protein Contents and Incorporation of Glycine-1-C^{14} into Straps and Mince of Pectoralis Muscle from Normal and Dystrophic 11-Day Hatched Chick. Measurements were made immediately after dissection of tissue (0 time) and after explantation of tissue preparations for 5 days.

Straps		DNA, μg/mg	PN, μg/mg	PN/DNA	Cts/μg DNA	Cts/μg PN	Cts/mg FW
0 Time	N (13)	1.4 ±.1	17.4 ± .5	13.4 ± 1.1	43.7 ± 7.4	3.2 ± .3*	54.8 ± 5.3*
	D (12)	2.1 ±.14	14.6 ± .6	7.4 ± .6	71.4 ± 11.2	9.5 ± 1.0	133.7 ± 13.4
5 Day explant	N (9)	.5 ±.03*	10.9 ± .4	23.9 ± 1.4	469.4 ± 47.3	20.3 ± 1.5*	218.3 ± 17.1*
	D (9)	.8 ±.08	8.3 ± .8	11.8 ± 2.0	598.3 ± 46.0*	64.5 ± 9.8	485.2 ± .9
Mince							
0 Time	N (34)	1.2 ±.1	17.8 ± .5	16.6 ± .9	31.5 ± 2.7	2.1 ± .2*	35.9 ± 2.8*
	D (28)	1.8 ±.1	14.7 ± .4	8.6 ± .5	60.0 ± 4.4	9.1 ± 1.5	105.5 ± 7.7
5 Day explant	N (16)	.2 ±.04*	10.6 ± .2	51.3 ± 7.2	433.4 ± 40.7	11.2 ± 1.8*	115.0 ± 18.5*
	D (15)	.6 ±.06	7.2 ±1.2	16.8 ± 2.3	782.3 ± 52.4*	57.0 ± 6.2	453.8 ± 43.8

* Significant difference between straps and mince ($p < .05$).
See Table I for other symbols.
Explants were maintained in synthetic medium(6) with 5% horse serum.

lated for the 3 bases of reference but the decline in dystrophic muscle was slower than in normal tissue.

Comparison of incorporation rates of glycine into proteins after *in vitro* culture for 5 days was carried out with 11 day (H) normal and dystrophic muscle which were explanted as mince or straps. Comparison of the values given for the beginning and the end of the explantation period (Table II) shows that DNA and protein were lost from the explants. Loss of DNA was greater than loss of protein. Also, in proportion to the initial values the loss of DNA was somewhat greater in normal than in dystrophic muscle explants and more pronounced in the minced samples than in the straps. Such differences were not apparent for the protein values.

At the beginning of the explantation period the dystrophic muscle showed a higher rate of incorporation than the normal muscle (Table II). In both muscle types a marked increase in incorporation rate occurred during the 5-day period of culture *in vitro*, and at the end of explantation the incorporation values for dystrophic muscle were still at a significantly higher level than for normal muscle. This held when calculated for all 3 parameters in the case of the minced explants, while the difference between normal and dystrophic muscle straps was not statistically significant when calculated as cts/μg DNA. As pointed out before, a somewhat greater decline occurred in DNA content of the normal than in that of the dystrophic explants which led to a relatively greater increase in the counts per DNA for the normal muscle and hence to a smaller difference between the values for the normal and dystrophic tissue.

Discussion. An increased DNA content has been observed in dystrophic chick and human muscle(5), in muscle from mice with hereditary dystrophy(8,9) and in rabbit muscle from tocopherol-deficient animals(9,10). The present data indicate that during embryonic development the measured parameters are similar in normal and dystrophic muscle up to the 18th day of development, but after this stage of development the DNA content of normal muscle is lower than in dystrophic muscle. The decline in DNA content which begins in normal muscle immediately after hatching is delayed in the dystrophic muscle for 4 days. At the time of hatching the chicks used in these experiments, which were bred for early onset of abnormal symptoms, showed muscular weakness in the flip tests. The number of nuclei in the muscle fibers was increased, but otherwise the muscle fibers looked normal in the light microscope and there was no indication of cellular infiltration.

The decline in amino acid incorporation into *in vitro* preparations of normal muscle begins 4 days after hatching. The dystrophic muscle maintains a high rate of incorporation for a longer period. Values for muscle from 11-day (H) old dystrophic chicks are similar to those for muscle from 7-day (H)

old normal chicks. Protein synthesis shows embryonic characteristics also in explants. When muscle from 11-day (H) chick is explanted for 5 days the amino acid incorporation increases sharply in strap and minced explants from both the normal and dystrophic muscle but reaches a higher value in the explants from the dystrophic muscle, similar to that of explants from embryonic muscle. A more rapid protein turnover in proteins from dystrophic muscle has been previously observed(11,12,13). In view of the observation of a more effective amino acid transport system in embryonic(14) and in dystrophic muscle(15,16) it remains undecided to what extent the elevated values for amino acid incorporation in embryonic and in dystrophic muscle are due to this more rapid accumulation of tracer amino acids in the pool or to the more rapid utilization of amino acids in peptide synthesis.

The data presented here corroborate earlier findings cited above which indicate a retardation of muscle maturation in dystrophic chick. Evidence is increasing that the dystrophic muscle cell retains abnormal characteristics under *in vitro* conditions and therefore the defect leading to dystrophy resides most likely within the muscle cell itself rather than in an insufficiency of the extramuscular controls, such as hormones. It has been pointed out before(1) that the immature state is perhaps only a concomitant, and certainly not a sufficient, condition for development of the dystrophic state. Nevertheless, the possibility exists that both retarded maturation and dystrophy are expressions of the same defect of the muscle cell. Therefore, mechanisms of muscle maturation may be pertinent to the problem of dystrophy as well. In this connection it should be pointed out that persistence of DNA accumulation in the developing leg muscle of the chick at 16 days (E) was found to result from the inactivation of the thyroid(17) and pituitary(18). An effect on muscle development by steroid hormones has been demonstrated(19), and several steroids have been found to alleviate the dystrophic condition in mice(20). A marked increase in amino acid incorporation into minced frog muscle of developing limbs from *Rana catesbiana* occurs in response to thyroxin(21).

The extensive cell death and the marked stimulation of amino acid incorporation in the explanted muscle show that the condition of the explants used in the present experiments limits a comparison between explants and intact tissue. In this connection it may be noteworthy that the differences between the mince and strap preparations in respect to loss of DNA and amino acid uptake at zero time and after 5 days of explantation are, even where significant, relatively small. This must mean that whatever changes are taking place in these tissue preparations cannot be the result of the more far reaching mechanical injury in the minced tissue. The nature of the cell alterations which lead to the loss of DNA and the increase in amino acid incorporation is obscure. Therefore, conditions will be explored for *in vitro* maintenance of the muscle cultures in which loss of DNA and stimulation of protein synthesis are suppressed.

Summary. 1. Evidence is presented to support the view that dystrophic muscle is retarded in its post-hatching maturation. DNA, PN, and incorporation levels were determined in developing chick normal and dystrophic pectoralis. By 11 days post-hatching, dystrophic muscle has a higher DNA content, greater incorporation of glycine-1-C^{14} into protein, and a lower PN content corresponding to the levels found in younger normal muscle. 2. After 5 days *in vitro,* normal and dystrophic muscle explants from 11-day hatched chick lost DNA and PN. In addition, the rate of protein synthesis was increased. Again, in explants the dystrophic muscle showed properties similar to those of normal muscle of a younger age.

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Spontaneous Leukemia in Germfree AK Mice.* (30446)

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The strain AK mouse was developed, by selective breeding, for high incidence of spontaneous leukemia(1); most of them usually die of lymphatic leukemia by 12 months of age. The nature of the "trigger" which initiates the leukemic disease is unknown; however, a virus has been clearly incriminated in pathogenesis of the disease. A virus recovered from leukemic AK mice has been adapted by passages to other strains of mice and to rats, and is designated Gross strain A leukemia virus(2). The adapted virus induces lymphatic leukemia in mouse strains which under normal circumstances have a low incidence of spontaneous leukemia. Mice, as well as rats, are most receptive to the virus if they are inoculated shortly after birth.

Lymphatic leukemia has been induced by X-irradiation in significant numbers of 6 strains of germfree and of conventional counterpart mice(3,4). They included Swiss-Webster, ICR, C3H, C57 BL, CFW, and Balb/C strains. The incidence of induced leukemia is highest in the Swiss-Webster and lowest in the Balb/C mouse strains. The lesions observed in them were basically the same: enlarged thymus and lymph nodes, and lesser numbers of them with lymphoblastic infiltration of visceral organs and blood. Virus-like inclusions were noted in and around thymus cells of the "normal" and of the leukemic germfree mice by electron microscopy. On this basis, these mice are designated "germfree."

Leukemogenic virus is thought to be disseminated to progeny AK mice by congenital route(s), where it rests in quiescent but observable condition until the spontaneous disease appears. It is likely that a defect develops in the normal homeostatic "cell control system," thereby permitting the disease to become manifest. The biochemical mechanism whereby spontaneous leukemia is initiated and is evolved in AK mice is unknown.

The observations presented here concern spontaneous leukemia in germfree AK mice.

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