Observations on Culture *in vitro* of Normal and Dystrophic Muscle Tissue.*† (26059)

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Efforts to demonstrate reproducible differences *in vitro* in growth patterns of cells from normal and dystrophic human tissue and thus to characterize the dystrophic condition have remained discouraging over the last 5 years of this study. As reported previously(1,2), some of the measured growth parameters were either widely scattered or markedly inconsistent. Other differences, *e.g.*, in DNA contents, which were more consistent were not necessarily characteristic for the dystrophic state of the muscle. Reports by other investigators(3,4) of pathologically significant differences could not be confirmed. Better defined and more uniform tissue samples were obtained from a strain of chicks with hereditary muscular dystrophy, primarily of the breast muscles(5). Quantitative differences in growth pattern of normal and dystrophic chick muscle cells in media with sub-optimal embryo extract concentrations were observed. These differences are statistically significant and may be related to the pathological state of the tissue. The data with chick muscle explants and a brief summary of the older experiments with human muscle are reported here.

Materials and methods. Chick muscle: The chicks, obtained from the flock at Univ. of California, Davis,(5) during the first week after hatching, were raised at our laboratory to an age of 7 weeks. Dystrophic manifestations (inability to stand up when put on back) became apparent during the third week after hatching. The animals were sacrificed at age intervals of 2 weeks by decapitation under ether anesthesia and hypothermia. Pectoral muscles were dissected and minced, 5-10 mg quantities, weighed on a "desicoted" pan, were transferred into roller tubes on a "desicoted" glass spoon and imbedded in a clot composed of .25 ml chicken plasma diluted 1:6 with Pannett-Compton saline(6) and 0.025 ml thrombin diluted 1:4. The nutritional media consisted of 40% horse serum, either 5% or 2.5% embryo extract and 20% synthetic medium (SM, 7) made up, to volume, with Pannett and Compton solution containing 100 mg % glucose, and penicillin and streptomyocin at a concentration of 100 units/ml and 100 μg/ml respectively. Embryo extract was prepared from 12-day chick embryos. Cultures were fed 3 times a week.

Protein nitrogen (PN) and deoxyribonucleic acid (DNA) determinations were made over a 3 week period. DNA was estimated by the Burton method(8). PN values, obtained by Nesslerization, were corrected for PN of the plasma.

Human muscle: A small piece (about 1 cm^3) of gastrocnemius was secured from dystrophic patients with the diagnosis of pseudo hypertrophic dystrophy made by the Pathology Department after examination of biopsies. Normal human skeletal muscle was obtained from patients not afflicted with muscular dystrophy.

Viability tests with human biopsies: The muscle was freed of connective tissue and cut into pieces of about 1 mm^3. Six to 8 pieces were imbedded in a single row in a clot of chick plasma in a roller tube. The medium was composed of 40% human serum, fetal pig brain or muscle extract, and other constituents made up as for the chick medium. The

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TABLE I. Deoxyribonucleic Acid (DNA) Content of Muscle Explants Expressed as μg DNA/mg of Original Wet Weight of Explants.

Each figure is mean of separate determinations on 6 cultures. N = Normal, D = Dystrophic, EE = Embryo extract.

Period *in vitro*	N	D	N	D	N	D	N	D
Series I				Age of donor chick (days)				
5% EE		4–5		14		21		42
0	1.8	1.6	.4	1.1	.4	.6	.3	.7
2	1.0	1.0	.3	.6	.2	.3	.1	.3
7	3.4	5.6		2.3	.6	1.5	.7	1.3
14	5.6	8.0	3.3	3.1	1.4	3.0	1.7	2.1
Series IIa				Age of donor chick (days)				
5% EE		7		21		35		49
0	.7	2.1	.4	1.2	.4	1.1	.4	.7
	.7	2.0	.4	1.1	.3	.7	.3	.7
2	.5	.9	.2	.3	.1	.4	.2	.3
	.4	.6	.2	.3	.2	.4	.1	.2
7	1.2	2.7	.2	.9	.3	1.4	.6	1.9
	1.5	2.5	.4	1.3	.4	1.2	.5	1.1
14	1.0	2.1	.5	.9	1.1	1.6	1.3	1.4
	1.7	1.2	.9	1.2	.7	2.5	1.0	2.7
Series IIb				Age of donor chick (days)				
2.5% EE		7		21				49
0	.8	2.1	.4	1.2			.4	.7
	.7	2.0	.4	1.1			.3	.7
2	.5	.8	.2	.3			.2	.2
	.4	.7	.2	.4			.1	.3
7	.8	2.0	.2	.6			.4	1.3
	.9	1.7	.3	.9			.3	.7
14	.7	.9	.2	.7			.7	.5
	.9	1.0	.4	.6			.4	.8

In Series I one normal and dystrophic donor animal each was used for each donor age. In Series IIa and IIb 2 normal and dystrophic donor animals each were used for each donor age. Tissue samples in Series IIa and IIb were obtained from the same donor animal and were identical in respect to all culture conditions except extract concentration.

fetal pig extracts were prepared by homogenization of the tissue for a few seconds in a blender with one half the tissue volume/weight of Pannett-Compton solution, centrifuged for 45 minutes at 3,000 × g. The supernatant was diluted (between 1 and 5 times) to contain 2 μg/ml of protein and 0.4 ml of the diluted extract was used in a total volume of 2 ml of medium. Explants were fed twice a week and sub-cultured at 4 week intervals. At this time the outgrowth was usually so extensive that only the tissue from one tube in a series was maintained.

Short term cultures of human biopsies. Biopsy samples were minced and placed into roller tubes similar to the chick muscle. Media containing various proportions of human serum and SM were tested. Addition of embryo extract was found to be unnecessary in the case of human muscle.

Results. Analyses of the explanted chick muscle cultures show (Table I) that beginning with donors of 7 days after hatching the freshly excised muscle samples (O period *in vitro*) from homozygous dystrophic chicks show a higher DNA content than normal controls. In the younger (4-5 day) donors in Series I this difference was not observed. During the first 2 days of culture of normal and of dystrophic muscle, about one half of DNA content of the initial explant is lost, but proliferation from the second to the seventh day of cultivation compensates for this loss. DNA content of either normal or dystrophic muscle showed variable changes during the 7-14 day growth period.

In both types of chick muscle, in contrast to human muscle, there is no loss, but actu-

TABLE II. Protein Content of Muscle Explants Expressed as μg Protein Nitrogen/mg of Original Wet Weight of Explants.

Each figure is mean of separate determinations on 6 cultures. N = Normal, D = Dystrophic, EE = Embryo extract.

Period *in vitro*	N	D	N	D	N	D	N	D
Series I				Age of donor chick (days)				
5% EE	4–5		14		21		42	
0	7.2	5.0	10.3	8.3	10.4	8.2	10.9	11.3
2	10.5	8.4	14.4	10.9	12.4	10.8	13.2	13.1
7	20.8	25.6	15.9	15.5	14.2	15.6	15.0	14.8
14	29.7	37.8	26.1	21.6	18.1	24.2	20.6	16.8
Series IIa				Age of donor chick (days)				
5% EE	7		21		35		49	
0	7.7	5.6	10.2	9.5	9.9	12.7	10.8	11.5
	8.3	8.6	11.3	12.1	8.5	9.3	11.2	10.3
2	11.1	10.5	12.4	11.7	12.2	12.3	13.6	13.1
	12.4	11.2	13.6	12.5	10.5	12.6	13.4	12.8
7	12.8	14.1	14.1	13.2	13.4	12.2	16.2	13.4
	16.3	16.4	15.3	14.1	12.9	16.9	15.7	16.2
14	10.2	14.9	15.7	10.2	16.4	13.0	16.5	12.1
	15.0	10.5	15.2	11.2	13.8	19.7	17.6	21.0
Series IIb				Age of donor chick (days)				
2.5% EE	7		21				49	
0	7.7	5.6	10.2	9.5			9.0	11.5
	8.3	8.6	11.3	12.1			11.2	10.3
2	10.1	9.5	12.5	12.0			12.8	14.0
	11.7	9.9	13.1	13.4			13.0	12.5
7	11.0	11.7	12.4	10.2			14.2	9.2
	13.4	12.0	12.1	11.7			14.9	12.4
14	7.6	7.6	12.3	6.3			12.9	5.8
	8.7	8.0	13.7	5.1			15.0	8.9

Conditions identical to those of Table I.

ally an increase, of protein content during initial culture period (0-2 days, Table II). A variable increase in protein content was observed during the 2-14 day culture period. In one of the series (I), embryo extract at a concentration of 5% leads to large increases in the PN in both normal and dystrophic animals. In another series (IIa) with the same extract concentration, the increases were smaller in cultures of both muscle types. The embryo extract used in the first series was obtained during the height of the laying season. Whether this was the reason for the difference in growth promoting quality of the extract remains undecided.

Replicate cultures of the same muscle samples as those of Series IIa were set up with 2.5% embryo extract (IIb). In this lower concentration of embryo extract, cultures from normal and dystrophic 7-day-old donors showed a decreased protein content during the later culture periods. Cultures from older, normal donors did not show this loss of protein, but in all cultures from the older dystrophic donors a marked decrease during the 2-14 day culture period was observed. Observation of these differences is based on cultures from only 4 donors. A statistical analysis of the figures for individual cultures showed that the probability of this difference occurring by chance was less than 5%. The fact that the samples for Series IIa and IIb were obtained from the muscles of the same donor increases the statistical significance of these findings. Such a result may indicate that maintenance of dystrophic muscle remains dependent on higher extract concentrations than do both muscle types up to the 7th day of donor age. In contrast, maturing normal muscle, after the 7th day of donor age, can maintain its protein content even with the lower extract concentration.

In freshly excised human muscle, just as in chick muscle, DNA contents of dystrophic tissue were found to be higher than in controls. In human tissue, both PN and DNA contents show a transitory decrease reaching a minimum between the second and fourth day of cultivation. After culture for 3 weeks, in a standard medium, both normal and dystrophic muscle reached PN and DNA values about 2 times higher than initial values. If SM was omitted, growth of the cultures in 20% or 40% human serum reached only the initial values. No consistent differences could be found in the growth pattern of normal and dystrophic human muscle in any one of these culture conditions. Cultures from 2 human dystrophic donors grew much slower than controls, in cultures from 3 donors the muscle gave higher DNA and PN values than controls, and in 4 series no significant difference could be detected.

In further tests with human muscle attempting to repeat the experiments of Geiger and Garvin(3,4) neither the number of cultures showing outgrowth after one month nor the maximal duration of *in vitro* maintenance revealed striking differences between growth characteristics of normal and dystrophic muscle tissue. It became apparent that, for no obvious reasons, viability of roller tube cultures varies greatly and that a statistically valid establishment of possible differences in onset of rate of growth would presuppose measurements on very large numbers of cultures under these conditions. In our series of 5 normal muscle samples, maximal survival periods for cultures from different individual donors varied from 3 to 6 months, and in the cultures from 8 dystrophic biopsies from 2 to 11 months. In cultures of normal muscle 67% to 97% survived the first month; in dystrophic muscle 42% to 98%.

Cell types obtained during cultivation of both normal and dystrophic muscle were of a wide morphological variety. They included fibroblast-like cells and sarcoblasts which interlaced to form a dense meshwork. Sometimes the cultures were surrounded by giant cells containing many nuclei and vacuoles. These cells are probably the same as those described by Geiger and Garvin(3,4) as specific for dystrophic muscle. However, in our investigation, they were found in both normal and dystrophic explants and usually indicated imminent death of the culture.

Discussion. The results of the measurements on chick muscle cultures suggest that normal and dystrophic cells grown *in vitro* may differ at least quantitatively in their requirements for a factor which is supplied by embryo extract. Confirmation of existence of such a difference would concentrate the search for the nature of the defect of the dystrophic cell and for factors which eliminate this defect on a well limited area of materials and technics. Harris and Kutsky(9) have recently isolated the protein of a nucleoprotein fraction from spleen which is essential for *in vitro* proliferation of chick muscle tissue cells, and Harris(10) has demonstrated a requirement of chick muscle cells for a dialyzable factor.

However, even if the defects in cultures of dystrophic chick muscle cells could be controlled it would not mean that human dystrophic muscle grown *in vitro* would respond to the same factors. From our data it is apparent that human muscle differs from chick muscle both in respect to its growth pattern under optimal growth conditions and in its growth factor requirement. Human muscle loses protein during the first days of explantation while chick muscle maintains its protein content during this explantation period. Also, human muscle can grow *in vitro* without embryo extract but requires addition of a mixture of synthetic growth factors. Chick muscle requires embryo extract and a synthetic mixture of growth factors cannot be substituted for this.

Summary. 1) The changes in protein nitrogen and DNA contents of normal chick wing muscle tissue and of tissue from chick with muscular dystrophy were compared after culture in roller tubes. 2) No significant differences in the 2 parameters were found for normal and dystrophic tissue when grown in a medium containing 5% embryo extract. 3) When grown in 2.5% embryo extract the cultures from donors of an age of 2 weeks and older showed a statistically significant difference. Protein nitrogen of the cultures

from dystrophic muscle decreased during the 7-14 day culture period, whereas no comparable decrease was observed in cultures of normal tisue. Changes in the DNA content did not indicate definite differences. 4) Differences between several parameters determined in normal and dystrophic human muscle tissue cultures were found to be highly inconsistent or of no apparent relevance for the pathological state of the tissue.

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Changes in Liver Tyrosine-α-Ketoglutarate Transaminase Activity During Growth of Walker Carcinosarcoma 256. (26060)

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The tyrosine oxidizing system of liver is a series of enzymes converting tyrosine to fumarate and acetoacetate in the presence of α-ketoglutarate(1). A decreased tyrosine oxidation was observed in liver of rats fed a low protein diet(2). More recently, evidence has been presented suggesting that the lower level of tyrosine oxidation resulted from a decrease in activity of the initial enzyme in the tyrosine-oxidizing-system, tyrosine-α-ketoglutarate transaminase(3).

In addition to low protein diet, a decrease in liver tyrosine-α-ketoglutarate transaminase activity has been reported in rats subjected to tourniquet injury(4). An increase in activity of the enzyme has been reported following injection of hydrocortisone(5). An adaptive increase in tyrosine transaminase activity followed tyrosine injection. However, tyrosine was an effective stimulus only if the adrenal glands or hydrocortisone also were present(5). This discovery suggested that adrenal steroids played a major role in regulation of activity of this enzyme.

A number of studies have suggested an altered activity of the adrenal gland in tumor-bearing animals(6). Also, tumor-bearing animals generally exhibit a loss of appetite, particularly during late stages of tumor growth. It was therefore of interest to study the effect of tumor growth on liver tyrosine-α-ketoglutarate transaminase activity.

Methods. Female Holtzman rats weighing 160-200 g were used in these experiments. The Walker carcinosarcoma 256 was transplanted by injecting a cell suspension of tumor into both *rectus femoris* muscles according to the method of Talalay *et al.*(7). All animals were fed a diet of Rockland rat chow and water *ad libitum*.

Livers to be used for transaminase determination were excised and chilled immediately in cracked ice. After chilling, livers were perfused with cold isotonic saline and homogenized in a Potter-Elvehjem homogenizer with sufficient 0.15 M potassium chloride to give a 10% homogenate. The homogenates were centrifuged at 15,000 × g for 20 minutes at 0°C. Tyrosine-α-ketoglutarate transaminase activity was determined on the supernatant according to the method of Lin and Knox(5). Enzyme activity was expressed as μmoles of *p*-hydroxyphenylpyru-