IN VITRO CULTURE OF MUSCLE TISSUE FROM

NORMAL, HETEROZYGOUS, AND DYSTROPHIC CHICKS

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ABSTRACT

The degree of histological deterioration of the original explant and the extent of cell spreading was evaluated in cultures of pectoralis muscle from 11-day chicks. Although the frequencies of these two parameters varied with the amounts of horse serum and embryo extract added to the medium, cultures from dystrophic chicks, in comparison to those from either normal or heterozygous animals, consistently showed the largest number of explants with the most extreme forms of histological deterioration and cell spreading. At 20 per cent horse serum the cultures from heterozygous chicks showed greater frequencies of the more extensive forms of deterioration and spreading than the normal muscle explants, but at 5 per cent horse serum these two groups appeared similar. Regardless of genetic background, cultures of the pectoralis muscle from 18-day embryos and of the latissimus dorsi muscle from 11-day chicks exhibited comparable high frequencies for the maximal degrees of deterioration and spreading.

The development of methods for the *in vitro* cultivation of isolated cells and tissues under controlled conditions has led to attempts to reproduce pathological responses in tissue culture. The occurrence of degenerative changes in cultured tissues has been reported by Bornstein (1) for cerebellum tissue exposed to serum from animals with allergic encephalomyelitis and by Sidman (2) in retinal explants from animals with inherited retinal dystrophy. The genetically retarded growth of the leg skeleton of the creeper chick and an apparent alleviation of this condition by tissue extracts from normal embryos could be demonstrated with organ culture technique (3, 4). Gluecksohn-Waelsch and Rota (5) carried out an *in vitro* analysis of a genetic defect in mice which led to a severe disturbance in the development of the kidney.

Some differences in the growth pattern *in vitro* between normal and dystrophic muscle tissues have been reported. Geiger and Garvin (6) observed differences in the cell types and in the maintenance of cultures from normal and dystrophic human muscle. According to O'Steen (7) and Pearce (8), outgrowth of myogenic cell elements such as multinucleate myotubes was greater in cultures of normal mouse muscle than in muscle cultures from mice with hereditary muscle dystrophy. In addition, Pearce (8) found that cultures of muscle from dystrophic mice contained larger numbers of small myoblasts than the cultures from normal mice. Herrmann et al. (9) reported that, in media containing low levels of embryo extract, explanted muscle from dystrophic chick loses some protein while the normal muscle maintains its protein level. However, in this latter study, it was not possible to obtain evidence for a direct equivalence between the degenerative condition of muscle *in vivo* and the abnormal growth pattern *in vitro*. Therefore, attempts were continued to develop culture conditions which would lead to more characteristic differences between the growth patterns of normal and dystrophic muscle tissue. The present report is concerned with an extension of the previous studies

(9) on the response of muscle cultures to varia-
tions in the nutrient medium. In addition, some
investigations have been carried out on the im-
portance of the source of the muscle with respect
to age and genetic background of the donor, as
well as to location of the muscle on the donor
animal.

MATERIALS AND METHODS

Animals Used

The strain of dystrophic chicks employed in the
present investigation was originally developed at the
University of California, Davis (10). A flock derived
from this strain is now being maintained at the De-
partment of Animal Genetics of the University of
Connecticut. No high degree of inbreeding was at-
tempted since propagation of chick in highly inbred
strains becomes very difficult. The dystrophic chicks
were tested for the onset of wing musculature weak-
ness by observing the promptness with which the
chicks could stand up after having been turned on
their backs. Muscle tissue used for the cultures was
obtained from chicks with a distinct delay in response
to this test. White Leghorn chicks obtained from a
local commercial distributor served as a source of
normal muscle, and White Leghorn-dystrophic
crosses were employed for heterozygote tissue.

Explantation of Muscle Tissue

The animals were decapitated, and the entire
breast area was swabbed with tincture of iodine. The
skin was severed over the sternum and pulled away
from the midline. Care was taken not to contaminate
the exposed muscle with feathers or iodine. The major
part of the pectoralis musculature was excised, placed
into a petri dish, and minced with fine curved scissors
for 5 minutes. The muscle fragments obtained in this
way are about 1 mm.³ In the present experiments,
minced muscle was used for several reasons. It is a
preparation which provides homogeneous material
suited for the setting up of a large number of culture
plates in a single continuous work period. The muscle
pieces are small enough for adequate exchange of
nutrients required for cell maintenance and for tracer
studies and other metabolic experiments to be carried
out with these cultures. The preparation of thin and
long muscle straps as a possible alternative tissue
preparation is much more laborious, and the uptake
of labeled amino acids into the proteins of minced
cultures or single, long muscle straps was found in
preliminary experiments to be approximately the
same. Therefore, the cells in the minced muscle show
still a high degree of organization and metabolic
integrity. However, for certain tests the use of other
types of tissue preparations is being contemplated.

Five to 10 mg portions of the mince were weighed out
on a 50-mg capacity Roller-Smith balance and were
quantitatively transferred with a glass spoon to
22 x 22 mm coverslips, which had been previously
covered with a thin layer of reconstituted collagen.
To facilitate the transfer of muscle tissue, the glass
spoon and the balance pan were covered with a thin
layer of silicone. The mince was spread evenly over
the entire coverslip area. The coverslips with the
mince were placed into 5-cm diameter petri dishes
and allowed to stand for 30 minutes at room tem-
perature before the addition of 5 ml of media per
dish. Tissue from the latissimus dorsi muscle was
handled in a similar manner as described for the
pectoralis muscle. However, it was necessary to re-
move the skin from the back to expose this muscle
and, because the latissimus dorsi is a smaller muscle
than the pectoralis, the mincing time was reduced
and the amount of tissue placed on each coverslip
was smaller.

The fluid phase of the cultures consisted of varying
amounts of a chemically defined mixture (11), horse
serum, embryo extract, and electrolyte solution (12).
After the medium was added, the small petri dishes
were placed into large plastic containers (25 cm
diameter, 9 cm height), which were kept in a hot
room maintained at 37.5°C for the experimental
period. A mixture of 95 per cent air and 5 per cent
CO_2 was passed through the containers, bubbling
first through a layer of water at the bottom of each
plastic container and out through two small openings
in the lid. The culture medium was replaced every
2 to 3 days.

Collagen Preparation

A solution of collagen was prepared from rat tail
tendons according to the method of Bornstein (13).
The solution (0.05 ml per coverslip) was spread
evenly over the coverslip area, and was exposed to
ammonia vapor in a desiccator for 6 minutes to
increase the pH to about 10.0, leading to gelation of
the collagen. The excess ammonia was removed by
washing with chick Ringer's solution three times at
20-minute intervals, giving a final pH of 7.3 to 7.5.
The coverslips with the neutralized collagen gels
were allowed to stand overnight in a moist chamber.

Protein Nitrogen and DNA Content of
Cultures

After various incubation periods, the medium was
removed and the cultures were washed three times
at half-hour intervals with 5 ml aliquots of chick
Ringer. The washed cultures, including the collagen,
were collected in 2-ml centrifuge tubes and extracted
first with 1 ml of cold 5 per cent trichloroacetic acid
followed by two extractions at 90°C with 1-ml por-

tions of 1 n perchloric acid. This treatment removed most of the collagen and nucleic acids. Coverslips with collagen, but without tissue, were placed into petri dishes with medium and were carried along in each series to correct for a small residue of non-cell protein remaining insoluble after hot perchloric acid treatment. The values presented for protein nitrogen of tissue were corrected for this value. Treatment with hot perchloric acid probably removed some easily hydrolyzable cell protein in addition to collagen, but it is assumed that this loss remains constant and that the remaining protein nitrogen gives a correct index of the relative increase in cell mass. After

RESULTS

I. Protein Nitrogen and DNA Content

In order to compare muscle explanted on collagen-coverslips with the results previously obtained (9) for plasma clot-roller tube cultures, changes in the DNA and protein nitrogen content of the explants during cultivation were determined. A mixture of 20 per cent chemically defined nutrients, 40 per cent horse serum, 1 per cent or 2.5 per cent embryo extract, and salt solution was used as the medium. The pectoralis muscle

TABLE I

DNA Content of Explants of Pectoralis Muscle from Normal and Dystrophic Chicks*

	8-Day donors				15-Day donors			
	Normal		Dystrophic		Normal		Dystrophic	
	Days of in vitro culture							
	0	11	0	11	0	11	0	11
	0.94	0.59	1.83	0.84	0.49	0.23	1.15	0.35
	0.78	0.23	1.18	0.28	0.49	0.52	0.91	0.55
	0.80	0.64	1.45	0.93	0.48	0.19	1.75	0.24
	0.52	0.32	1.12	0.57	0.72	0.52	1.28	1.06
	0.53	0.27	1.49	0.24	0.33	0.33	0.96	0.93
	0.78		1.18		0.52	0.42	1.17	0.55
					0.34	0.16	0.88	0.51
					0.49	0.23	1.75	0.57
Mean	0.73	0.41	1.37	0.57	0.48	0.32	1.23	0.56
±SE	0.07	0.09	0.11	0.13	0.04	0.05	0.12	0.09

* Expressed as μg/mg wet weight of the original explant.

treatment with hot perchloric acid, lipids were removed by washing with alcohol-ether and the remaining protein was digested with sulphuric acid containing selenium catalyst. The protein nitrogen was quantitated by Nesslerization with ammonium sulfate standards. DNA was determined by the method of Burton (14).

Fixation and Staining of Cultures

The tissues were fixed in a mixture of three parts 100 per cent methyl alcohol and one part glacial acetic acid for about 1 hour following the procedure described by Newcomer (15), and stained with Harris hematoxylin following the procedure given by Paul (16).

tissue was obtained from chicks 8 days and 15 days after hatching, and determinations were carried out on cultures kept for 2 hours on the medium (zero time) and on cultures maintained in vitro for 11 days. Twelve cultures from each animal were analyzed; 6 after 2 hours and 6 after the 11-day period. DNA and protein nitrogen determinations were carried out on individual cultures.

Similar to the previous observations (9), the DNA content of the dystrophic muscle based on wet weight was higher than that of the normal muscle (Table I). After 11 days of cultivation, the DNA content for both the normal and dystrophic muscles from either 8- or 15-day chicks was lower

than the zero time levels. Although this DNA decrease was usually greater for the explants of dystrophic tissue, considerable variation was observed in the extent of the decrease.

The cultures of normal tissue from both 8- and 15-day donors showed no marked changes in protein nitrogen during the cultivation period, while the dystrophic cultures when grown in medium containing 1.0 or 2.5 per cent embryo extract showed decrease in their protein content (Table II). In the 8-day donor chicks, this loss amounted to about 22 per cent of the 2-hour value and was not significant (p > 0.1), but the 31 per cent

II. Culture Patterns of Normal and Dystrophic Muscle Cell Explants

In the previous series of experiments (9), differences in culture patterns between the normal and dystrophic tissues were not observed. However, in the present series of experiments, comparisons of normal and dystrophic tissues indicated clear differences. The various culture patterns were readily distinguished by the degree to which the histological fiber structure of the original explant was maintained and the extent of cell spreading (or outgrowth) from the original explant. The

TABLE II

Protein Nitrogen Content of Explants of Pectoralis Muscle from Normal and Dystrophic Chicks

	8-Day donors				15-Day donors			
	Normal		Dystrophic		Normal		Dystrophic	
				Days of *in vitro* culture				
	0	11	0	11	0	11	0	11
	13.6	15.0	11.8	8.9	15.5	22.5	21.0	17.1
	11.4	12.1	9.6	4.9	18.6	16.8	16.2	8.7
	15.5	13.9	15.4	12.9	15.2	13.9	13.9	12.3
	13.9	11.2	11.7	7.4	17.0	16.3	13.6	7.2
	11.6	17.4	10.8	15.3	18.6	16.4	13.8	8.7
	17.4	19.8	20.2	13.3	17.0	15.9	13.6	10.8
	11.3	12.2	11.6	7.6	15.7	17.1	14.5	9.6
	15.5	14.1	15.4	12.6	15.2	15.2	13.7	7.6
Mean	13.8	14.5	13.3	10.4	16.6	16.8	15.0	10.3
±SE	0.8	0.7	1.2	1.7	0.5	0.8	0.9	1.1

* Expressed as μg/mg wet weight of the original explant.

protein nitrogen loss in the cultures from the 15-day donor was found to be significant (p < 0.02). The loss of protein nitrogen in the present experiments was found to be somewhat smaller than that previously reported (9). However, in the earlier series protein nitrogen was determined after 14 days of culture, and some additional loss would have occurred during the 3 days of extended cultivation.

In agreement with the earlier findings (9), the marked loss in DNA in both culture types and the loss of protein nitrogen in the dystrophic cultures were observed only in the presence of embryo extract concentration of 2.5 per cent and below, and did not occur in cultures growing with 5 per cent embryo extract.

photographs presented in Figs. 1 to 4 were taken from pectoralis muscle tissue derived from 11-day chicks which had been cultured for 11 days *in vitro* and are representative of the three categories employed in the classification. Fig. 1 shows an example of an explant form which would be considered minimal for both deterioration and spreading. The histological fiber structure as well as the sharp outline of the original explant was maintained and no cells have appeared outside of the explant. Fig. 2 represents an explant in which the histological fiber structure was beginning to deteriorate. The sharp irregular contour of the original explant has been lost, giving the explant a smooth, confluent outline (intermediate deterioration). A noticeable, but still sparse, layer
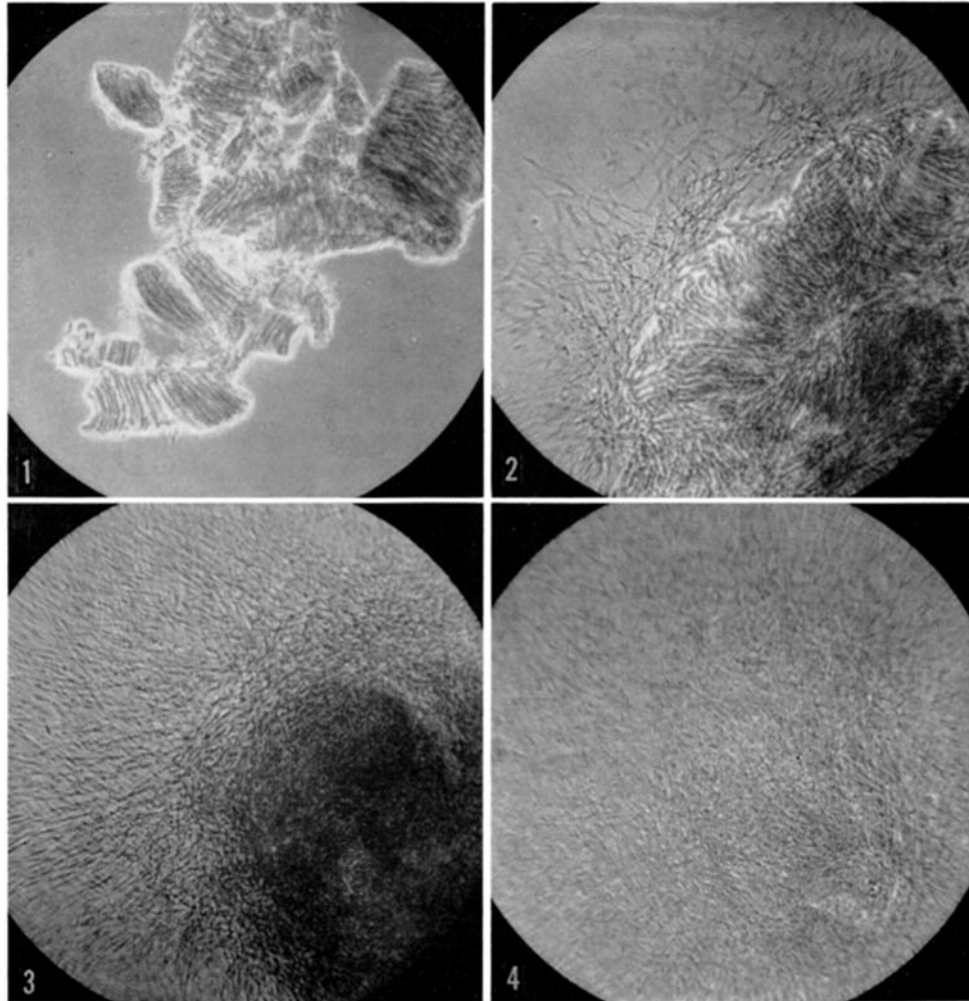
FIGURE 1   Muscle explant showing minimal cell spreading and minimal histological deterioration. × 100.

FIGURE 2   Muscle explant showing intermediate cell spreading and intermediate histological deterioration. × 100.

FIGURES 3 AND 4   Muscle explants showing maximal cell spreading and maximal histological deterioration. × 100.

of cells has appeared outside the original explant (intermediate spreading). In Figs. 3 and 4, the histological structure of the original explant has virtually disappeared (maximum deterioration). In Fig. 3, a dense amorphous mass remains in place of the original explant, and in Fig. 4 the outline of the original explant has been lost. The dense layer of cells which has appeared in addition to or in place of the original explant was indicative of maximum spreading. The extent to which the

appearance of these cells outside the explant results from cell migration or actual proliferation has not been clearly determined. The single term, cell spreading, will be used here.

The distinctions between these classes has made it possible to determine the frequencies of the different histological patterns for each coverslip. The first series of such counts was made on the cultures which were also used for the nitrogen and DNA determinations. In these cultures, the

medium contained 1 or 2.5 per cent embryo extract and 40 per cent horse serum, and only the cell-spreading criterion was recorded (Table III). Maximum spreading was encountered only in the explants derived from dystrophic chicks, with an incidence of 27 per cent of the total muscle fragments evaluated. Intermediate spreading was found in cultures of both muscle types, but with slightly higher frequency (48 per cent) in dystrophic than in normal muscle (35 per cent). The frequencies for minimal outgrowth were 65 per cent for normal and 25 per cent for dystrophic explants. About 40 per cent of the dystrophic muscle explants classified as maximal spreading exhibited muscle straps in the cell spreading area. In this series, muscle straps were not observed in explants of normal muscle.

TABLE III

*Cell Spreading in Explants of Pectoralis Muscle from Normal and Dystrophic Chicks**

Spreading	Normal	Dystrophic
	per cent	*per cent*
Maximal	0	27
Intermediate	35	48
Minimal	65	25

* Explants were cultured for 11 days and a total of 510 explants were counted. The values obtained for 8- and 15-day donors were combined. The medium consisted of 40 per cent horse serum, 20 per cent chemically defined nutrients, 1 or 2.5 per cent embryo extract, and salts.

During the subsequent fall and winter season, variations in the composition of the medium were studied in order to evaluate the range of conditions under which differences in the culture pattern between normal and dystrophic muscle explants would become apparent. It can be seen from the data presented in Table IV that increased amounts of horse serum and embryo extract enhanced the frequencies of the maximal and intermediate classes for both cell spreading and deterioration. However, under almost all conditions, the intermediate and maximal degrees of spreading and deterioration were greater in the cultures of dystrophic muscle. In the absence of horse serum and embryo extract, essentially all of the explants from normal muscle showed minimal spreading and deterioration, but in this medium between 30 and 40 per cent of the dystrophic explants ex-

hibited intermediate degrees of spreading and deterioration. The normal cultures at 1 per cent horse serum still showed only 8 per cent intermediate and no maximal spreading; deterioration was limited to a total of 12 per cent for the maximal and intermediate categories. However, with this same level of horse serum, 69 per cent of the dystrophic cultures showed intermediate spreading and 52 per cent showed maximal deterioration. Even with 5 per cent horse serum, the normal cultures showed cell spreading of 83 per cent minimal and 17 per cent intermediate; and deterioration of 59 per cent minimal, 28 per cent intermediate, and 13 per cent maximal. In contrast, the dystrophic explants in 5 per cent horse serum gave an incidence of 42 per cent maximal and 58 per cent intermediate and essentially no explants with minimal spreading, and an incidence of 73 per cent maximal and 27 per cent intermediate deterioration.

Increasing the horse serum concentration to 20 per cent did not appreciably change the incidence of the spreading and deteriorating categories, in either the normal or dystrophic cultures, from that observed at 5 per cent horse serum. The addition of 40 per cent serum and 1 or 5 per cent embryo extract to the medium resulted in more extensive spreading and deterioration in the normal muscle explants but did not appear to further influence the dystrophic cultures. In a majority of cultures with maximal spreading of both normal and dystrophic explants the typical multinucleate muscle straps and occasionally cross-striations could be detected.

Although chicks heterozygous for genetic muscular dystrophy do not exhibit the inability of dystrophic chicks to return to an upright position after being placed on their backs, an intermediate expression of the dystrophic condition has been observed in the extent of muscle hypertrophy (17, 18) and in the serum activity levels of aldolase and glutamic-oxalacetic transaminase (18). A series of studies were carried out in which explants from the pectoralis muscle of 11-day normal, heterozygous, and dystrophic chicks were directly compared (Table V). It can be seen that with either 5 or 20 per cent horse serum the explants of dystrophic muscle consistently showed higher frequencies for the intermediate plus maximal degrees of spreading and deterioration than did the explants derived from either heterozygous or normal animals. Furthermore, in both series of

TABLE IV

Cell Spreading and Histological Deterioration in Explants of Pectoralis Muscle from Normal and Dystrophic 11-Day Chicks*

Medium			Normal								Dystrophic						
				Spreading			Deterioration					Spreading			Deterioration		
HS	CDN	EE		+	±	-	+	±	-			+	±	-	+	±	-
per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
	100		(66)	0	0	100	0	3	97	(41)		0	39	61	7	32	61
1	99		(72)	0	8	92	6	6	88	(80)		1	69	35	52	20	38
5	95		(46)	0	17	83	13	28	59	(104)		42	58	0	73	27	0
10	90		(41)	0	54	46	15	32	54	(57)		46	53	1	83	17	0
20	80		(78)	0	45	55	13	45	42	(128)		61	38	1	88	12	0
40	20	1	(129)	7	48	45	30	31	39	(152)		44	44	12	66	28	7
40	20	5	(172)	12	62	26	42	37	22	(136)		59	36	4	75	23	2

* Explants were cultured for 11 days. Numbers of explants counted are shown in parenthesis. Components of medium: horse serum (HS), chemically defined nutrients (CDN), embryo extract (EE). Degree of spreading or deterioration: Maximal (+), intermediate (±), minimal (−).

experiments with 20 per cent horse serum, the frequencies for the two higher degrees of spreading and deterioration were greater for muscle from heterozygous chicks than for explants from normal donors. This intermediate position of the frequencies observed for the explants from heterozygous chick relative to the normal or dystrophic tissue was not apparent when the level of horse serum in the medium was reduced to 5 per cent. The frequencies for spreading and deterioration were the same for explants from either normal or heterozygous donors in two separate experiments with 5 per cent horse serum, while in two other experiments with this amount of horse serum the

plants, but with 8-day chicks the difference between normal and dystrophic culture patterns appeared as great as that observed with 11-day donors.

Kaplan and Cahn (19) recently reported that both normal latissimus dorsi muscle and pectoralis muscle from dystrophic chicks contained the embryonic heart lactic dehydrogenase instead of that form which is characteristic of mature skeletal muscle. In view of this observation, explants were prepared from the latissimus dorsi muscle of 11-day normal, heterozygote, and dystrophic chicks. Similar to the results obtained with the 18-day embryo pectoralis muscle, explants of this

TABLE V

*Cell Spreading and Histological Deterioration in Explants of Pectoralis Muscle from Normal, Heterozygous, and Dystrophic 11-Day Chicks**

Medium					Spreading			Deterioration		
HS	CDN	Salts	Donor		+	±	−	+	±	−
per cent	per cent	per cent			per cent	per cent	per cent	per cent	per cent	per cent
20	20	60	Normal	(22)	0	9	91	0	9	91
			Heterozygous	(52)	0	19	81	8	38	54
			Dystrophic	(51)	0	43	57	77	21	2
20	80		Normal	(60)	0	8	92	0	7	93
			Heterozygous	(49)	2	27	71	11	25	63
			Dystrophic	(62)	24	63	13	66	29	5
5	95		Normal	(270)	0	1	99	0	1	99
			Heterozygous	(233)	0	6	94	0	5	95
			Dystrophic	(403)	11	54	35	38	33	29

* Explants were cultured for 11 days. Numbers of explants counted are shown in parenthesis. Components of medium: horse serum (HS), chemically defined nutrients (CDN), embryo extract (EE). Degree of spreading or deterioration: maximal (+), intermediate (±), minimal (−).

intermediate degrees for spreading and deterioration of heterozygous explants were only slightly greater than those of normal muscle.

In an attempt to evaluate the significance of the age of the donor chick, cultures were prepared from the pectoralis muscle of 18-day embryos and chicks at 1, 8, and 11 days. The results obtained for only the 18-day embryo and 11-day chick studies are presented in Table VI. Explants from 18-day embryos showed a similar high incidence of maximal deterioration and maximal spreading in cultures from either normal, heterozygous, or dystrophic donors. In the explants from 1-day chicks, the normal muscle showed slightly less outgrowth and deterioration than the dystrophic ex-

tissue showed high frequencies of maximal cell spreading and maximal deterioration regardless of genetic background (Table VI).

DISCUSSION

The results from earlier experiments (9) with cultures of normal and dystrophic chicks maintained with low embryo extract concentration differ in several respects from the results obtained in the present series of experiments. Previously, no differences in growth patterns were observed. The protein loss in the dystrophic cell cultures was more marked and a more complete recovery in the DNA content of both dystrophic and control cultures was obtained after a loss during the

TABLE VI

*Cell Spreading and Histological Deterioration in Explants of Pectoralis Muscle from 18-Day Embryos and of Latissimus Dorsi Muscle from 11-Day Chicks**

Medium			Donor		Spreading			Deterioration		
HS	CDN	Salts			+	±	−	+	±	−
per cent	*per cent*	*per cent*			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
20	20	60	Pectoralis 18-Day embryo							
			Normal	(73)	87	13	0	100	0	0
			Heterozygous	(48)	58	31	11	100	0	0
			Dystrophic	(53)	58	36	6	100	0	0
			Latissimus dorsi 11-Day chick							
			Normal	(20)	15	50	35	100	0	0
			Heterozygous	(17)	6	53	41	100	0	0
			Dystrophic	(30)	0	40	60	100	0	0
20	80		Pectoralis 18-Day embryo							
			Normal	(52)	75	17	8	88	12	0
			Heterozygous	(48)	42	56	2	92	8	0
			Dystrophic	(64)	75	13	12	100	0	0
			Latissimus dorsi 11-Day chick							
			Normal	(22)	0	73	27	100	0	0
			Heterozygous	(19)	21	68	11	100	0	0
			Dystrophic	(10)	10	50	40	60	40	0

* Explants were cultured for 11 days. Numbers of explants counted are shown in parenthesis. Components of medium: horse serum (HS), chemically defined nutrients (CDN). Degree of spreading or deterioration: maximal (+), intermediate (±), minimal (−).

initial explantation period. In the earlier experiments, the cultures were grown on plasma clots in stoppered Roller tubes without 5 per cent CO_2 gassing and the donor chicks were 14 days or older. In the present series, a collagen substratum was used with a large volume of medium in repeatedly gassed petri dishes, and most of the studies were with 11-day chicks. However, there is no clear indication that these differences in culture conditions or donor age are the ones responsible for the differences in the observed growth patterns of the earlier and the present experiments.

The main observation reported in this paper is the quantitative difference in the incidence of the histological deterioration and cell spreading which is noted when a comparison is made of explants of muscle tissue from 11-day normal, heterozygous, and homozygous dystrophic chicks grown under the same culture conditions. That histological deterioration is not necessarily followed by cell spreading is indicated by the much higher incidence of the former after culture in 20 per cent horse serum with 20 per cent chemically defined nutrients and of the latter in cultures receiving 1 per cent horse serum and 99 per cent chemically defined nutrients.

The degree to which deterioration and cell spreading could be elicited by certain culture conditions varied somewhat. During the fall and

winter, the extent of cell spreading and deterioration under the same conditions of *in vitro* maintenance was in general more marked in both normal and dystrophic; during the later part of the year the changes were less distinct, in particular in media containing low serum concentrations. Whether this variation was due to some undetermined difference in the constituents of the media or to some intrinsic seasonal change in the muscle has not been fully established. However, media which promoted a good response during the earlier part of the year and were stored in the frozen state did not give an equally good response at a later time. Hence, a change in the properties of the muscle itself would seem more likely as an explanation of this variability. Seasonal changes affecting the development of the chick are well known. With reference to muscle development, it should be pointed out that marked seasonal variation in the incidence of a muscle hypoplasia in response to nicotine has been observed by Landauer (20). The observed variability may deserve further study, but it seems of little relevance to the present main problem. The pertinent fact is the consistency in the difference between normal and dystrophic muscles which was found to be always apparent in the explants from the 11-day chick when grown under culture conditions which elicited any histological deterioration or cell spreading.

In attempting an interpretation of the different responses of normal and dystrophic muscle *in vitro*, one has to take into account the following facts. In the chick embryo, as late as the 18th day of development, the responses of the normal, the homozygous, and the heterozygous dystrophic muscles are alike. The three explant types show equally maximal spreading and deterioration in essentially all explants. Shortly after hatching, the normal muscle loses its capacity to respond in this maximal fashion to the *in vitro* culture conditions while the heterozygous and dystrophic explants retain this responsiveness for a longer time, to a lesser or greater extent, respectively. This could mean that the difference in the culture pattern of the dystrophic explants is due to retention of some of the embryonic properties. Such a suggestion seems to be corroborated by the high content in the dystrophic muscle of the embryonic

form of lactic dehydrogenase (19). However, a closer relation between embryonic and dystrophic properties seems to be negated by the results obtained with the latissimus dorsi. Explants of this muscle, obtained on the 11th day after hatching, also show a full expression of the embryonic culture patterns as well as the high level of the embryonic lactic dehydrogenase (19). Although this muscle retains its embryonic properties, it does not show *in vivo* the symptoms of dystrophy. Therefore, the factors which lead to a prolonged maintenance of embryonic properties in the *in vitro* cultures of dystrophic muscle are, at most, a necessary but, by no means, a sufficient condition for dystrophic degeneration *in vivo*. This apparent impasse could be explained by assuming that the similar forms of histological deterioration and spreading *in vitro* observed with explants from dystrophic pectoralis and normal latissimus dorsi and embryonic pectoralis are general cell reactions to different defects. The *in vitro* defect in the dystrophic muscle may thus be more or less directly related to the dystrophic degeneration as suggested by the parallelism of the *in vitro* and *in vivo* defects in normal homozygous and heterozygous muscle tissue. In the normal latissimus dorsi, a different condition may lead to *in vitro* deterioration and spreading, but not to dystrophy *in vivo*. Possible differences between the latissimus dorsi, the embryonic pectoralis, and the dystrophic pectoralis muscle which may be investigated are decreased cell retentiveness with respect to enzymes or creatinphosphate, differences in the rate of protein turnover, as well as other parameters which have been found to differ in normal and dystrophic muscle (21). The examination of some of those aspects of the problem which are facilitated by *in vitro* systems will be attempted as a continuation of the reported experiments.

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