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INTRINSIC AND EXTRINSIC FACTORS IN SKELETAL MUSCLE ORGANOGENESIS: REAGGREGATION OF DISSOCIATED CHICK MYOGENIC TISSUE IN VITRO.

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INTRINSIC AND EXTRINSIC FACTORS IN SKELETAL MUSCLE ORGANOGENESIS: REAGGREGATION OF DISSOCIATED CHICK MYOGENIC TISSUE IN VITRO

by
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of the requirements for the degree of
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INTRODUCTION

Skeletal muscle tissue in the adult vertebrate is organized into gross bundles or fasciculi separated from one another by connective tissue condensations. Each of these bundles is composed of a multitude of fibers oriented in one and only one direction. Collectively, different bundles exhibit a variety of fiber orientations. With few exceptions, this highly patterned architecture is derived from a single sheet of cells, the mesodermal germ layer of the embryo. By various subdivisions within this sheet and by proliferation of cells from this sheet, the anlagen of the definitive adult muscles are formed.

McMurrich (1923) has summarized the basic processes responsible for the establishment of patterning in myological tissue from its mesodermal primordium. He cites the following developmental phenomena to be operative in the organogenesis of definitive skeletal muscles:

1. the association of pre-muscle cells in closely associated aggregates.
2. the oriented elongation of developing fibers within aggregates into parallel rows.
3. the apparent migration of primordial masses.
4. the superficial fusion of neighboring aggregates.
5. the disjunction of masses longitudinally into subdivisions.
6. the tangential splitting of aggregates to form sheets.
7. the partial or complete degeneration of masses.

Modeling in myogenic tissue is accomplished by one or more of these phenomena. Each of these may be briefly documented as follows:

The aggregation of cells. Pre-muscle cellular accumulation into localized foci is one of the earliest indications of myogenesis. This phenomenon is most apparent in the cells of the dorsal mesoderm, which proliferate rapidly and subsequently form aggregates termed somites. Further subdivision of the somite itself yields the dorsomedial myotome, the site of striated muscle formation. Aggregation of pre-muscle tissue is also seen in the subepidermal proliferation of mesenchymal cells to form the limb anlage (Byrnes, 1898; Lewis, 1910; Saunders, 1948; Tschumi, 1957). Bardeen and Lewis (1901) report further differential accumulations within these limb buds, presaging the formation of flexor and extensor masses and eventually the definitive limb musculature.

The parallelism of elongation cells. The cells of the myotome elongate into spindle shapes (myoblasts) and align themselves with their long axes in a craniocaudal direction (Boyd, 1960; Blechschmidt, 1961). Secondary fusion of neighboring myoblasts, according to most recent investigations, results in the formation of elongated and multinucleated muscle straps or myotubes (Holtzer, Abbott and Lash, 1958; Wilde, 1959; Stockdale and Holtzer, 1960, 1961; Konigsberg et al, 1960; Capers, 1960; Cooper and Konigsberg, 1961a,b). As they emerge, these myotubes exhibit a well-organized parallelism with respect to others in the immediate vicinity.

The migration of cellular masses. Migratory activity is another mechanism employed by muscle tissue in the establishment of primordia. As is evidenced by the variety of muscle fiber orientations in the adult, differential re-arrangement of aggregates does occur. Thus, the original parallelism is retained only with respect to the aggregate itself.

Movement of cellular masses is widespread throughout the body and may be quite extensive. In the formation of trunk musculature, a ventro-lateral extension of cervical and thoracic myotomes, accompanied by a downward growth of lateral plate mesoderm, has been observed (Rawles and Straus, 1948; Detwiler, 1955; Liedke, 1958). The two classical examples of migration of muscle masses are the tongue and diaphragm. Tongue musculature has been traced from occipital and upper cervical somites into the oral region (Hunter, R.M., 1935; Hunter, R.P., 1935; Bates, 1948; Deuchar, 1958). Diaphragmatic muscle is thought to be originally situated in upper cervical myotomes and secondarily relocated by a downward growth into the lower thoracic region (Wells, 1954).

The fusion of aggregates. Secondary fusion of neighboring cellular assemblages is seen throughout the body. Bardeen and Lewis (1901) reported that the primordial back and neck musculature is formed by the superficial fusion of cervical and thoracic myotomes. Prior to the migration of the intrinsic tongue musculature, a fusion of occipital and cervical myotomes occurs (Bates, 1948). The ventro-lateral abdominal and thoracic walls in the chick result from the fusion of hypaxial and lateral plate mesoderm (Straus and Rawles, 1953). Also in the formation of M. sternomastoideus and M. trapezius, there is the combined contribution of upper cervical myotomes and head mesenchyme (McKensie, 1955, 1960).

The longitudinal subdivision of cellular masses. Longitudinal splitting of fused aggregates is responsible for the creation of definitive muscles. During the development of fore and hind limbs, a subdivision within the mesenchymal

collar surrounding the central skeletal core yields flexor and extensor masses. Further partitioning results in the creation of muscles from these primitive aggregations. Similarly, in the cervical and thoracic myotomes, there is a schism into dorsal (epaxial) and ventral (hypaxial) segments. Superficial amalgamation of epaxial cells produces the muscles of the back; hypaxial cells contribute to the prevertebral muscles and to the ventrolateral thoracic and abdominal wall musculature (Bardeen, 1900). Pre-somite segmentation forms dorsal and ventral masses from which are developed the ocular muscles (Adelmann, 1929).

The tangential splitting of masses. Additional subdivision of primitive muscle masses is achieved by tangential cleavage into superficial and deep layerings seen in the formation of the deep back muscles and the superficial respiratory muscles (Bardeen and Lewis, 1901). A similar split in hypaxial masses contributes to the layered ventral abdominal wall (McMurrich, 1923).

The degeneration of muscle tissue. Partial or complete regression of muscle to form connective tissue elements (tendons, aponeuroses or fasciae) is reported to be a factor in the organogenesis of definitive muscles ("retrogressive metamorphosis", Bardeen, 1900). In the development of M. levator ani, Power (1948) has reported the physiologic degeneration of muscle tissue into fascial sheets. Also, the elongation of tendons during development has been attributed to the degeneration of muscle tissue by Carey (1922b) and by Haines (1932).

All of the organogenic phenomena briefly described above are thought to be caused by factors both intrinsic and extrinsic to the cell masses involved. As expressed by Holtfreter and Hamburger (1956),

> "... a cell which ... has become determined to differentiate in a certain direction acquires simultaneously the tendencies of mobility and adhesion characteristic of this cell type. Varying with the type, the cell may exhibit the tendencies of elongating or spreading, of invagination, aggregating, dispersing, etc. Proper execution of these tendencies is a prerequisite for the final arrangement of the cells into typical tissues and organs."

Superimposed on this intrinsic capacity for self-organization, it is added that

> "... the marshaling of the cells into tissue patterns is controlled by a variety of environmental factors ..."

and that

> "... to manifest these tendencies, environmental conditions ... are required.".

Thus, it may be expected that the cyto-architecture of adult myological tissue also results from the operation of both intrinsic and extrinsic determinants. Moreover, these extrinsic environmental parameters cannot be divorced from the intrinsic morphology and differentiative character of the muscle cell itself.

The role of extrinsic modalities in the genesis of body musculature has been given considerable attention in the literature. The most commonly implicated agent responsible for the organogenic behavior of developing muscle is the set of mechanical forces in the environment of this tissue. Carey (1922a,b) has advanced the idea that the architecture of every skeletal muscle in the body may be defined in terms of an intermittent tension acting on the tissue during its ontogeny. He suggests that there are dominant and subdominant regions of tension which are responsible for cellular

rearrangements in the embryo. Theoretically, the areas of dominant tension draw out the cells of the subdominant regions. Carey conceived three factors to be responsible for the intermittent induction of these tractional stimuli, all of which are extrinsic to the myogenic population itself. These are the growth in thickness of epithelial sheets, the accumulation of fluids and the accelerated linear growth of skeletal elements. Thus, the direction of muscle fiber orientation is determined by the tensile stresses induced in the pre-muscle mass by the above growth processes. To explain atypically oriented fibers, which are never found in normal adult muscle, Carey suggests that all strands not aligned in correspondence with the more permanent tensile stress lines degenerate.

This concept of loci of differential tension is also incorporated into the explanations of Blechschmidt (1961), who describes "dilation fields" or regions of decreased tension into which developing muscle tissue may expand. These dilation fields are contrasted to "retention fields" in which there are compressive forces and in which connective tissue develops. Thus, Carey's regions of subdominant tension would be termed "dilation fields" by Blechschmidt; areas of dominant tension would be "retention fields".

Blechschmidt singles out the growth of embryonic skin as well as the straightening of the embryonic axis as the formative agents in the creation of these fields, and thus, in the establishment of muscle patterns. The alignment of the intrinsic musculature of the tongue into bundles of three different orientations he attributes to the generation of several dilation fields, each of which develops strength

equal to that of any other. Supposedly, the growth of the oral epithelium creates these fields. He explains the development and orientation of M. rectus abdominis in a dilation field created by the ribs lifting ventrally and drawing away from the symphysis pubis. The alignment of myotomic cells in a cranio-caudal direction he correlates with the growth in length of the neural tube and with the straightening of embryonic flexures. In all dilation fields, he states, the muscle fibers develop as straight as possible.

The longitudinal and tangential splitting of tissue aggregates is attributed by Blechschmidt to the formation of "gliding surfaces" within dilation fields. Along these surfaces of greater mobility, there are created subdivisions of a muscle mass into single muscles.

Migratory activity is explained as the result of differential growth processes rather than as an active cellular migration (Carey, 1922a). As seen in the development of the tongue, this behavior is thought to be the resultant of combined mechanical forces acting in the area. The rapid growth of the pharynx and the forward expansion of all anterior structures of the head are the causative factors in the movement of occipital myotomes (Bates, 1948). The movement of diaphragmatic pre-muscle masses is considered by Blechschmidt (1961) to be due to the straightening of embryonic body flexures during development.

In vivo experimental verification of these factors responsible for myological organization has yielded information concerning the effects of the physical environment upon myogenic tissue and the capacity for self-organization of developing muscle cells. By the extirpation of normal sites

of origin and insertion of a myogenic mass; by the ectopic re-location of the normal origin and insertion; and by the substitution of ectopic cartilaginous structures for normal attachments, Hall (1950) was able to show that muscle organization occurs in the absence of specific trophic influences between the muscle tissue and its skeletal attachments. Such work has also demonstrated that contact with structures affording origin and insertion for a muscle mass is of minimal importance in the organization of the fibers, in contradistinction to Blechschmidt's claim. The highest degree of structural organization, i.e., parallel fiber orientation, was achieved in the absence of skeletal attachment. Hall did find that the structural elements in the vicinity of the developing tissue mass were not totally irrelevant to the latter's organization, since removal of neighboring cartilaginous tissue produced disorganization of the muscle population. The compaction of the muscle tissue and its orientation, then, are dependent upon the presence of the "mesostratum", as shown in work with other tissues by Weiss (1933).

Although grafting of populations of developing skeletal muscle onto the chorio-allantoic membrane has shown that the fibers group into bundles, strands have been observed to have an entirely atypical position with regard to the rest of the population (Hunt, 1932). Even fibers extending from the central skeletal element of the graft to its surface, at right angles to the expected direction, were found. These fibers were normal in morphology and exhibited no signs of degeneration. Not only does this dispute Carey's contention that abnormally oriented fibers will degenerate, but it renders unlikely his claim that tensile force produced by the growth

of the skeletal element is sufficient to explain the fiber orientation in these grafts.

Other interesting aspects of skeletal muscle organogenesis are derived from regeneration studies of transplanted limbs. Pietsch (1962a) has reported that chondrogenesis and myogenesis in Amblystoma are mutually independent events, that is, the development of a typical skeletal core is not necessarily followed by the normal organization of the muscle elements. He draws from such results the view that the

> "... basic reactions in differentiation of the limb regenerate are inherent functions of the particular cells involved in production of each tissue; that environment in which they develop - while important - serves in an ancillary and non-specific capacity."

Thus, he implicates the intrinsic capacity of myogenic tissue to effect its own organization.

Further transplantation studies by Pietsch (1962b) have characterized this self-organizing behavior of muscle populations. In transplanted limbs of Amblystoma larvae, one muscle, the M. ulna carpalis, seems to form prior to the appearance of other definitive limb muscles. Moreover, this mass plays an organizing role, initiating and regulating the segmentation of adjacent muscle tissue. In its absence, total disorganization of myological elements occurs in the transplants.

Observations of the in vitro behavior of skeletal muscle were first made by W.H. and M.R. Lewis (1915, 1917). Since then, many investigators have cultivated differentiating skeletal muscle. An analysis of the in vitro support of myogenesis by a variety of nutritive media in a monolayer system has been presented by Resseguie (1961). Konigsberg (1960, 1961, 1962) has applied cloning techniques to myogenic tissue in his cultures. Other work on the differentiation of muscle

tissue in plasma clot organ culture has been reported by Engel and Horvath (1960), who have employed the fluorescent antibody technique for the study of myofibril formation. Additional studies by Wilde (1950, 1952) have indicated that multinucleation of myogenic cells occurs *in vitro* by the fusion of myoblasts. Reviews including other studies of myogenesis *in vitro* are reported by Holtzer (1961) and Murray (1960).

The intrinsic capacity for self-organization of myogenic tissue *in vitro* is well documented. Holtfreter (1947) has reported that explanted myogenic tissue masses have no tendency to adhere in a compact group, but the cells themselves have an inherent polarity. The parallel fiber orientation in the myotome as well as in muscle bundles, then, has been explained as the expression of an original intracellular axiality; the bundling itself, an expression of the spatial conditions for growth as supplied by the mesodermal matrix. As a result of his reaggregative studies, Moscona (1958, 1959) concluded that pre-muscle tissue requires a solid substrate on which to organize. In suspension, he claims, myogenic behavior is inhibited due to the lack of a suitable stratum for organization. In contrast, Wilde (1959) has reported that in suspension, myogenic activity proceeds and that the muscle cells aggregate into spherical masses in which fibers are oriented in a radial direction.

Correlation between the size of the explanted mass and the degree of parallelism of its fiber development may be inferred from additional *in vitro* studies. Muchmore (1957) has observed a more compact and orderly arrangement of cells and a more regular orientation of cells if comparatively larger masses of explanted tissue are utilized. It is the population

size and not the density which is responsible for this organization. No better order was seen if explanted tissue were maintained in balanced saline for twenty-four hours prior to cultivation, thus effecting a more closely compacted assemblage. Other work indicates that there is a maximal mass which, if exceeded, increases the variety of differentiated cell types within the developing tissue (Lopaschov, 1935). Thus it may be concluded that the mass of a tissue is an important factor in determining the differentiation of its component cells.

Not only the mass of tissue available, but the proportional share of different histogenic types of cells in the population is apparently critical. According to Moscona (1956), when chondroblasts and myoblasts are cultivated together, the continued differentiation of one will be favored over that of the other unless a certain "liminal proportional concentration" is present.

The reaggregation of previously dissociated cell masses is a technique which has been used recently to study the cellular and environmental factors responsible for the attainment of tissue patterns (Cohen, 1942; Leighton, 1951; Moscona, 1952, 1957; Trinkaus and Groves, 1955; Weiss and Moscona, 1958; Auerbach and Grobstein, 1958; Auerbach, 1960; Curtis, 1962; Steinberg, 1962a,b,c).

Following the dissociation of tissues or organs by cation depletion or enzymatic digestion of intercellular protein, the cells, if suspended in growth medium, settle in the liquid and aggregate. These aggregates increase in size by accretion and adhesion of cells. Moscona (1952) reported that the rate of aggregation varies with cell type, cell concentration, and the conditions of the culture medium and environment. He

demonstrated that the characteristic tissue patterns reside in the cells themselves.

Moscona and others (Moscona, 1961a,b, 1962; Steinberg, 1962a,b,c) have extended this work by growing cells in the presence of environmental forces. Under these conditions, the initial amassing of cells is not dependent on active cellular movement, but is dependent on the rotation of the culture vessel and medium. In the presence of a rotation-generated centripetal force, cells floating in a liquid medium are found to adhere to one another, forming spherical aggregates whose size is dependent on the temperature, speed of rotation of the culture vessel, and hydrogen ion concentration. The pattern evoked in these masses is characteristic for the tissue type involved and is secondarily effected by selective localization and sorting of cells due to differences in mutual adhesiveness.

The present investigation has been conducted to characterize <u>in vitro</u> the effects of the physical environment upon the organization of myogenic populations. That physical forces have an effect on the organization of non-myogenic tissue systems <u>in vitro</u> was first investigated by Harrison (1914) and has since been shown by Glucksman (1939) with chondrogenic cells and by Weiss and co-workers with neurogenic cells (1934, 1956, 1959) and with connective tissue cells (1929, 1933). However, no <u>in vitro</u> analysis of the effects of mechanical forces upon the organization of myogenic tissue has been reported. Thus, in view of the strong claims made by Blechschmidt and Carey that physical forces are operative and are of prime importance in the organogenesis of vertebrate skeletal muscle, and in view of the lack of <u>in vitro</u> experimental verification of such claims, this study seemed warranted.

Since one method for studying the capacity of a tissue to organize has been established, i.e., to dissociate the cells and allow them to reaggregate, that procedure was employed in this investigation. The virtue of such in vitro experimentation is that variation of population size as well as of culture environment can be easily effected. The utilization of a variety of cultivation techniques, in each of which the tissues's environment differs, provides a system for elucidation of the response of reaggregating cells to the presence of qualitatively estimable physical forces. From such an experimental program might be developed a better understanding of the role of mechanical factors in the establishment of patterns within vertebrate skeletal muscle tissue during embryogenesis in vivo.

METHODS AND MATERIALS

White Leghorn chicken embryos of Hamburger and Hamilton (1951) stages 36-40 (10-14 days of incubation) were utilized as the tissue source for this investigation. At this period in development, myogenesis has begun and a spectrum of cellular differentiation is present. In addition, general tissue organization has been initiated and primitive skeletal elements are well defined.

Embryos were aseptically removed from the eggs and repeatedly washed in an isotonic calcium-magnesium free solution (GKN)[1] to which penicillin G (200 units/ml.) and streptomycin (200μg/ml.) were added. The hind limbs were removed at the proximal end of the femur. The limb was then bisected at the tibio-tarsometatarsal joint and the proximal portion transferred to fresh GKN. The epithelium was peeled off and the skeletal element extracted, leaving a collar of soft tissue. This tissue was transferred to fresh GKN and repeatedly minced by the scissors-like manipulation of two scalpels until tissue fragments approximately 1-2 $mm.^3$ were obtained.

The tissue mince was transferred to 100 ml. of dissociating medium in a 250 ml. screw-capped Erlenmeyer flask containing a teflon-coated magnetized stirring bar. Three media at pH 7.2 were used for dissociating the cell populations: (a) 0.25% trypsin in GKN, containing 100 units of penicillin G and 100μg of streptomycin per milliliter; (b) GKN with the same concentration of antibiotics as above; and (c) 0.02% versene[2] containing 0.12% methylcellulose and antibiotics as above

[1] 80.0 gms. of NaCl, 4.0 gms. of KCl and 10.0 gms. glucose per liter of distilled water.

[2] Ethylenedinitrilotetraacetic acid, disodium salt (Eastman Organic Chemicals, Rochester, New York).

(Merchant, Kahn and Murphy, 1960). The suspension was slowly and constantly rotated at constant temperature (37°C, 25°C or 4°C) for varied periods of time. The degree of dissociation was established by microscopic examination and viability counts were made by the vital dye exclusion technique with 0.4% erythrosin B (Phillips and Terryberry, 1957).

Following disaggregation, the remaining large tissue fragments were allowed to settle and the supernatant, containing small tissue fragments as well as cells was decanted into a sterile centrifuge. The cells contained in the supernatent were packed by centrifugation at 500 RPM for 10 minutes and subsequently washed once in nutritive medium and resuspended in a fresh solution. Medium #199 (Morgan, Morton and Parker, 1950) supplemented with 10% calf serum and antibiotics (penicillin G, 100 units/ml; streptomycin, 100µg/ml) served as the growth medium. Phenol red (.001-.002%) was added to all solutions as a pH indicator (Merchant, Kahn and Murphy, 1960). Total cell counts were taken on a Coulter Electronic Counter, Model A (Coulter, 1956) and culture vessels were seeded with 1.5×10^5 to 4.5×10^7 cells/ml.

Four cultivation techniques were utilized in this study. The first was a modification of Trowell's (1959) raft technique of organ culture. As shown in Figure 1, the culture vessel consisted of a 40 mm. watch glass in the concavity of which was placed a stainless steel wire mesh.[1] This watch glass was placed in a 60 mm. petri dish on top of 4-5 doughnut shaped filter paper discs. These discs served two purposes: a) to stabilize the convex surface of the watch glass; and b) to provide a humid atmosphere to the vessel, since 2-3 ml.

[1]United Foundation Wire Mesh, #46

of sterile distilled water were added to these prior to cultivation. This unit was wrapped in Patapar paper[1] and dry heat sterilized for two hours at 250°C. Medium was added to the sterile set-up, filling the space between the wire mesh and the concave surface of the watch glass. Because of the porous nature of the wire mesh, the tissue fragments had to be compacted prior to cultivation. For this reason, the tissue fragments and cells were centrifuged into a mass at 500 RPM for 10 minutes and then placed on the stainless steel raft. The explant was maintained at the interphase between the liquid medium and the atmosphere within the petri dish during cultivation. Because the cells were aggregated by centrifugation, this technique shall be referred to as the packed aggregation technique.

The second method of culture was the monolayer technique. In this procedure, as described by Earle and co-workers (1950), cells are cultivated on a glass surface with a fluid growth medium as an overlay. Ten milliliters of cell suspension were introduced into a large French square bottle as shown in Figure 2. The bottle was laid on its side and the cells allowed to settle during incubation. Since the cells were suspended in an unagitated medium and since assemblage was effected independently of medium agitation, this shall be termed the free aggregation technique.

The remaining two culture methods utilized extrinsic forces to keep the medium in motion and the cells in suspension were permitted to aggregate in this moving field. The first of these is a modification of a method originally introduced by Earle et al (1954) and recently adapted to and

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[1]A.J. Buck and Son

characterized for aggregation studies by Moscona (1961a,b; 1962). Thirty to fifty milliliters of the cellular suspension were added to a sterile 250 ml. screw-capped Erlenmeyer flask as shown in Figure 2. This vessel was mounted by clamps onto a rotary shaker table. Continuous rotation at 100 RPM induced a centripetal motion to the fluid and cells therein. This method shall henceforth be designated the centripetal aggregation technique.

The fourth procedure involved the use of a modified Microspinner flask[1] in the center of which was mounted a vertical teflon rod (Figure 3). At the top of this rod was a horizontally oriented teflon-coated magnetized metal bar. The central shaft was firmly mounted on a short spike in the bottom of the cylindrical flask and fitted into a tubular structure at its top. By means of a motorized magnetic stirrer positioned above the culture vessel, the central rod was rotated on its axis, thus generating a centrifugal movement of the cell suspension. The diameter of the central rod was 13 mm. and the radius of the medium chamber was 7.5 mm. Six to ten milliliters of cell suspension were cultivated in this vessel, producing a column of fluid approximately 20-35 mm. in height. This system was studied further by (a) varying the speed of rotation of the central rod from 150-450 RPM as determined by means of a Stroboscope[2] and (b) modifying the viscosity of the medium by the addition of Dextran[3], a high molecular weight (15-20,000) and inert molecule. The relative viscosity of each medium was calculated from readings with an Ostwald viscometer, using distilled water as the standard

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[1]Bellco Glass Company
[2]Strobotac type 1531-A, General Radio Company
[3]Pharmachem, Inc.

liquid (Bull, 1943). As discussed below, this method shall be designated as the centrifugal aggregation technique.

Theoretically, the two suspension techniques conform to 1) a free vortex (centripetal) system and 2) a forced vortex (centrifugal) system (Binder, 1962).

As shown in Figure 4, on any spherical particle, "A", in such suspension systems, there are three forces: 1) W: the weight of the particle due to gravitational pull; 2) CF or CP: the opposing centrifugal or centripetal forces imparted to "A" by the rotation of the fluid medium; 3) P: a force resulting from pressures exerted upon "A" by neighboring particles and equal in magnitude and opposite in direction to the resultant of CF(CP) and W. V is the velocity imparted to "A" by the rotating fluid. These systems differ from one another and are characterized by the net forces on the particle, as represented in Figure 4 by the solid arrow. In the centripetal system, the net force on suspended particles is centralizing; in the centrifugal system it is peripheralizing.

Incubation temperature for all cultures was 37°C. The time of cultivation varied with the technique. Organ cultures were maintained for 4-7 days, with medium changes on the third day; monolayers were cultivated for 8-10 days with medium changes every third day. Since medium changes could not be done without disrupting the fluid movement in the suspension cultures, these were only carried for 2-3 days.

Control tissues of the original explanted limb; the untreated mince; the residue after disaggregation; and the cellular suspension (smeared preparation) prior to cultivation were fixed. All cultured material was fixed immediately and embedded in paraffin as discussed below, with the exception of the free aggregation cultures. Both Bouin's and Formalin-

aceto-alcohol fixations were used. For histochemical staining, the latter fixative was preferred. The dehydrated tissues were cleared in carbon disulphide, embedded in paraffin, and sectioned at 4, 6 or 8μ thicknesses.

Free aggregation cultures were fixed, dehydrated and embedded *in situ*, using the collodion stripping technique (Reisseg *et al*, 1956; Enders and Peebles, 1954). The principle of this technique is to embed the cellular sheet in an overlay of collodion which, when hardened, could be peeled off the glass surface. Aggregates were cut from the collodion sheet and re-embedded in paraffin for sectioning. The remainder of the sheet was mounted on glass slides.

Routine histological staining was done with Harris' hematoxylin and eosin (3% eosin Y and 3% eosin B in 95% ethanol). For specific muscle staining, the acid alizarin blue modification of Conn *et al* (1960) and a Masson's trichrome procedure, wherein light green yellowish (2% in distilled water) replaced Masson C, were done. Neutral mucopolysaccharides were stained by the periodic acid-Schiff technique (Mowry, 1958); sulfated mucopolysaccharides were stained by the aldehyde fuchsin technique (Gomori, 1950; Cameron and Steele, 1959). Alcian blue (Mowry, 1956) or colloidal iron procedures (Steedman, 1950; Ritter and Oleson, 1950) were followed to localize acid mucopolysaccharides.

Since early stages in myogenesis are difficult to identify by histological staining, especially after cultivation, the immunochemical technique of Coons and Kaplan (1950) was used to identify the presence of myosin within cells as employed by Holtzer and co-workers (1957, 1959, 1960, 1961); Engel and Horvath (1960); Winnick and Goldwasser, (1961).

Myosin was extracted from the musculature of white rats according to the modification of Szent-Gyorgyi's original technique, as reported by Mihalyi (1950). The animals were decapitated, skinned, eviscerated and rapidly immersed into an ice water bath for ten minutes. The muscles of the legs and back were excised, cleaned of connective tissue and minced in a pre-chilled meat grinder. The mince was then suspended in 0.15M potassium phosphate buffer (pH 6.5) to dissolve the myosin. Actin contamination was minimized by keeping the temperature at which the extraction was conducted at 4°C. The addition of cold distilled water, followed by straining to remove the solid residue, left a liquid containing myosin in a crystalline state. This precipitate was collected by settling, decantation and centrifugation. By adding 0.6M KCl, the myosin was redissolved and the insoluble residue removed by centrifugation. Second and third precipitations were done and the final precipitate suspended in a 0.5M NaCl solution. The yield was a solution with 25.5 mg/ml. of antigen in 0.5M NaCl. This solution was stored at -10°C.

Following the technique of Klatzo et al (1958), the antigen, suspended in NaCl, was injected into 4-month-old New Zealand white rabbits in doses of 8-10 mg. Injections were administered in multiple sites, either intravenously via the marginal vein of the ear, intraperitoneally, or sub-cutaneously, the location varying with each injection. An incomplete Freund adjuvant[1], mixed in equal volume with the antigen, was used to increase the immune response (Freund and co-workers, 1942, 1947, 1951). Innoculations were given on alternate days for ten weeks. Six out of seven possible

[1]Difco, Bacto-adjuvant

injections during a two-week period were given, allowing a four-day rest period before the next injection. A dilution assay on samples of the immune serum was made at various intervals, using serial dilutions of the antiserum to assay for the antibody titer as described by Klatzo et al (1958). Maximal antibody titer was reached when a visible and stable precipitate was obtained with a 1:32 dilution of the antibody. Two days following this, the rabbit was exsanguinated by heart puncture. This immune serum was recovered by centrifugation for twenty minutes at 20,000 RPM. Sterilization was effected through membrane[1] and sintered glass filters. Ammonium sulphate fractionation of the globulin proteins from this serum was followed by the biuret procedure for total protein determination. Tetra-methyl rhodamine isothiocyanate was used for conjugation according to Riggs and associates (1958). The conjugated protein was then fractionated on a diethyl-aminoethyl (DEAE) cellulose column (Levy and Sober, 1960; Riggs et al, 1960). The antimyosin was eluted from the column by 0.125M NaCl, dialyzed against pH 7.2 phosphate buffered saline and stored at 4°C. Paraffin-embedded tissue sections of 2μ thickness were stained with fluorescent antibody for one-half hour and then studied with a Leitz Ortholux fluorescent microscope.

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[1]Millipore, HA type, .45μ pore size (±.2μ), Millipore Filter Corporation

RESULTS

Control Tissues

In intact as well as in de-epithelialized and de-skeletalized hind limbs of chick embryos of stages 36 through 40, myogenic tissue was organized into distinct bundles of four or more multinucleated and elongated cells, separated by a loose network of interstitial tissue (Figs. 5-7). The only observable difference in the myogenic population between these stages was in the relative proportion of cellular types. These multinucleated cells have been termed myotubes (Boyd, 1960) myosymplasts (Kliskow, 1960) or sarcoblasts (Cooper and Konigsberg, 1961a). These myotubes contained as many as 10-15 centrally located and rounded nuclei irregularly distributed within the sarcoplasm of the rod-shaped primitive muscle fiber and compressed in areas of nuclear clumping (Fig. 9). Their cytoplasm was highly refractile and exhibited heightened acidophilia in contrast to surrounding mononucleated cells of the interstitium. In longitudinal section, acidophilic intracytoplasmic fibrils (myofibrils) were clearly observed to parallel the length of the myotube and in some regions cross-striations were apparent (Fig. 8). Viewed transversely, the myofibrils occupied the peripheral or subsarcolemmal cytoplasm, leaving a centrally clear sarcoplasmic area in which the nuclei were located (Fig. 12).

Although the cytoplasm of myotubes was generally very acidophilic, variation in the intensity of this staining reaction among different cells, even within the same bundle, was marked. The regular appearance of this variable staining plus its appearance irrespective of both the location of the bundle in the thickness of the tissue and of the myotube

within the bundle suggest that it was not a fixation artifact but that it represented varying degrees of muscle fiber differentiation or functional contraction.

Staining with acid alizarin blue (Fig. 10) and Masson-light green (Fig. 11) also demonstrated the presence of myofibrils. These fibrils appeared as rounded condensations of acidophilic material within the peripheral sarcoplasm of some cells. Whereas other myotubes exhibited condensed masses in the sub-sarcolemmal cytoplasm, discrete fibrils were not evident (Fig. 11). This distinction may reflect a difference in degree of differentiation of the myotube.

Histochemical characterization of muscle cells showed a diffuse and weakly PAS-positive sarcoplasmic reaction with granular condensations of PAS-positive material (Fig. 14). These granules were possibly deposits of glycogen. Staining for acid mucopolysaccharides with alcian blue (Fig. 14) or colloidal iron (Fig. 15) demonstrated a uniformly negative muscle cytoplasm. Colloidal iron-positive fibers appeared between muscle cells at higher magnification. Aldehyde fuchsin staining of developing muscle was also negative, implying the absence of sulphated mucopolysaccharides (Fig. 16).

Stained with tetramethyl rhodamine isothiocyanate-labelled antimyosin, myosin-containing cells were readily distinguishable. The myotubes and the mononucleated myoblasts fluoresced brilliantly, in contrast to the interstitial tissue which exhibited a low-order background fluorescence. At high magnification, as is shown in Figure 13, the cytoplasmic condensations and discreet myofibrils within the myotubes were found to be the sites of intense fluorescence.

Connecting muscle bundles and filling the interstices between myotubes were mononucleated cells of varying morphology.

The first of these cell types was found closely applied to the surface of but not incorporated within the myotube. This cell was roughly spindle-shaped with a single round nucleus that was indistinguishable from those of the myotube (Fig. 8). The nucleus usually posessed one large nucleolus but might, in addition, contain several smaller satellite nucleolar bodies. Its cytoplasm manifested uniform diffuse to slightly granular basophilia. This cell has been described in the literature as a primitive muscle cell or myoblast (Boyd, 1960).

A second cytological type was found associated in loose networks in the intervals between muscle bundles. These cells appeared stellate or spindle-shaped in morphology with ovoid nuclei and weakly acidophilic cytoplasms. Their nuclei contained multiple small nucleoli, in contrast to those of the myoblast (Fig. 8). A definite orientation of these cells perpendicular to the myotubes and encircling the developing muscle was apparent (Fig. 5). Such an orientation of connecting tissue has been previously reported by Kitiyakara (1961).

Other cells distinguishable in the vicinity of the muscle fibers were erythroblasts. Endothelium-lined vessels were observed, containing these large mononucleated and rounded cells. These had a clear, watery cytoplasm containing a densely-clumped basophilic nucleus and were easily identifiable (Fig. 7).

Staining with Masson-light green and acid alizarin blue further substantiated the decreased acidophilia of the connecting tissue elements and the absence of fibrils. Histochemically, the cytoplasm of these interstitial cells was

completely PAS and aldehyde fuchsin-negative, indicating the lack of neutral and sulfated mucopolysaccharides, respectively. Compressed between these cells as well as between myotubes were short colloidal iron and alcian blue-positive fibrils and within the cytoplasm of some erythroblasts and mesenchymal cells were found acid mucopolysaccharide granules.

Staining of these interstitial cells with the fluorescent antimyosin was of a low intensity in contrast to the myogenic tissue (Fig. 13). Thus, differential staining with antimyosin is a sensitive technique which was used in conjunction with histochemical and cytological stains to aid in the identification of myogenic tissue both in control and in cultured material.

Observation of sectioned minced tissue fragments prior to dissociation revealed that they ranged from 1-2 mm. in size and that histologically they resembled the intact and de-epithelialized,de-skeletalized control limbs. In contrast to these controls, however, this mince which was centrifuged and fixed prior to treatment with a dissociating medium exhibited small quantities of an extracellular mucoid material. This substance was intensely acidophilic as shown by its staining with light green and eosin. It was also inconsistently PAS-positive, indicating the presence of neutral mucopolysaccharides, and does not contain acid mucopolysaccharides.

As expected, the post-dissociation fragments were smaller and the dissociation supernatant contained loose cells and tissue fragments, thus confirming the disintegration of the tissue. Histological changes during dissociation did occur. The residual minced tissue was found to contain increased quantities of extracellular mucin and the prevalance of this

material varied with the dissociation medium utilized and with the length of exposure to these media. After tryptic digestion for one-half hour at 25°C, little of this mucin was found, but the degree of dissociation of the tissue was low. Following a one hour treatment with trypsin at 36°C, the material was more prevalent and after six hours at a lower temperature (4°C), the mucin was pooled throughout the interstitial tissue of the mince. In contrast, exposures from one to 18 hours with GKN at 36°C yielded no increase in the presence of this material. Longer exposures to GKN resulted in increased dissociation and yielded less of this mucinous substance than with trypsin.

The greatest degree of disaggregation in the shortest time was obtained using versene. However, treatment for one-half hour at 36°C with versene resulted in an amount of extracellular mucin greater than that in the residual mince exposed similarly to trypsin. Therefore, .12% methylcellulose was added to the versene medium to reduce the possibility of injurious effects of the chelating agent upon the cell surface.

Following trituration, washing and resuspension in nutritive medium, samples of the dissociation supernatant were taken for total counting, viability counting and microscopic examination and the remaining suspension was used to seed the culture vessels. As mentioned above, higher total counts were obtained in a shorter exposure period with versene than with GKN or trypsin treatment. The viabilities obtained on the suspension resulting from trypsinization for one hour at 36°C were below 70%. With versene, viability counts ranged between 70-75% and with GKN treatment for 1-2 hours, counts were routinely above 75%. Extended exposures of the remaining minces in all media produced dissociated cells and tissue

fragments of decreasing viabilities. Individual cells as well as small tissue fragments which could not be separated by trituration were present in all dissociate supernatants. All cultures were seeded with this mixed suspension of tissue fragments and cells (Figs. 17 and 18). In the supernatants of all media, extracellular mucin was observed to surround tissue fragments.

Cultivated Tissues

Packed Aggregation Technique.

The tissue fragments and cells recovered from the dissociation medium were washed once with nutritive medium and randomly compacted by centrifugation. Part of this mass was fixed as a post-dissociation mince control and smaller segments were cultivated for 4-7 days aboard a stainless steel raft at the medium-vapor interphase of the culture vessel. Eight trypsinized and twenty GKN-treated cultures were studied. Following cultivation, these masses were compared with the pre-cultivation controls.

The post-dissociation mince control exhibited clusters of myogenic tissue in which all fibers of any one bundle were aligned similarly, but because of random packing a mosaic of bundle orientation was evident. After maintenance *in vitro*, as shown in Figure 19, the same mosaic appearance of fascicles was observed. It follows that fiber re-orientation was not effected during cultivation. In contrast to control tissue, the muscle bundles of cultivated tissue were compacted and the interstitial space was proportionately reduced.

Histologically, some myotubes exhibited degenerative changes but others appeared normal. These pathological alterations, when seen, were not restricted to specific

bundles but were found in transversely, obliquely and longitudinally sectioned myotubes. Pyknotic nuclei were found both intra-cellularly and extra-cellularly and were apparently derived from the enucleated myotube "ghosts" present and from erythroblast nuclei which appeared pyknotic even in control tissue (Fig. 7). Other myotubes exhibited typical morphology and even retained their longitudinal and cross-striations. Normal mononucleated myoblasts with basophilic cytoplasmic granulations could be seen. These retained their position around the myotubes. Interstitial tissue cells were present, but were not as prevalent as in control tissues. The loss of these cells can be attributed either to degeneration as evidenced by pyknotic nuclei seen in some of these cells or to their migration from the explant into the surrounding culture medium. Extracellular mucin was present in trypsin-treated tissue fragments but less of this material was found in GKN-treated tissue.

Free Aggregation Technique.

Cells and tissue fragments were recovered from the dissociation supernatant and maintained for 7-10 days under an overlay of unagitated nutritive medium on the plane glass surface of the culture vessel. Seventeen bottles were cultivated: 3 from trypsinized tissue; 6 from GKN-exposed tissue; and 8 from versene-treated tissue. Multicellular formed aggregates as well as isolated mononucleated and multinucleated cells of several types were observed in all of these cultures. Some of these cells had 3-4 cytoplasmic processes and a single central or peripheral nucleus with several nucleoli. Their cytoplasm was faintly staining with eosin and in general they resembled the mesenchymal cells seen in the intact control

limbs. Other mononucleated cells were highly attenuated and spindle-shaped with a centrally located round nucleus and a large single nucleolus. On the basis of their morphology and their highly refractive cytoplasm, they were classified as mononucleated myoblasts (Fig. 20). Capers (1960) has observed such myogenic cells of this character in vitro. Peculiarly, these myoblasts occasionally had knob-like cytoplasmic formations at either end, giving them a dumbell shape which was not observed in other mononucleated cells. Because this peculiarity is only seen in cells of myoblast morphology and only terminally in these cells, it is not considered a generalized "cytoplasmic bubbling" consequent to cultivation.

In addition, multinucleated cells which were spindle-shaped with a dense cytoplasm and knob-like termini were present (Fig. 21). These cells contained 3-6 round nuclei, each with single nucleolus, and were longer than the mononucleated myoblasts. Such cells exhibiting a highly acidophilic cytoplasm corresponded to the myocyte or the multinucleated myoblast.

A third cellular type exhibiting the highly refracting and deeply eosinophilic staining reactions of the myotube in vivo was observed. These gigantic cells (myotubes) were strap-shaped and were usually found to span the interval between two or more clusters of cells. They contained as many as 50 nuclei which were clumped together in the center of the hammock-shaped mass (Fig. 22). The nuclei were round with a single large nucleolar mass and a clearly defined nuclear membrane.

Spherical multicellular aggregates were also common in these cultures. Spindle-shaped multinucleated cells (myotubes)

radiated from these aggregates where they contacted the glass surface. When sectioned, these masses revealed at high magnification multinucleated cells oriented in a radial fashion with stratified squamous epithelioid cells at the periphery of the aggregate (Fig. 23). When stained with Masson-light green, these mononucleated epithelioid cells were characterized by a weak cytoplasmic affinity for light green in contrast to the central cells which displayed a greater acidophilia. On the basis of this differential staining and in view of a similar distinction in the control tissue, the central multinucleated cells were identified as myotubes and the peripheral ones, interstitial cells. Staining with fluorescent antimyosin further corroborated this interpretation, since an intense fluorescence was observed only in the central cells.

In addition to the cytology described above which closely resembled stages in muscle differentiation in vivo, a number of interstitial tissue cells could be seen. These were distinguishable from the myogenic cells on the basis of staining reaction and morphology. The interstitial cells possessed a less refractile and acidophilic cytoplasm than the muscle cells (Fig. 22). Morphologically, the former were mononuclear in these cultures. Where it was difficult to ascertain the presence of multinuclearity, the nuclei were not clumped together as described in the giant muscle cells. Secondly, the nucleoli of the muscle cells were always large and single while the non-myogenic nucleoli were multiple. Interstitial cells never exhibited the high degree of attenuation seen in the myoblast and usually had several cytoplasmic processes in contrast to the constantly spindle-shaped myoblasts.

A close correlation existed between the cell population density and the presence of myoblasts, myocytes, myotubes or muscle aggregates. In areas of very dense cell accumulations muscle aggregates were formed, whereas myotubes were found at the periphery of these areas, spanning the interval between two or more clusters of cells. Multinucleated myocytes and myoblasts were always seen in sparsely-populated areas. This suggests that the degree of differentiation of these cells was restricted or conditioned by the cell density of the immediate vicinity. The myoblasts and the myocyte did not differentiate further due to the lack of cells with which to fuse. The giant myotube in turn probably represented the unrestricted fusion of mononucleated cells consequent to an inadequate population size. The formation of several smaller myotubes occurred where cell density was sufficient, as seen in the muscle aggregate as well as <u>in vivo</u>.

Centripetal Aggregation Technique.

After cultivation for 1-2 days in the presence of a centripetal force, dissociated tissue fragments showed striking reorganization of muscle bundles. Although this myogenic tissue still exhibited the grouping of myotubes into small clusters of similarly aligned fibers, these fibers were organized in a definitely concentric or onion-skin arrangement within the newly-formed spheroid or ovoid explant (Fig. 24). Comparison with control tissue recovered from the dissociating flask, washed, centrifuged and fixed demonstrated that the mince did not exhibit this characteristic curving of muscle bundles. It follows that such reorganization must have been effected during exposure to the centripetal force.

A second change in tissue organization which is attributed to the centripetal technique is the high degree of

compaction of muscle tissue. This change may be the result of the loss of tissue from between and within groups of myotubes, since a definite accumulation of interstitial cells peripherally and the formation of a stratified epitheliod coat from these cells is observed in these explants (Fig. 25). The interstitial networks remaining between muscle bundles seem partially disrupted in contrast to the well-formed and organized meshwork of the control series.

The only degenerative changes seen in these cells were found at the periphery of the explant. Extracellular mucin was found around the periphery of tissue fragments in small amounts in all cultures.

It is important to emphasize that in the presence of the centripetal force, all culture vessels contained not one but many individual rcunded explants ranging in size from a fraction of a millimeter to slightly more than one millimeter in diameter. In no case was there observed the mass clumping of fragments into a single central explant. The fusion of smaller fragments to others did probably occur but, in general, these tissue masses acted as units and organized themselves internally in response to their new environment.

Considerable turbulence was observed in the swirling fluid suspension during cultivation. Although surface foaming was not great, fluid flow was definitely not streamlined, and the surface of the medium was in continual disorganized agitation. These results are based on 10 cultures: 2 from trypsinized tissue; 4 from GKN-treated tissue and 3 from versene-treated tissue.

Centrifugal Aggregation Technique.

Suspensions of cells and tissue fragments recovered from the supernatant dissociation medium were cultivated in the

presence of a rotation-generated centrifugal force. Theoretically, such a force would be expected to push cells peripherally and thereby disperse them. However, a single large aggregate was formed around the central teflon rod. The first gross change in the cultures was the formation of small cellular aggregates below one millimeter in diameter, whose size increased with time. The appearance of these small clusters began to occur within one-half hour after cultivation was initiated.

Subsequently, these small aggregates were observed to fuse with one another, forming increasingly larger masses (Fig. 26). As fusion progressed, a string-shaped mass was formed which eventually became continuous by wrapping itself around, but not adhering to, the central rotating rod. Ultimately a toroidal (torus) or doughnut-shaped single explant was constructed within the culture vessel. The only subsequent gross change to be observed was an increase in the cohesiveness of this central explant. When first constructed, the torus could be disintegrated by interrupting the field of force. In time, however, the integrity of the explant was sustained even in the absence of rotation of the central rod. Thus, within a period of 12-24 hours, a self-sustaining toroidal explant was constructed (Fig. 27).

Microscopic examination of the early spherical masses revealed that they were multi-cellular bodies whose fusion was effected by the intermingling of and mutual adhesion of surface cells. By the progressive fusion of individual masses, an elongated chain of cells was formed, which eventually met itself and established a doughnut-shaped (toroidal) explant by end-to-end fusion.

Interestingly, the explant was never found to be cylindrical since it had very little height and occupied a single horizontal locus around the central teflon rod. In different cultures, the locus of explant formation varied. In an attempt to characterize the determinants of this positioning, measurements of the height of torus formation relative to the height of nutritive medium and variation in this position with speed of rotation and cell population number (cells/ml.) were recorded. Analysis of this data revealed that the location of the explant along the height of the vertical rod varied directly with the speed of rotation, with total column height and cell number as constants. A direct variation with the cell population density, when column height and speed of rotation were held constant, was also apparent. The location of the torus relative to the total height of the column of medium was independent of variation in the quantity of cell suspension cultivated.

It was difficult to remove the explants from the teflon rod as an intact mass. Although the tissue was not adherent to this structure, it was tightly wrapped around the rod. Consequently, the tissue was usually removed from the rod as an interrupted string-shaped mass and fixed as such. Histologically, the torus was composed of a densely packed mass of cells, which when examined along certain planes of section were aligned in curving parallel rows that extended in a radial direction. These rows were separated by loosely organized cells (Fig. 28). Examination of sections stained with anti-myosin indicated that the rows were composed predominantly of mononucleated with some multinucleated myosin-containing cells. Contrastingly, the cells of the interstices stained lightly with the antibody. Regions of differential cell

density were observed in transverse sections of these aggregates (Fig. 29). In some areas dense cellular accumulations were evident, as distinguished from areas with less cellular compaction, suggesting the establishment of myogenic foci. Around the periphery of the torus, cells were found associated in a stratified squamous epithelioid covering. Such cells were non-myogenic and completely encircled the explant.

Degenerative cellular products were found in these aggregates. Pyknotic nuclei, stripped of cytoplasm, were evident and may be attributed to either degenerated erythroblasts or enucleated "ghosts" of myotubes as discussed above. Infrequently, pools of hyaline acidophilic material were observed in these aggregates. This substance was most prevalent in the trypsinized tissue aggregates and, even with the trypsin-treated cells, less material appeared than was found in the concurrent centripetal cultures. In view of the nuclear degeneration, this material was considered the product of corresponding cytoplasmic degeneration or leakage and not a normal cellular exudate. Thirty-three such explants were studied (12 trypsinized, 7 GKN-exposed and 14 versene-treated).

Since the centrifugal aggregation technique was designed specifically for this investigation, the reliability of this procedure was ascertained by subjection of the system to a series of modifications. Variation in the density of the cell population, the speed of rotation and the viscosity of the cultivation medium did not alter the microscopic anatomy of the explants. However, alteration of the time required for cellular assemblage was noticed. All of the cells used for these modifications were obtained from stage 40 embryos.

Cell Population Density and Aggregate Formation. A series of six cultures of cell numbers ranging from 2×10^5 through

$3x10^6$ was maintained for 48 hours. The rotation frequency for these was kept at 300 RPM and the formation of aggregates was grossly observed at 12 hour intervals.

A unified aggregate was not observed in the culture of $2x10^5$ cells/ml. Even after 48 hours of rotation only small clumps, slightly larger than those of the original suspension were seen at the termination of the culture period. In contrast, formed doughnut-shaped explants appeared in cultures of $4x10^5$, $6x10^5$, $8x10^5$, $1x10^6$, and $2x10^6$ cells/ml. Large spherical conglomerates were present in the culture of $3x10^6$ cells/ml. after 12 hours, but confluence of these into a torus was not apparent until after 24 hours had elapsed.

Such information suggests that the formation of a doughnut in this system can occur over a 10-fold range of cell population densities. However, a critical minimum population number is needed for the formation of such an aggregate, and above a certain level the time for doughnut formation is retarded.

Speed of Rotation and Aggregate Formation. In three simultaneous cultures of $1x10^6$ cells/ml., the rotation frequency of the teflon rod was set at 150, 300 and 450 RPM as determined by the use of a stroboscope. Cultivation was conducted for 24 hours which was sufficient for aggregation at 300 RPM as described above. The apparent sizes of the aggregates were observed after 1 hour and at approximately 4 hour intervals thereafter. The data obtained indicates that the rate of formation of small spherical aggregates, the rate of fusion of these aggregates and the degree of cohesiveness of the toroidal explant are all dependent on the speed of rotation of the central rod.

The size of the small spherical aggregates was graded from (-) to (+++), where (-) was the approximate size of clumps in the suspension at the initiation of the culture. Cohesiveness of the torus was established by observing its disintegration consequent to interruption of the rotation-mediated centrifugal force. All explants were found to re-form at their previous locus on the central bar shortly after resumption of rotation.

As seen in Table 1, all rotation speeds resulted in the formation of a toroidal explant. However, with higher frequencies (1) the increase in size of the original spherical aggregates was delayed and consequently (2) the fusion of these initial clusters into a single toroidal aggregate was retarded but (3) the time required for the establishment of the cohesiveness of the torus was reduced.

Culture Medium Viscosity and Aggregate Formation. A series of three cultures was conducted, wherein increasing concentrations of Dextran, an inert molecule of a molecular weight of 15-20,000, were added to the nutritive medium. The viscosities of these solutions as well as others are plotted in Figure 30. Figure 31 shows the change in viscosity of the culture with increasing cell number. As anticipated, the viscosity of the cultivation medium increased directly with augmentation of the cell population density. Maintaining a constant cell number, the viscosity of the cell suspension increased beyond this baseline viscosity for that population as increasing quantities of Dextran were added.

Cultures of $3x10^6$ cells/ml. were maintained at 300 RPM for 48 hours during which gross observations were made at varied intervals as shown in Table 2. The size of spherical aggregates was recorded with (-) representing the size of

clumps in the suspension when rotation was initiated. The cohesiveness of the toroidal explant was determined by interrupting the rotation of the central bar. Rotation frequency was maintained at 300 RPM.

Although increased viscosity temporally hindered aggregation of cells, once a toroidal aggregate was established cohesion was more rapidly acquired than in solutions of lower viscosities (see Table 2). Despite alterations in viscosity, toroidal explants were generated in all cultures.

Table 1: The Formation of Aggregates as a Function of the Frequency of Rotation in the Centrifugal Aggregation Technique ($1x10^6$ cells/ml.)

Cultivation time (hours)	Gross appearance of explant at rotational frequency of:		
	150 RPM	300 RPM	450 RPM
0	-	-	-
1	++	+	-
5	+++	++	+
8	torus	+++	++
12	torus	torus	+++
16	torus	cohesive torus	cohesive torus
24	cohesive torus	cohesive torus	cohesive torus

Table 2: The Formation of Aggregates in Solutions of Varying Viscosity in the Centrifugal Aggregation Technique ($3x10^6$ cells/ml.; 300 RPM)

Cultivation time (hours)	Gross appearance of explant with concentration of Dextran of:		
	0 mg/ml	4 mg/ml	12 mg/ml
0	-	-	-
2	++	+	-
7	++	+	-
14	+++	++	+
24	torus	+++	++
38	torus	torus	++
48	cohesive torus	cohesive torus	cohesive torus

DISCUSSION

I. The *In Vitro* Aggregation of Dissociated Skeletal Muscle Tissue.

That disaggregated embryonic cells reassemble *in vitro* is a well documented phenomenon. Moreover, these reaggregations are not random conglomerations of cells. Rather, the cells sort themselves by mutual interaction so that patterns typically seen *in vivo* are established (Moscona, 1957, 1962). The mutual architectural relationships between these cells is determined by intrinsic properties of the cells themselves (Townes and Holtfreter, 1955; Moscona and Moscona, 1952).

The present investigation has been undertaken (1) to examine the reaggregative behavior of dissociated embryonic skeletal muscle cells *in vitro* and specifically (2) to analyze the effect of mechanical forces upon the establishment of patterning within this tissue. To test these forces, different cultivation techniques were utilized. The virtue of these techniques was that each was characterized by a different physical environment. If the component cells intrinsically dictate the architecture of the reaggregated tissue as suggested by Holtfreter and Hamburger (1956), then any deviation in patterning within the explants effected during cultivation should be a direct reflection of the physical environment upon cellular behavior.

As expected, disaggregated myogenic cells do reassemble during maintenance *in vitro* even in suspension and more importantly, microscopic examination of these aggregates reveals that the cyto-architecture established varies with the cultivation technique employed. Although there is variation

in pattern, the morphologies of all aggregates bear resemblances that are independent of the technique utilized. In review, two sets of characteristics are evident in the cultures: First, there is a constantly appearing set which is considered the intrinsic expression of myogenic cellular behavior and which is unmodified by the in vitro technique; secondly, there is a set of characteristics which is superimposed on the intrinsic qualities of all cells in vitro which varies with the method of cultivation and which is considered the extrinsic effect of the physical environment upon these cells.

Intrinsic Determinants:

Common to the cultured myogenic tissue is, in all techniques, its association into discrete foci. In the packed aggregates, formed by centrifuging tissue fragments into a mass which was subsequently maintained as an organ culture, grouping of muscle tissue is retained during cultivation. Despite the reduction of interstitial space which accompanies the relocation of connecting cells, no disintegration of the muscle bundles occurs. Local accumulations of myogenic tissue are also retained during cultivation in the presence of a centripetal force, despite a shift in muscle bundle orientations and despite the relocation of interstitial cells between these fascicles. Those aggregates generated in unagitated medium (free aggregates or "self aggregates" [Moscona, 1952]) reveal the accumulation of myogenic cells in spherical arrays coated with an epitheliod assemblage of non-muscle tissue. When cultivated in a centrifugal field, cells also compact into aggregates containing foci of myogenic cells enveloped by a loose network of interstitial tissue. Hence, in vitro these embryonic cells mimic the bundling of myological tissue

characteristically observed *in vivo* and they do so regardless of the mechanical nature of their environment.

Further evidence of intrinsic cellular behavior is derived from the parallel alignment of cells within myogenic *fasciculi*. Even where bundle re-orientation was effected in the centripetal system, muscle cells retain their parallelism. It seems that this histological rearrangement is not a shuffling of independent cells but a reorganization of multicellular assemblages acting as units. Similarly, cellular alignment is maintained in the packed aggregates. In the free and centrifugal aggregations a radial organization of muscle fibers is seen, corroborating the work of Wilde (1959) and demonstrating that the organogenic behavior of myogenic tissue can proceed in suspension. This radial patterning is perhaps an expression of parallelism by the component cells, although a slight deviation from a strictly mathematical definition of this arrangement exists.

In summary, the data indicates that myogenic tissue aggregates retain organizational qualities which supercede the effects of the *in vitro* physical environment. The similarity of such inherent cellular organization to that seen *in vivo* substantiates the intrinsic quality of this behavior.

Extrinsic Determinations:

An analysis of the effect of physical factors on patterning in reaggregating tissue must be predicated on a qualitative knowledge of the forces characterizing the *in vitro* techniques utilized. The following forces are exerted upon all aggregates: (1) the force of gravity, (2) the intercellular forces of cohesion which are responsible for the maintenance of explant integrity and (3) surface tension.

Although these three are the major forces on the cells in organ culture (packed aggregates), the following additional forces are exerted on explants formed in the remaining culture systems. Free aggregates are exposed to the pressure exerted by the fluid overlay. For the two suspension systems, vectorial diagrams of the forces on a hypothetical particle are presented in Figure 4. Common to these is an influence contributed by neighboring cellular and fluid particles in suspension. Another is generated by the rotatory action of the vessel, imparting a velocity to the contained mixture. However, the primary forces in these systems are the centripetal and centrifugal ones.

The variability in tissue pattern seen in different cultures can be directly correlated with the mechanical characteristics of the physical environments as described above. Macroscopically, the globular shape of all aggregates is a reflection of those pressures exerted upon the explants' surface plus the cohesive interaction between component cells. Modifications of this common gross structure are found in the free and packed aggregates which have one flattened surface ascribable to the compressing gravitational force. The uniformly toroidal (doughnut-shaped) centrifugal aggregate and the spheroid or ovoid centripetal aggregate also owe their gross shape to the physical environment. In both, pressure exerted by the medium has rounded these masses.

Not only variation in the gross appearance of these explants, but also their microscopic cyto-architecture reflects the qualitative character of the forces to which they were exposed. Packed aggregates are to be contrasted with the other aggregates, since they were not submerged in and consequently not subject to forces applied by a fluid medium. The

mosaic of myogenic bundle orientations in the organ cultures is apparently the unaltered remnant of the pre-cultivation packing.

Cytological organization effected in the other aggregation techniques definitely reflects the nature of the physical environment. The spheroid shape assumed by the centripetal aggregates during cultivation reflects a histologic rearrangement of muscle bundles into concentric layers. Elongated myotubes in these centripetal assemblages are generally curved in accord with the curvilinear shape of the bundles. On the other hand, myogenic tissue in the free and centrifugal aggregates is organized into radiating rows of cells, but these rows in the latter are curved. Fluid flow in the centrifugal system is streamlined in concentric circular paths in which the tangential velocity of medium flow at any point is inversely proportional to the distance of that point from the surface of the central rotating rod. Hence, the cells in suspension at the periphery of the torus are in a field of decreased velocity in contrast to the more centrally positioned cells. Whereas the cells can align in radiating columns, they are unable to overcome this velocity differential and assume curving rows.

Only the effects of qualitatively varying forces have been considered above. Quantitative alterations, which were possible only in the rotation-mediated centripetal and centrifugal systems, were not found to alter the gross or histological structure of aggregates. In the centripetal system, Moscona (1962) has reported an inversely proportionate relationship between aggregate size and the speed of rotation. In contrast, changing the speed of rotation in the centrifugal system only prolonged the formation of the aggregate.

From the preceding discussion, it may be concluded that two sets of factors operate on the myogenic tissue aggregate. Superimposed on the pattern of development inherent to the cultured cells themselves, which bears a remarkable similarity to *in vivo* skeletal muscle tissue patterns, is the effect of the physical environment upon cyto-architecture. The close degree of correlation between the forces to which the tissue are exposed and the resultant tissue organization leads to the conclusion that myogenic tissue patterning is susceptible to control by extrinsic forces *in vitro*.

A review of previous reaggregative studies indicates that such work with dissociated embryonic cells has constantly disregarded a fundamental determinant of tissue organization. Steinberg (1962a) concludes that:

> "... the mutual architectural relationships assumed by cells in vertebrate tissues and organs are determined by intrinsic properties of the individual cells themselves ...".

Also, Moscona (1961b) demonstrates that:

> " ...by using rotation-mediated cell aggregation, it is possible to synthesize *in vitro* tissue-like structures from discrete embryonic cells ..."

and that these "aggregation patterns" effected

> "represent a typical, reproducible and measurable expression of cell behavior."

In all of this work the effect of *extrinsic factors* inherent to the cultivation technique is completely ignored. Although the present study corroborates an intrinsic control of tissue patterning in the aggregates, it also demonstrates that modifications may be superimposed upon this basic pattern by varying the cultivation technique to which the tissue is exposed. Thus, previous reaggregative work should be

reappraised with a recognition of extracellular determination of tissue structure.

Returning to a consideration of muscle tissue specifically, the existence of a set of organogenic factors intrinsic to the myological cells themselves as suggested by Holtfreter (1947) and Pietsch (1962a,b) is confirmed. Furthermore, the results of the present study substantiate an influence of extrinsic environmental factors upon myogenic patterning as reported by Carey (1922b), Blechschmidt (1960) and Hall (1950). But above and beyond verifying the existence of these determinants, this study has demonstrated their concomitant interaction in vitro. It is emphasized, in contrast to previous work, that an understanding of the architectural genesis of vertebrate skeletal musculature must be an expression of the effect of physical forces upon the organizational behavior of the tissue.

McMurrich's (1923) set of fundamental processes of myological organogenesis may now be reconsidered. The association of muscle cells, the parallel orientation of elongating fibers, the differential relocation of adhesive and unified masses as well as the fusion, subdivision or degeneration of masses seem best interpreted as the activity of the cells themselves. Modifications of gross muscle patterns which are superimposed on the fundamental behavior of the cells themselves may then be considered the expression of extrinsic determinants. Thus, the direction of fibers in the gross muscle as well as the shape of the muscle and its location in the body can be attributed to the environment in which the cells developed.

The present investigation is by no means exhaustive. Not only is further in vitro investigation on the organogenesis

of embryonic skeletal muscle still to be desired, but the critical evaluation of such *in vitro* experimentation must rest on correlative studies *in vivo*. The basic organization characterizing this tissue *in vitro* is exemplified also by embryonic skeletal muscle in the embryo. However, the effects of the environment as shown in this study cannot yet be correlated with physical conditions of *in vivo* organogenesis. Such information is presently undetermined and can only be obtained from a more intimate acquaintance with the intact and viable embryo itself.

II. The Centrifugal Aggregation Technique

It is not completely clear why aggregation is enhanced in the presence of a dispersing centrifugal force beyond that seen in a centralizing centripetal field which would seemingly promote cellular adhesion. One possible explanation for this distinction is that the centrifugal force induces increased cellular adhesiveness. Moscona (1962) has correlated the size of aggregates in the centripetal system with cellular cohesion. When decreasing the temperature or increasing the rotational frequency in this system, smaller aggregates were generated. He attributes this observation to a decrease in cellular cohesiveness. Accepting the fact that environmental conditions do modify the degree of intercellular cohesion, the centrifugal field might operate to augment this behavior.

A mechanism for this augmentation has been suggested by Moscona (1961b). He states that cells possibly excrete "aggregating supporting moieties" or cementing substances. The mucoid material found around cells after trypsin disaggregation is singled out as such an exudate. In contrast, Rinaldini (1959) has reported that trypsin hydrolyzes intercellular mucoprotein and thus generates large amounts of this viscous residue. He considers this material not as an exudate or a physiological substance but as a jelly-like hydrolysate consequent to tryptic action. Steinberg (1958) has further suggested another mechanism for the production of this material. He reports that it might be a colloidal substance leaking from the cells due to a permeability change consequent to pH alterations or to the loss of extracellular calcium. The trypsin, he suggests, merely digests this material and does not elaborate the leakage at all.

If such a material were a normal exudate from the cells as suggested by Moscona, one would expect an increase in adhesiveness concomitant with a quantitative or qualitative change in this material. No such quantitative increase was observed in the centrifugal aggregates to explain the increased degree of cellular clumping in comparison to the centripetal technique. Qualitative variation was not ascertained in this study. Furthermore, the material Moscona describes was found to be less prevalent with the use of other dissociating media. Consequently, it was considered to have no normal physiological importance, but rather to be a degenerative product. Since the extracellular mucin was found to be decreased with the use of versene or GKN and since it was more prevalent with increased exposure to trypsin, undoubtedly this material reflects tryptic activity. In view of the staining reactions of this mucinous material which closely resembles that of cytoplasm, the substance might be due to leakage (Steinberg, 1959) and might also reflect tryptic digestion of intercellular mucoprotein (Rinaldini, 1959) which normally maintains cell membrane integrity.

A second explanation for enhanced clumping in the centrifugal technique might be due to an increase in cellular necrosis and cytoplasmic leakage. Since the aggregates from the centripetal and centrifugal procedures exhibited comparable amounts of necrosis and apparent cell leakage, this explanation would not fully account for the differences in aggregability.

A third explanation for the comparatively decreased degree of aggregability in the centripetal technique might be inherent to the technique itself. As observed in this investigation, and as noticed by Moscona (1961b), turbulence exists in this

system. Moscona reports this as "shearing forces of the circulating liquid acting to keep them [the cells] dispersed". In contrast, fluid flow in the centrifugal system is more streamlined, as evidenced by both a smooth meniscus at the surface of the medium and also by the orderly movement of grossly observable aggregates during cultivation.

Another characteristic of the centrifugal technique is the acquisition of adhesiveness of the toroidal explant secondary to its formation. This suggests the "sorting out" of cells, resulting in a stable association of similar cellular types from a previous condition of unstable, rotation-dependent and non-selective attachment of cells. Such would support the "specific adhesion theory" originally proposed by Holtfreter (1939) and recently supported by Moscona (1960, 1961b) and Steinberg (1958).

Modification of the centrifugal aggregation technique reveals that the viscosity, the cell population density and the speed of rotation of the cultivated suspension are all important determinants of aggregate formation. In the presence of culture media of increased viscosity, the initial assemblage of cells is temporally retarded. Such might be expected since one would have to exert a greater effort to approximate two gross spherical bodies immersed in a medium of molasses than in an aqueous medium. It follows that any two cells coming together in a fluid medium of low viscosity would do so with greater frequency than if in a highly viscous medium. Since intercellular contact is undoubtedly a prerequisite for adhesion, and assuming no mechanism for increasing the range of action of intercellular forces, the speed of aggregation would be less in the presence of heightened viscosity.

A mechanism for the impedance of cellular assemblage with increased cell population density might be suggested by specific adhesion of cell types (Holtfreter, 1939). Although cells would tend to meet other cells with greater frequency in a densely populated solution, the establishment of adhesion of like cell types might be hindered by virtue of the increased viscosity of the suspension.

Torus formation in the centrifugal system is delayed with increased speeds of rotation. In the centripetal system, Moscona (1962) has observed that cellular assemblage is diminished in the presence of increased rotational speeds. In both the centripetal and centrifugal systems, this decrease in aggregation probably is attributable directly to the force applied. Increasing the rate of rotation is thought to augment the mechanical field and increase cellular agitation. Such might be disruptive to adhesion of cells, thus retarding aggregate formation.

In conclusion, the centrifugal technique has provided information on the effect of a particular environment upon the aggregability of myogenic cells. The contrasting character of the physical forces associated with this system to that of the centripetal technique indicate that the two methods employed concurrently might provide a desirable tool for future studies with reaggregating cell systems.

SUMMARY

It is commonly accepted that vertebrate skeletal muscle organization *in vivo* is characterized by discreet *fasciculi* of similarly aligned cells surrounded by connective tissue condensations. The factors responsible for this patterning during embryogenesis are obscure.

The reaggregation of chick embryonic muscle tissue has been investigated *in vitro* to determine the factors responsible for myological organogenesis. Dissociated tissues were maintained (a) as an organ culture at the surface of the nutritive medium (packed aggregation); (b) as a monolayer on the surface of a glass culture vessel with an overlay of nutrient medium (free aggregation); (c) as a shaker suspension exposed to a centripetal force (centripetal aggregation) and (d) as a spinner suspension exposed to a centrifugal force (centrifugal aggregation).

The results of this investigation reveal that regardless of the technique utilized for cultivation *in vitro*, dissociated chick myogenic cells aggregate to form a tissue exhibiting bundles of cells aligned in parallel array and united by an interstitial cellular network. Superimposed on this architecture are modifications reflecting the physical character of the cultivation technique. Consequently two sets of determinants govern muscle tissue organogenesis: (1) an intrinsic set which is inherent to the cells themselves and (2) an extrinsic set attributable to the environment in which the cells develop.

Furthermore, these results indicate that *in vivo* the organization of muscle tissue may also be explained in terms of these two factors. The aggregability of embryonic cells

into fascicles, the secondary subdivision or fusion of masses and the parallel alignment of elongated cells within a fascicle are all intrinsic expressions of cellular behavior. Although susceptible to modification, this intrinsic behavior is never eradicated. Any variation in gross myology seen in the adult body as well as any variations seen among individuals must be attributed to the imposition of environmental factors upon the cells. The anatomical orientation of fibers within a muscle, the shape of that muscle and its location within the body are direct reflections of the environment in which this tissue has developed.

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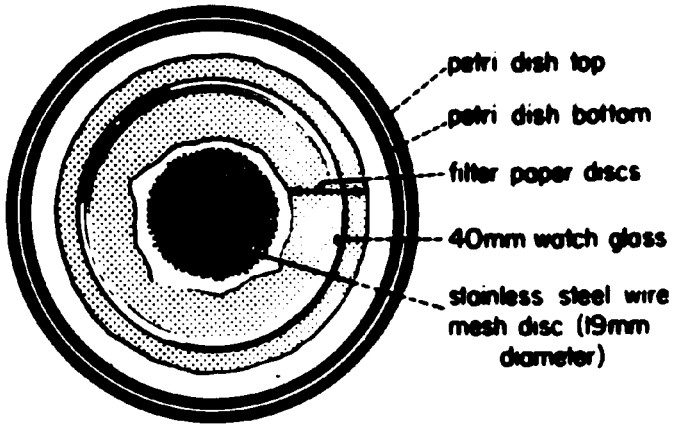
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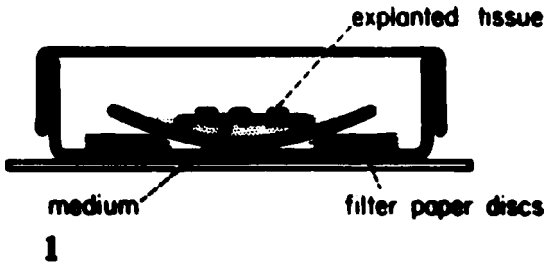
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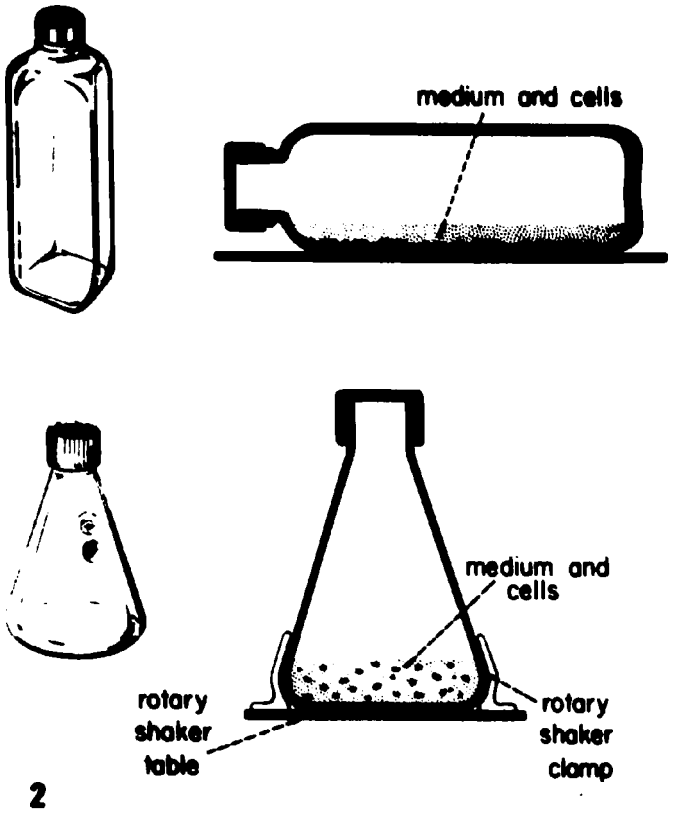
Figure 1. Top and mid-sagittal views of packed aggregation (organ culture) system.


petri dish top
petri dish bottom
filter paper discs
40mm watch glass
stainless steel wire mesh disc (19mm diameter)


explanted tissue
medium
filter paper discs

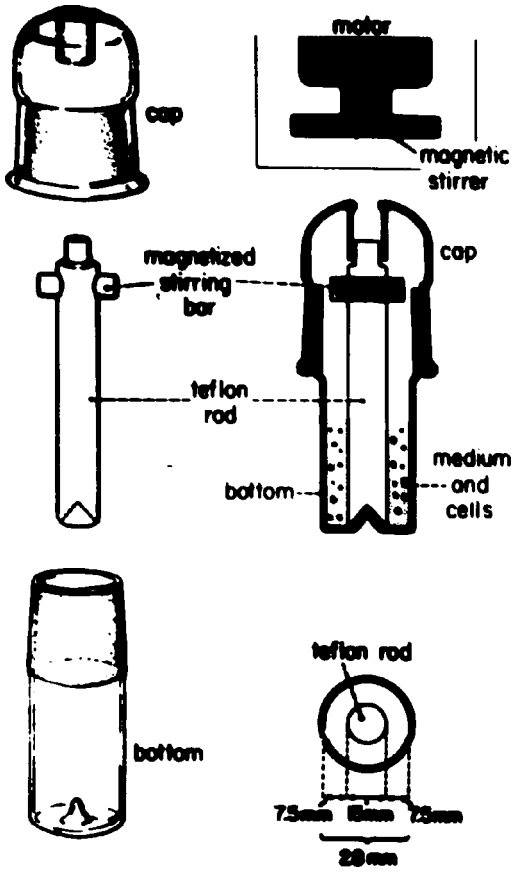
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Figure 2. Mid-sagittal section views of free aggregation (monolayer) system (top) and centripetal aggregation (shaker) system (bottom).


medium and cells
medium and cells
rotary shaker table
rotary shaker clamp

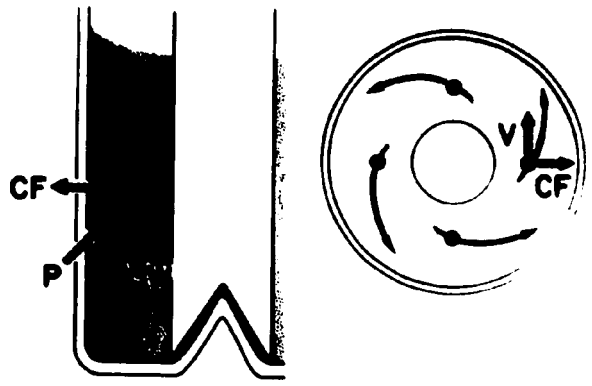
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Figure 3. Exploded view (left), mid-sagittal view (right top) and cross-sectional view (right bottom) of centrifugal aggregation system.

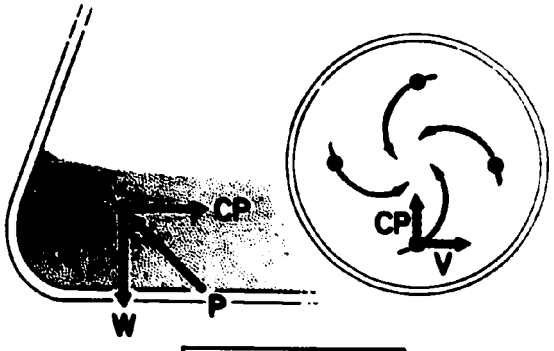

cap
motor
magnetic
stirrer
magnetized
stirring
bar
cap
teflon
rod
medium
and
cells
bottom
bottom
teflon rod
7.5mm
15mm
7.5mm
30mm

3

Figure 4. Vector diagrams showing forces on a theoretical particle (A) in centrifugal and centripetal systems. CF = centrifugal force; CP = centripetal force; W = gravitational force; P = pressure of neighboring particles; V = velocity.



Centrifugal System



4

Centripetal System

Figure 5. Longitudinal section through the intact limb musculature of a stage 40 (14d) chick embryo demonstrating the orientation of interstitial tissue perpendicular to muscle fibers in some areas. Hematoxylin and eosin; 350x.

Figure 6. Longitudinal section through the intact limb musculature of a stage 40 (14d) chick embryo. Notice the fasciculation of myotubes separated by a loose network of interstitial tissue. Hematoxylin and eosin; 350x.



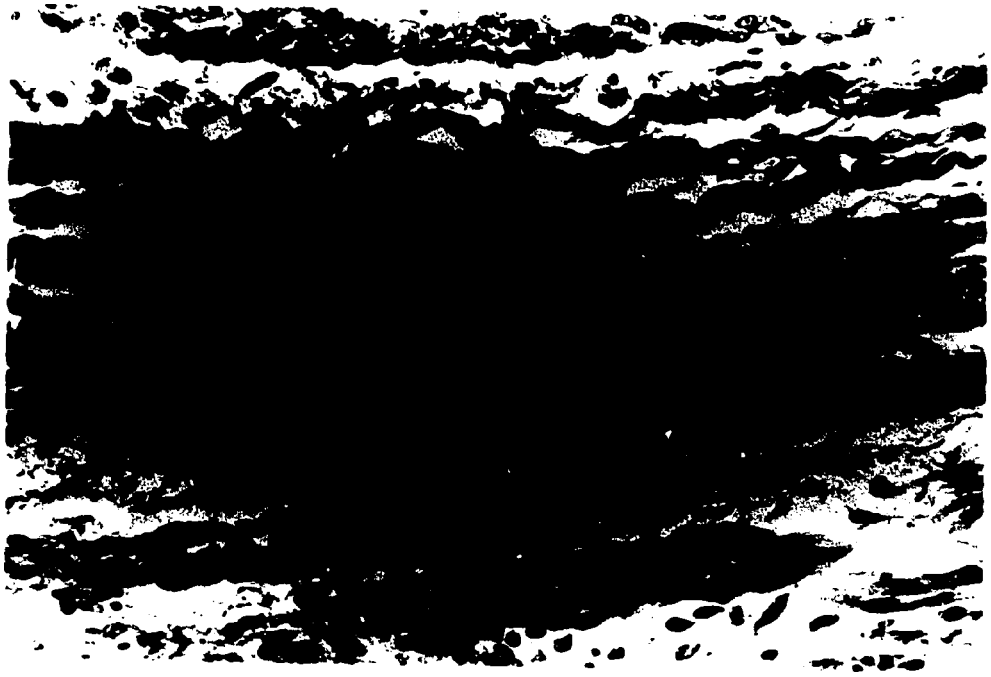


Figure 7. Transverse section through the intact limb musculature of a stage 40 (14d) chick embryo. Note the definite fasciculation of muscle tissue; also note the presence of myofibrils in some myotubes (short arrow) and the presence of erythroblasts (long arrow). Hematoxylin and eosin; 350x.

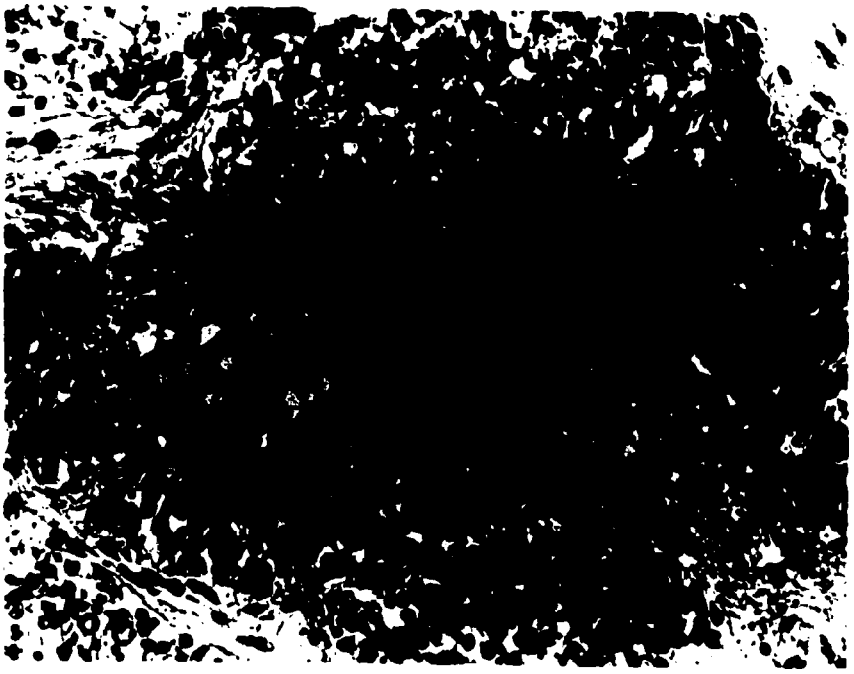


Figure 8. Longitudinal section through the intact limb musculature of a stage 38 (12d) chick embryo stained with PAS-alcian blue and hematoxylin. Note the clumping of nuclei and the mononucleated cells surrounding the myotubes. Also notice the dense cytoplasm of some of these mononucleated cells (long arrow) in contrast to the cytoplasm of some stellate mononucleated cells of the interstitial networks (short arrow). 1420x.

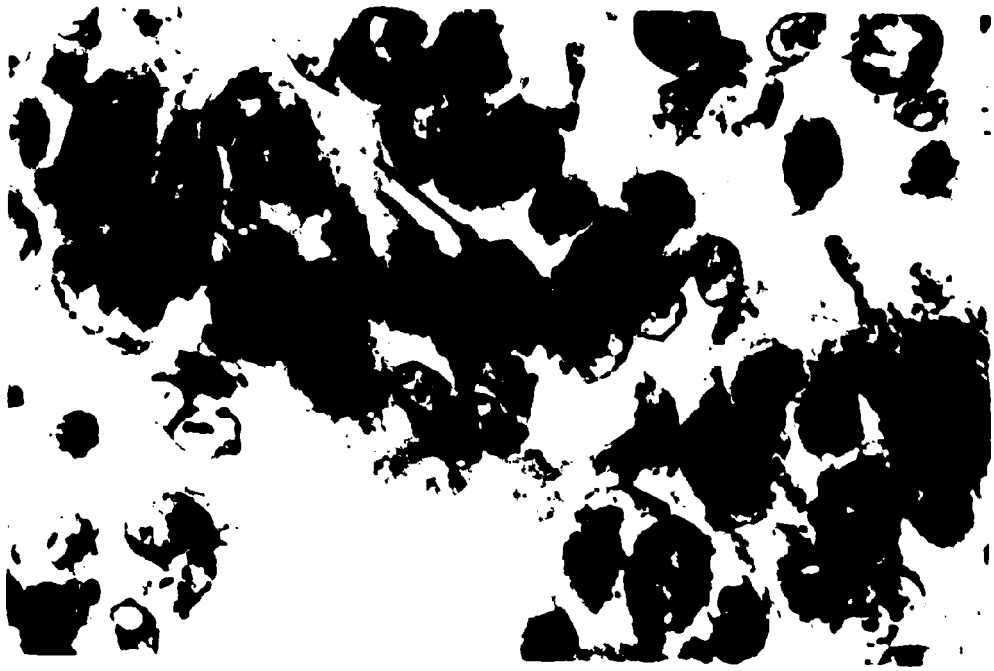
Figure 9. Longitudinal section through the intact limb musculature of a stage 40 (14d) chick embryo showing the presence of longitudinal striations (long arrow) and cross striations in areas (short arrow). Hematoxylin and eosin; 1420x.





Figure 10. Transverse section through the intact limb musculature of a stage 38 (12d) chick embryo, showing the presence of acidophilic clumps in the cytoplasm of myotubes (arrow). Acid alizarin blue; 1420x.

Figure 11. Transverse section through the intact limb musculature of a stage 38 (12d) chick embryo; notice the dense condensations of acidophilic material in the cytoplasm of the myotubes. Masson-light green; 560x.



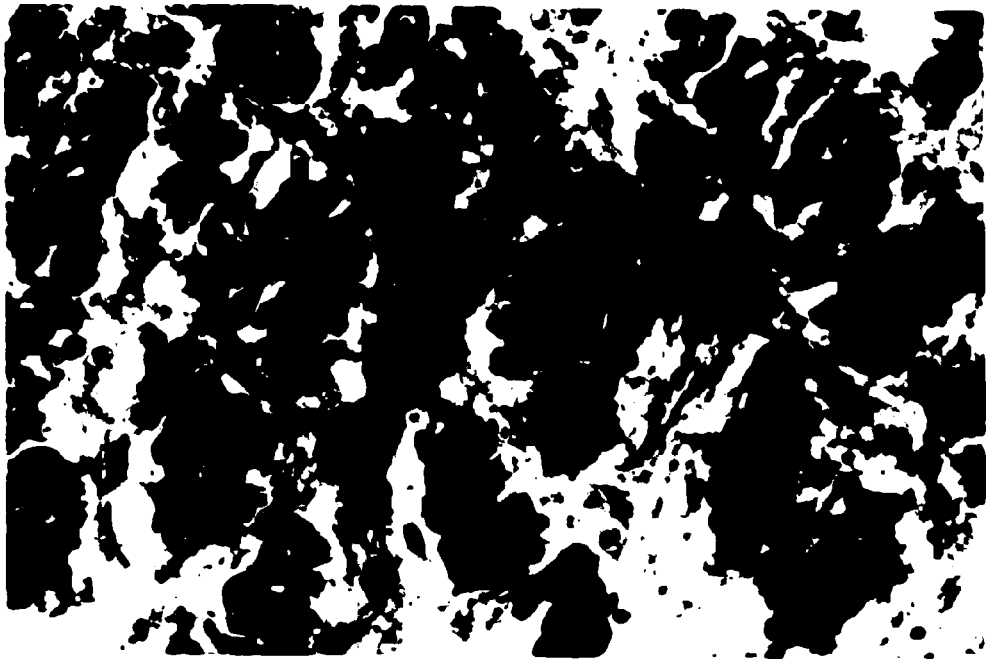
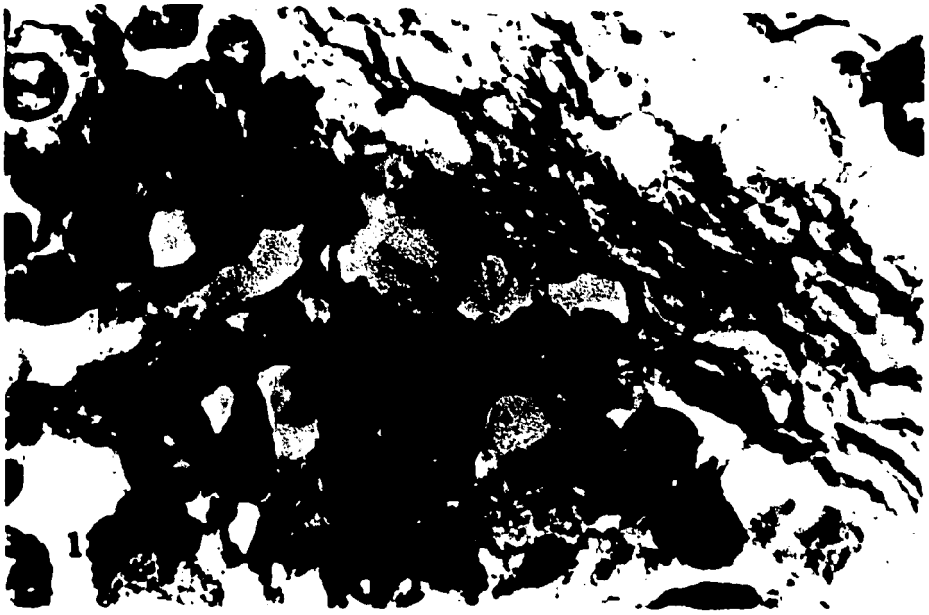


Figure 12. Transverse section through the intact limb musculature of a stage 40 (14d) chick embryo. Arrow points to cytoplasmic myofibrils. Hematoxylin and eosin; 1420x.

Figure 13. Transverse section through the intact limb musculature of a stage 40 (14d) chick embryo stained with tetramethyl rhodamine isothiocyanate-labelled antimyosin. Note the localization of the antibody in the peripheral cytoplasm of the myotubes (arrow) versus its low-order appearance in the interstitium between myotubes. 700x.



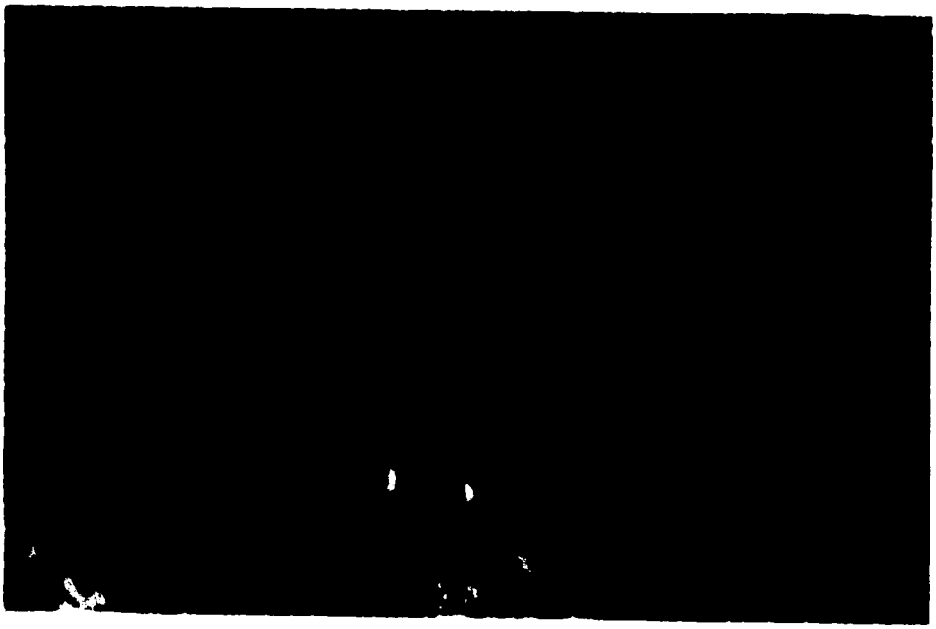


Figure 14. Longitudinal section through the intact limb musculature of a stage 38 (12d) chick embryo stained with periodic acid-Schiff, alcian blue and hematoxylin. Observe the PAS-positive granules in the cytoplasm of some myotubes (arrow). 1420x.

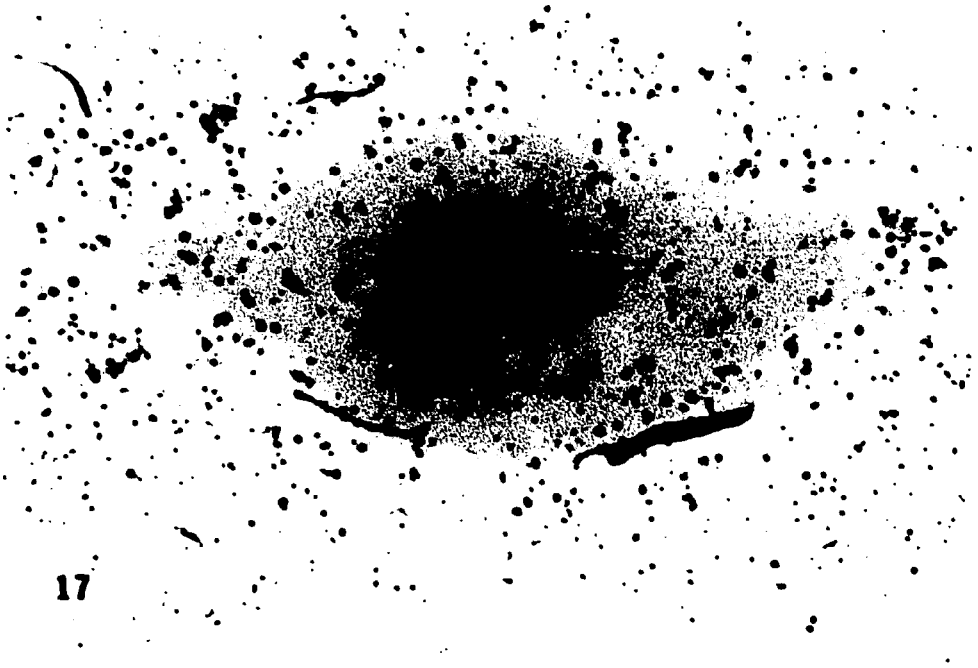
Figure 15. Section through the intact limb of a stage 38 (12d) chick embryo stained with colloidal iron and hematoxylin. The darkly staining chondroblasts (upper right) are CI-positive in contrast to the CI-negative muscle tissue (lower left). 140x.

Figure 16. Aldehyde fuchsin, Masson-light green and hematoxylin stained intact limb of a stage 38 (12d) chick embryo. The cartilage matrix (upper right) is AF-positive and the muscle tissue (lower left) is AF-negative. 140x.



Figure 17. Smeared preparation of cells recovered from the versene dissociation supernatant, indicating the presence of both individual cells and small tissue fragments. Hematoxylin and eosin; 140 x.

Figure 18. Smeared preparation of versene dissociated tissue. Hematoxylin and eosin; 450x.



17



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Figure 19. Sectioned four day organ culture (packed aggregate) demonstrating the mosaic of fiber orientations and the reduction of interstitial tissue between muscle bundles. Hematoxylin and eosin; 350x.

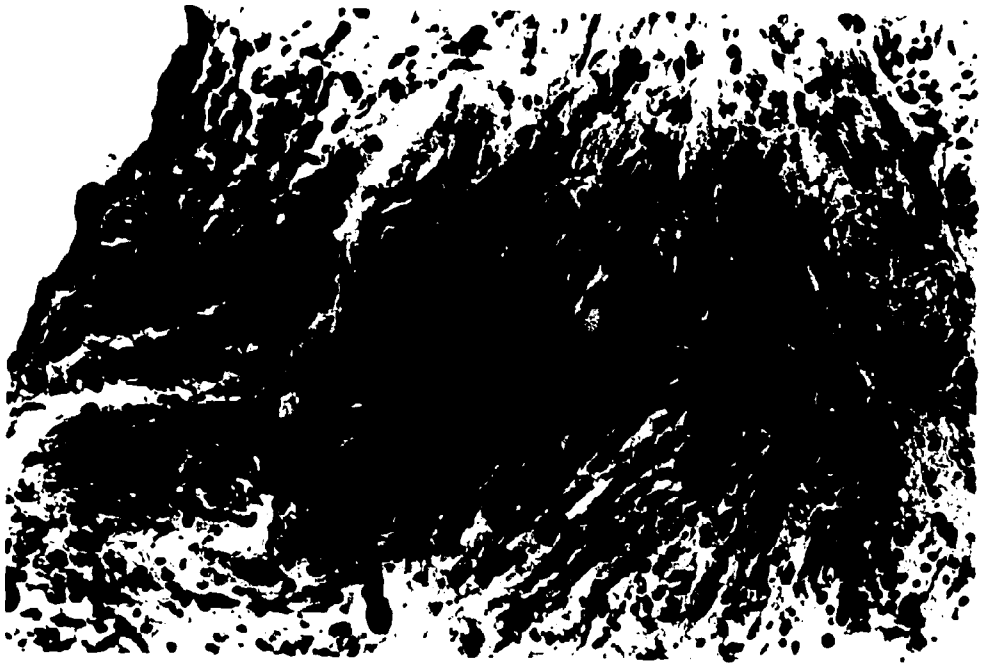
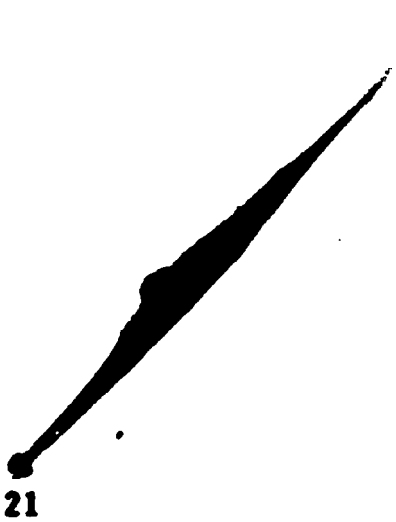


Figure 20. Two myoblasts from a seven day monolayer culture (free aggregate). Hematoxylin and eosin; 224x.

Figure 21. Myocyte from a seven day monolayer culture. Notice the dense cytoplasm of this cell and the cytoplasmic bubbling at its surface. Hematoxylin and eosin; 350x.



20



21

Figure 22. Multinucleated muscle strap (myotube) from a seven day monolayer culture. Note the increased acidophilia of this giant muscle cell in comparison to the surrounding mononucleated cells. Also note the pronounced nucleoli (arrow). Hematoxylin and eosin; 350x.

Figure 23. Section through a multicellular aggregate from an eight day monolayer. Notice the peripheral epitheliod cellular layer with a central accumulation of radially-oriented cells. Hematoxylin and eosin; 350x.


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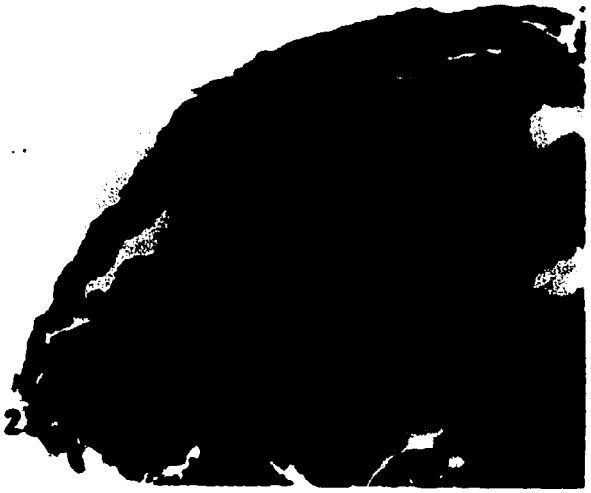
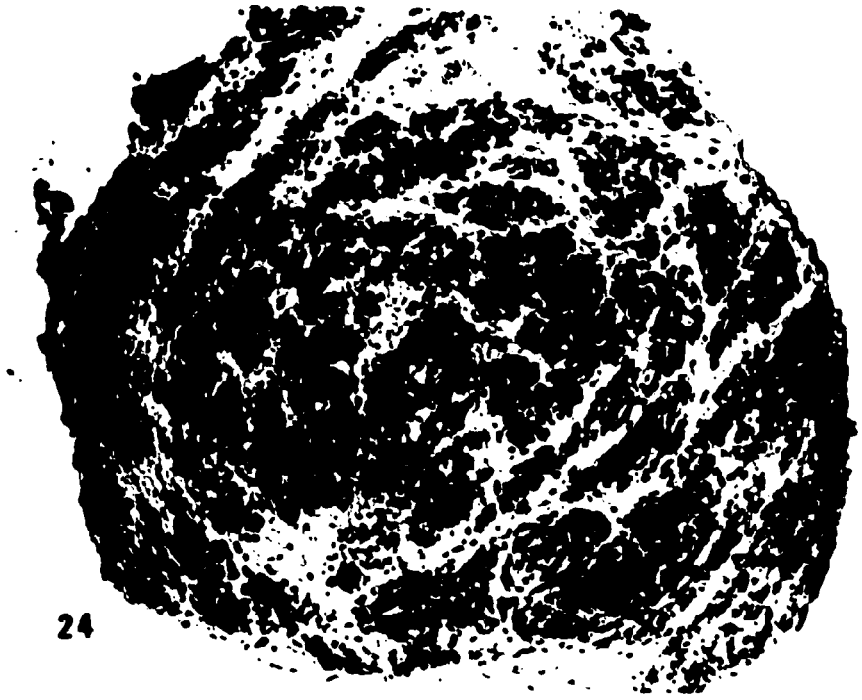


Figure 24. Section through a centripetal aggregate cultivated for two days. Note the spherical shape of the explant and the concentric layer of muscle bundles. Hematoxylin and eosin; 140x.

Figure 25. Section through a centripetal aggregate cultivated for two days. Observe the peripheralized interstitial tissue. Hematoxylin and eosin; 350x.



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Figure 26. Fused spheroid aggregates recovered from the centrifugal aggregation technique. Hematoxylin and eosin; 350x.

Figure 27. Longitudinal section of a portion of a torus from the centrifugal aggregation technique. Hematoxylin and eosin; 140x.

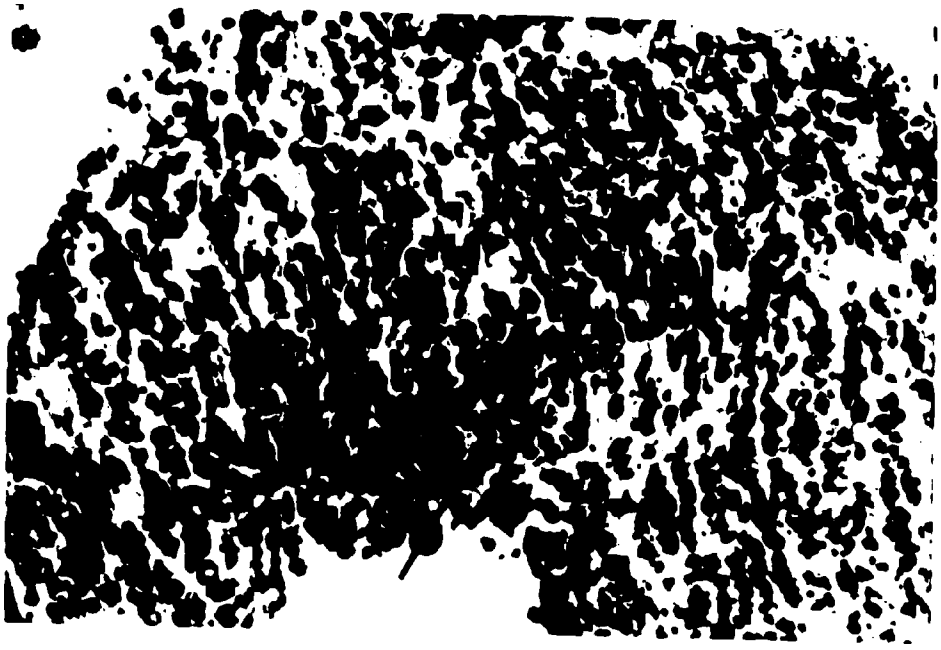


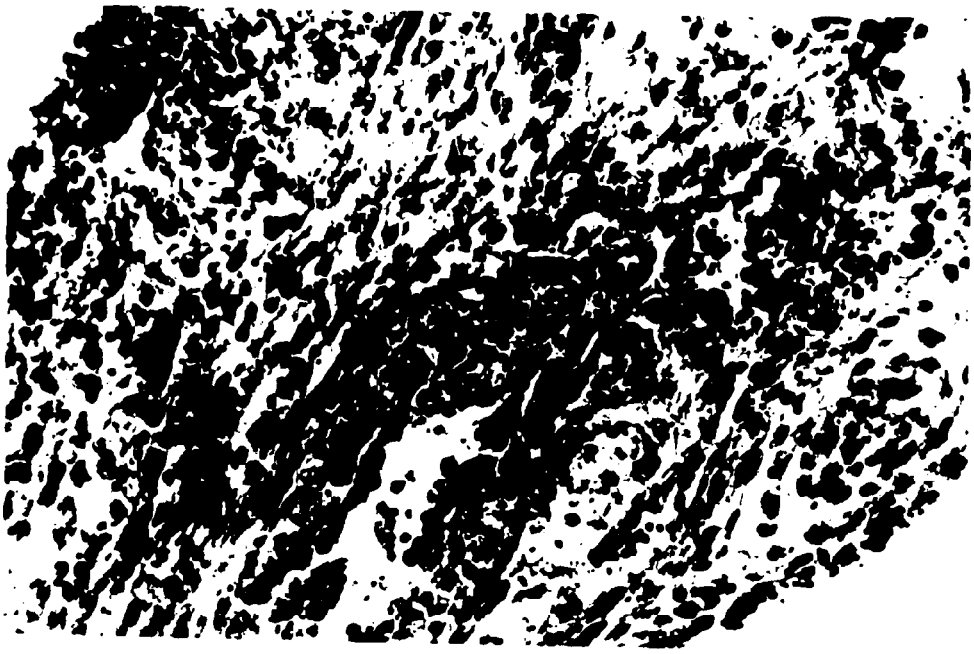
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Figure 28. Longitudinal section of torus from centrifugal aggregation technique. Note the apparent alignment of cells into curving rows extending from the inner surface of the explant (arrow) peripherally. Hematoxylin and eosin; 350x.

Figure 29. Transverse section through a torus from the centrifugal aggregation technique, showing the differential in cellular density throughout the explant. Hematoxylin and eosin; 350x.





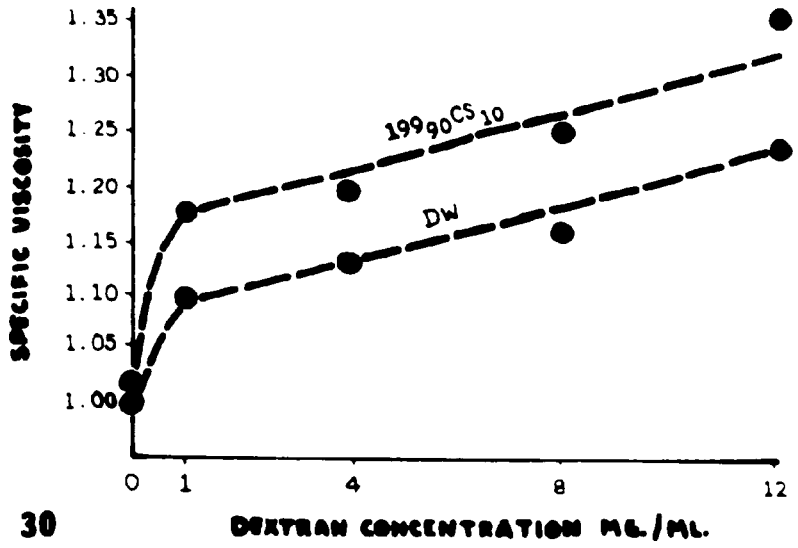


Fig. 30. Variation in the viscosity of solutions of distilled water and culture medium with the addition of Dextran

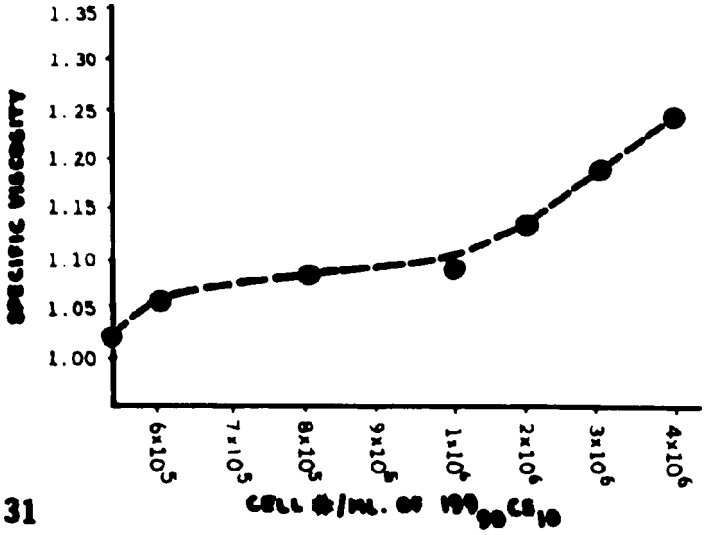


Fig. 31. Variation in culture viscosity with cell population density.