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Fusion mechanism of the myoblasts in the myotome of the chick embryo

R. González Santander, M.V. Toledo Lobo, F.J. Martínez Alonso and G. Martínez Cuadrado
Department of Morphological Sciences and Surgery, Faculty of Medicine, Histology Division, University of Alcalá de Henares, Madrid, Spain

Summary. We have studied the fusion process of myoblasts in the mytome corresponding to H.H. stages 22, 23 and 24 from calcitonin-treated chick embryos and their untreated controls. The micrograph images of this process were studied to detect the ultrastructural changes in myoblast morphology that could be associated with the known hormonal and biochemical changes that take place in preparation of fusion. Once actin and myosin myofilament differentiation and sarcomerogenesis had begun, the myotome myoblasts fused in bundles of 10-14 myoblasts, and the following was observed during this process: plasmatic membrane interdigitation and invagination; the appearance of cytoplasmic flaps covering other myoblasts and fading at the point of contact; plasmatic membranes that fade and disintegrate; membrane rupturing; double closed lamina; small ringed lamina; large disperse vesicles; small vesicles (liposomes), and semi-dense amorphous material. Seven stages were established: 1.- Membrane rupture; 2.- Double closed and elongated lamina; 3.- Small ringed lamina aligned lengthwise; 4.- Large dispersed vesicles; 5.- Imprecise boundaries with amorphous material in diffuse areas; 6.- Cytoplasm fusion; and 7.- Prefunctional syncitium.

Answers to the questions: «how», «when», «where», «why» and «for what purpose» the fusion of myoblasts takes place are suggested.

Key words: Myogenesis, Myotome, Chick embryo, Myoblast fusion, Muscle cytodifferentiation

Introduction

In vertebrates, striated muscular tissue originates in the mesoderm, directly from the embryonic mesenchyma (in the case of the head muscles) or through the somites (for the rest of the body). We know that the histogenesis of striated muscular tissue begins with the differentiation of a small mononucleate cell known as the presumed myoblast and ends in a large multinucleate cell known as muscle fibre.

Several possibilities have been suggested to explain «how» a mononucleate cell can become a multinucleate cell, during the myogenesis of striated muscular tisue, and finally end up as an extremely elongated cell with several nuclei.

In the 1960s the origin of the multinucleate cells was controversial (Boid, 1960), but at present the subjects of study are the biochemistry and molecular genetics of fusion (Miller, 1992).

There are three possible types of explanation (theories or hypotheses):

1.- The multinucleate muscular fibres originate due to the fusion of several myoblasts (multicellular origin).

2.- The multinucleate muscular fibres originate due to cellular multiplication, with no separation of their cytoplasms, where the nuclear division (karyokinesis) is not followed by the division of the cytoplasm (cytokinesis). The muscular fibre is a «syncitium» (unicellular origin) (Remark, 1845).

3.- Both of the above theories combine simultaneously, alternatively or at different times during development.

The first hypothesis (multicellular origin due to fusion) is supported by the following:

Studies carried out using fixed tissues: Eycleshymer, 1904; Heidenhain, 1911; Konigsberg, 1965.

Studies carried out on tissue cultures: Lash et al., 1957; Capers, 1960; Holtzer, 1959; Konigsberg et al., 1960; ; Stockdale and Holtzer, 1961; Betz et al., 1966.

Studies on myoblast cultures in chick embryos after 12 days incubation: Shimada et al., 1967, Shimada, 1971.

Auto-radiographic studies on the regeneration of the skeletal muscle: Bintliff and Walker, 1960.

Studies with tritiated thymidine show the elongation of the muscular cells in the myotome on the third day of incubation of the chick embryo: Stockdale and Holtzer, 1961.

Studies on the muscle of growing rats, with tritiated thymidine marking the nuclei of the mononucleate cells

Offprint requests to: Dr. R. González Santander, Facultad de Medicina, Universidad de Alcalá de Henares, Madrid, Spain

and 24 hours afterwards, the nuclei of multinucleate cells: Moss and Leblond, 1971.

Studies under the electron microscope:

a.- In 16-somite and myotome (H.H. stage 12 and 36 hours after incubation), and 20-somite (H.H. stage 20 and 60 hours after incubation) chick embryos. Fusion of the cytoplasm was observed in several myoblasts, since there were discontinuous membranes in some areas: Dessouki and Hibbs, 1965.

b.- In H.H. 20-25 stage somites in chick embryos, on the third and fourth day of incubation: Przybylski and Blumberg, 1966.

c.- In chick embryos after 12 days of incubation: Fischman, 1967.

d.- On the skeletal muscle development: Hay, 1961, 1963; Bergman, 1962; Shafiq, 1963; Price et al., 1964; Heuson-Stiennon, 1965.

e.- In the sartorius muscle in 65-old pig foetuses: Campion et al., 1981.

f.- In the intercostal muscle of 16-, 18- and 20-day-old rat foetuses: Kelly and Zacks, 1969.

g.- In the straight ocular muscle in 12 cm.-long human foetuses: Gamble et al., 1978.

h.- In rat muscle regeneration: Robertson et al., 1990.

The second hypothesis (multicellular origin due to syncitial division) is supported by the following:

In optical microscopy studies carried out by the first authors, it was established that «during the gradual growth of the muscular tissue, the nuclei increase in number due to mitosis and during the final stages, perhaps due to amitosis»: Bloom and Fawcett, 1964.

Other authors are of the opinion that «the resulting cells are multinucleate, possibly due to amitotic division, since the mitosis figures are rare»: Hamilton et al., 1964.

Studies carried out on fixed tissue establish that multinucleation comes about as a result of short nuclear divisions without being accompanied by the ensuing cytoplasmic division: Remark, 1845; Bardem, 1900; Naville, 1922; Weed, 1936.

Other authors also believe this supported by work carried out on tissue cultures: Chevremont, 1940; Pogogeff and Murray, 1946; Godman, 1955, 1957.

«*In vitro*» studies by other authors confirm that «the nuclei of the new generations of striated muscular cells come from the central nuclei of the myotubes, which are constantly multiplying due to mitosis»: Pogogeff and Murray, 1946.

Contrary to this theory is the observation that «mitotic figures are common among mononucleate cells, but have not been observed in cells containing more than one nucleus» (Fischman, 1967), and that «mitosis or amitosis has not been observed in multinucleate muscular cells» (Shimada et al., 1967).

The third hypothesis, in which both the above hypotheses occur simultaneously, is self-supporting, in the face of the pros and cons of the other two, and also because «the striated muscles grow as the result of the grouping of new myoblasts from the adjacent mesenchyma due to division of the young myoblasts, before the myofibrils are formed»: Hamilton et al., 1964.

Currently, researchers accept the multicellular origin theory, due to fusion, but the «fusion processes» must still be determined: its stages; its mechanisms («how»); at what point of muscle fiber develops; at what point of embryo development («when»); and «why» and «for what purpose» the cells multiply and then merge again (purpose).

In reply to some of these questions, we have conducted an electron microscope study of myoblasts in the myotome of the chick embryo, virtually at the commencement of their formation, at H.H. stages 22, 23 and 24 (from 84 hours to 108 hours of incubation), in control embryos and embryos subjected to the action of calcitonin, which modifies the calcium concentration. In our opinion, this ion could take part in the fusion of the myoblasts (David et al., 1981).

Materials and methods

We used White Leghorn chick embryos, incubated at 38 °C, after selecting several series of Hamburger and Hamilton (1951) stages 22, 23 and 24, from 84 to 108 hours of incubation. Two series were established: a control series; and a series subjected to the effects of calcitonin («Calsynar-50» manufactured by Rorer Laboratories = lyophilized salmon calcitonin), injected into the amniotic cavity at a dosage of 0.25 IU in 0.125 cc. The embryos were injected at H.H. stage 17 (56 hours and 25 minutes of incubation) and were fixed at H.H. stage 24 (around 103 hours and 40 minutes). For the removal of the embryo and preparation of the calcitonin dosage, 9 per thousand saline solution was used and the Millonig buffer was prepared without calcium chloride. The embryos were removed, submerged in Ringer fluid at a temperature of 38 °C, insulated in Millonig buffer and fixed in 2% glutaraldehyde at 4 °C for three hours. Some samples were fixed in 8% tannic acid in 2.5% glutaraldehyde for 2 h (Van Deurs, 1975). A microdisection was then made in order to obtain part of the embryo trunk at the level of the outline of the upper limbs. Three or four pieces were obtained by cross section of the embryonic trunk, which contained: the neural tube; the notochord; and the brachial somites. These pieces were then postfixed in 1% osmium tetroxide for an hour and a half, at 4 °C, stained in block with 2% uranyl acetate and embedded in araldite. Two types of incisions were made, all crossing the neural tube: some semi-thin, which included the whole transversal section of the embryo, these being stained with toluidin blue and being observed under the light microscope; thin sections, made after trimming the inclusion plug, in order to include only the «somite», mainly the «myotome». These were stained with lead citrate, using the Reynolds method (Reynolds, 1963), and then observed under the transmission electron microscope.

Results

The myotome was observed under the light microscope as a lamina, bounded on the outside by the dermatome and on the inside by the undifferentiated somitic mesenchyma. The myotome was formed by fusiform myoblasts, apparently mononucleate, with clear cytoplasm, interphase nucleus and a well-defined nucleolus.

Observed under the electron microscope, the myotome was formed by clear cells, grouped into clumps of several myoblasts, among which were some thin cytoplasmic flaps from other «dark» cells (filled with ribosomes»), that could be «presumed myoblasts» (without myofilaments), or presumed fibroblasts, since several short, isolated collagen fibres were observed at the side, in the interstitial space. The «clear myoblasts» were shaped like elongated spindles, grouped into clumps (parallel bunches) and were not surrounded by basal membrane, except for the side forming the internal boundary of the myotome lamina, where there was a thin basal membrane. This was absent at the external boundary facing the dermatome.

Under the electron microscope, we observed (in controls and in series treated with calcitonin) that all the clear myoblasts showed ultrastructural differentiations of actin and myosin myofilaments in their cytoplasm (in some cases, initiating sarcomerogenesis) and that the plasmatic membranes in some areas were faded (imprecise, disintegrated) or discontinued due to the dissolution of some areas of the membrane, leaving fusion passages among the myoblasts with a junction of cytoplasms. At different stages, we observed the following ultrastructural modifications:

Stage 22

It was observed that the relationship between the myoblasts belonging to the same group or clump was closer when the well-defined structure of some areas of their plasmatic membrane disappeared. The following was observed: areas of double discontinued membranes, which were disintegrated, ruptured membranes in several places, forming double, closed, elongated lamina or small ringed lamina aligned lengthwise (Fig. 1); ruptures of these rings and fragmentation of the same; large vesicles (from 140-160 nm in diameter) (Fig. 2a) and small vesicles (23.4 nm in diameter), aligned at the intercellular boundaries. Sometimes, at the side of the fragmented membranes and in the adjacent cytoplasm, there were several dictyosomes with a cloud of Golgi vesicles (from 50-100 nm in diameter) (Fig. 3). The following was also observed: communication passages (small, large and wide) among the myoblasts; imprecise boundary areas between two myoblasts, with areas faded by amorphous material which was located in the projection of the discontinued membrane line (Fig. 2a-c); wide communication passages between myoblasts with fusion of cytoplasm in these areas; and differentiation of myofilaments crossing the two merged myoblasts lengthwise.

We had already observed all the above modifications of the plasmatic membrane at the ends of the myoblast (sharp tips) and in the lateral contact areas.

Stage 23

Within the clumps of 2-6 myoblasts, we observed the fusion of the cytoplasm in different areas, giving rise to different communications between myoblasts, where thin and thick myofilaments could be distinguished which continued into the two merged myoblasts. In other areas, double membranes were observed, which were discontinued, as well as elongated ringed lamina, discontinued and aligned (Fig. 2d).

Stage 24

We observed groups of 2-3 myoblasts joined together by wide areas, where the plasmatic membrane was not well defined (disintegrated, imprecise, faded) and in its place was amorphous material, which tended to establish the boundary between two cytoplasmic areas of different myoblasts, but with some difficulty (Figs. 2e, 10, 11). In other places, there were wide communication passages which gave rise to true fusions of cytoplasms. Here, we began to observe myofilaments that were passing from one myoblast to another.

Neither the isolated nor the grouped myoblasts were surrounded by basal membrane, except for the side forming the internal boundary of the myotome lamina. The grouped myoblasts had clear cytoplasm, but the clumps were separated peripherally by thin flaps of darker cytoplasm (filled with ribosomes), that could be presumed myoblasts or presumed fibroblasts (Fig. 6). Only in one of these cells did we observe mitosis. It was also common to see a clear myoblast surrounding or partly attached to another, at which point of contact the membranes of both were faded. There were also some invaginations of the plasmatic membrane between two clear myoblasts (Figs. 10, 11) with adjacent areas of faded membrane (imprecise, disintegrated). In a group of several myoblasts which had merged (Figs. 7, 8) there were wide areas of faded, imprecise or disintegrated membranes, formed by semidense amorphous material (Fig. 9) between which there were small aligned vesicles (23.4 nm in diameter) and other larger vesicles (from 140-160 nm in diameter) (Fig. 8). We had already observed these areas of faded membranes mainly between clear myoblasts (Figs. 8, 9), but also in some cases, between a clear myoblast and a dark one.

The differences found in the embryos treated with calcitonin were as follows

There were groups or clumps with a greater number of grouped myoblasts, up to 5 and 14 myoblasts (Fig. 5)

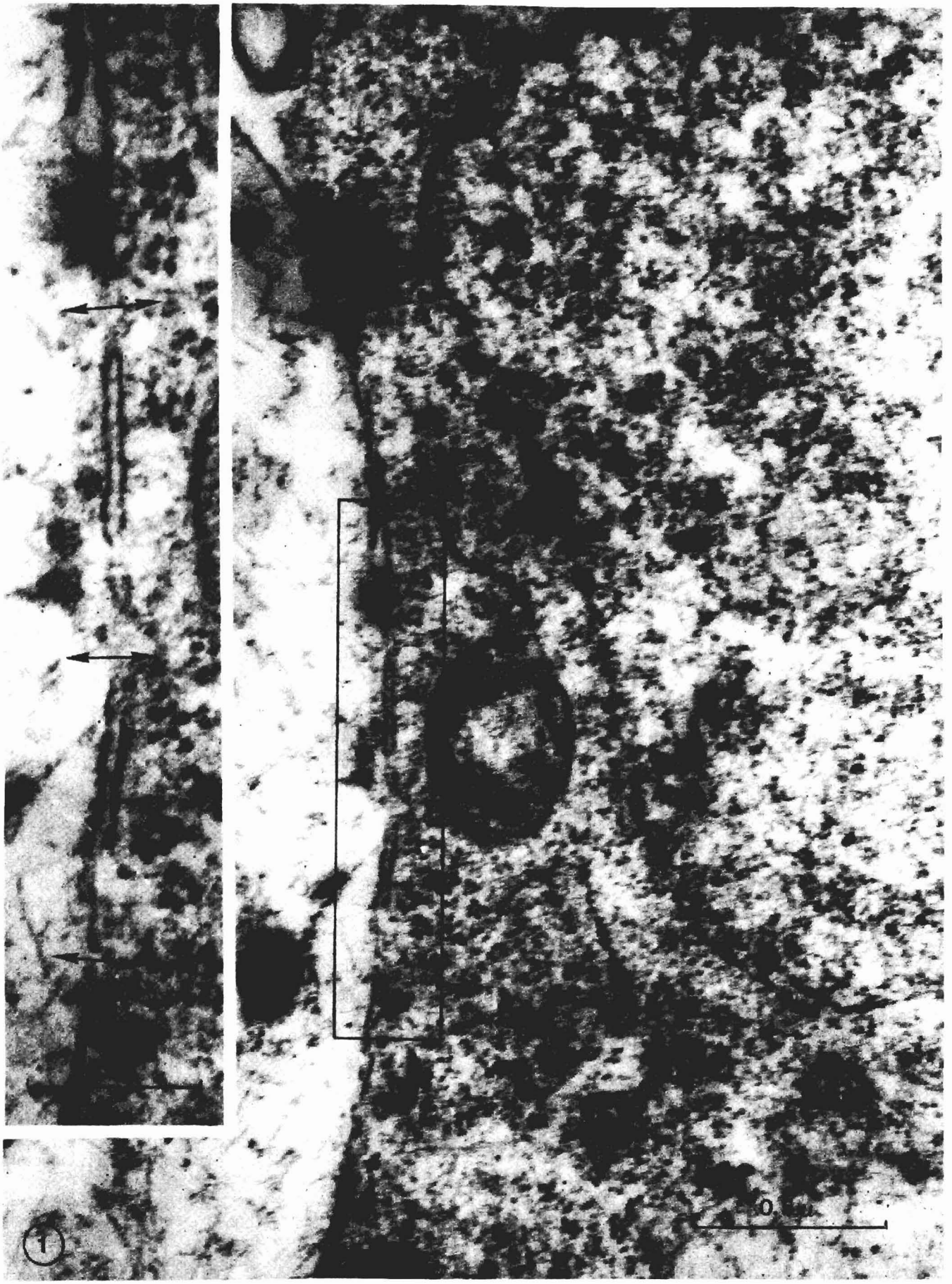

Fig. 1. H.H. Stage 22. Rupture of membranes separating clear and dark myoblasts. (double arrow= passages), x 20,000. Square magnified x 50,000

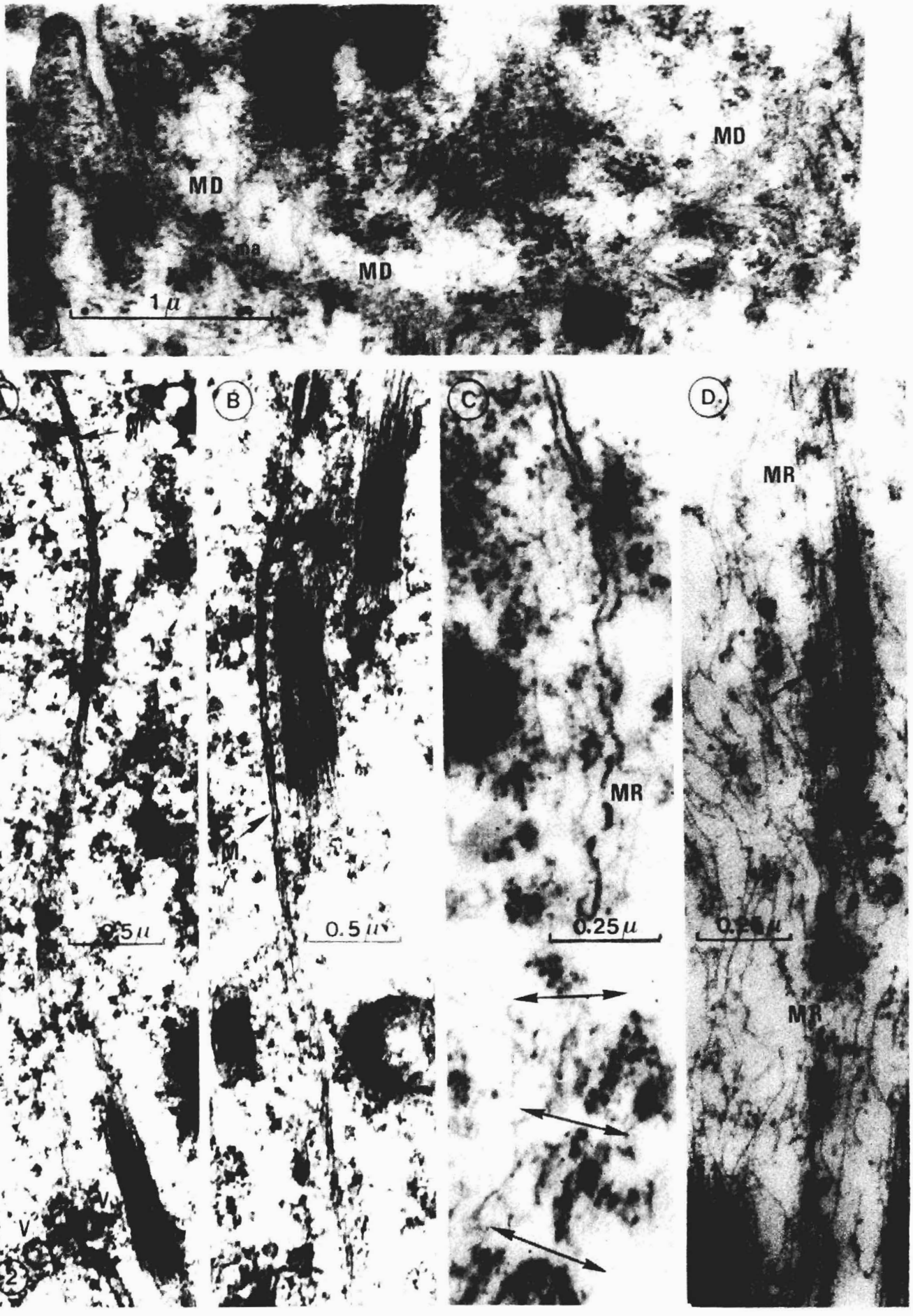
MD
MD
MD
1 μ
B
0.5 μ
C
MR
0.25 μ
D
MR
MR
V
V
2

Fig. 2. Several areas of development in the fusion between clear myoblasts (double arrow= passages): Intact membrane (M); ruptured membrane (MR); faded membrane (MD), large vesicle (V); amorphous material (ma); **a.** Stage 22. x 12,000. **b.** Stage 22. x 12,000. **c.** Stage 22. x 30,000. **d.** Stage 23. x 20,000 **3.** Stage 24 (calcitonin), magnified x 12,000 taken from the square in Fig. 6.

joined by cytoplasmic bridges or wide faded, imprecise or disintegrated membranes. Deep invaginations were observed in the plasmatic membrane between neighbouring myoblasts. The cytoplasmic flaps of the myoblasts were more frequent, attached to other myoblasts (Figs. 6, 7). Many more disintegrated membranes were observed, and a greater number of small vesicles in the membrane areas faded with amorphous material (Figs. 6-8). At the sides there were also more large vesicles. Apparently the smooth endoplasmic reticulum was somewhat more developed.

Discussion

Our observations, as well as the studies of many authors we have consulted, show without doubt that multinucleate skeletal muscle fibre originates as a result of the *fusion of mononucleate myoblasts*, which gives rise to a true syncitium. The *cytoplasmic fusion* is shown by *«in situ»* studies and *«monolaminar cultures»* under the electron microscope using additional biochemical, ultrastructural and histo-autoradiography techniques, which have clarified our knowledge regarding the process of differentiation and development of muscle fibres (Eycleshimer, 1904; Heidenhain, 1911; Lash et al., 1957; Firket, 1958; Holtzer et al., 1958; Bintliff and Walker, 1960; Capers, 1960; Holtzer, 1959; Konigsberg et al., 1960; Hay, 1961, 1963; Stockdale and Holtzer, 1961; Bassleer, 1962; Bergman, 1962; Shafiq, 1963; Strehler et al., 1963; Price et al., 1964; Stockdale et al., 1964; Dessouki and Hibbs, 1965; Heuson-Stiennon, 1965; Konigsberg, 1965, 1971; Betz et al., 1966; Przybylski and Blumberg, 1966; Fischman, 1967; Shimada et al., 1967; Bischoff and Holtzer, 1969, 1978; Kelly and Zacks, 1969; Shainberg et al., 1969, 1971; Bodemer, 1970; Richler and Yaffe, 1970; Moss and Leblond, 1971; Yaffe, 1971; Rash and Fambrough, 1973; Vertel and Fischman, 1973; Holtzer et al., 1975; Moss and Strohman, 1976; Nameroff and Munar, 1976; Bloom and Fawcett, 1978; Gamble et al., 1978; Kalderon, 1980; Campion et al., 1981; Couch and Strimatter, 1983; Ham and Cormack, 1983; Caplan et al., 1988; Darnell et al., 1988).

In agreement with other hormonal and biochemical research, our ultrastructural findings show that hormonal and biochemical changes prepare myoblast fusion (A) and once prepared, ultrastructural morphological fusion (B) takes place immediately in seven steps:

A.- Hormonal and biochemical preparation of the myoblasts prior to fusion

Due to reasons still unknown, a segregation of similar mesodermic cells take place, (through the somites and the mesenchyma) which group together. These are identified as «presumed myoblasts»; mononucleate cells which are subjected to repeated proliferation. Mitosis is often observed in these mononucleate cells (Herrmann, 1963; Przybylski and Blumberg, 1966; Fischman, 1967; Kelly and Zacks, 1969). The size of the proliferative «pool» is reduced as the age of the embryo increases (Marchok and Herrmann, 1967).

The presumed myoblasts leave the proliferative «pool» in order to prepare themselves for fusion. The decision to leave the cellular cycle is a step in the programmed sequence of cytodifferentiation, as a necessary prerequisite for myogenic differentiation (Bischoff and Holtzer, 1969). The presumed myoblasts thus enter into stage G1 of the intermitotic period, when they are competent to begin fusion (Strehler et al., 1963). The «G1» stage is lengthened, which may be the condition necessary for discriminating between myoblasts which are able to merge and those which are not. The latter would have to re-enter the mitotic cell cycle (Buckley and Konigsberg, 1974). There seems to be a «G1 sub-stage» which would be the minimum time necessary for the primitive myoblasts to accumulate certain cellular products necessary for reaching the specialization level, as has been observed in cultures (Konigsberg, 1971). The synthesis of specialized products can only commence after the minimum time in G1, «G1 sub-stage»; the window hypothesis (Buell and Fahey, 1969; Buell et al., 1971).

Fusion only occurs during the G1 stage, as has been seen in cultures (Strehler et al., 1963). The fusion process «per se» requires the synthesis of proteins, so that the presumed myoblasts synthesize RNAm in the «pre-fusion» time (Shainberg, 1971). The calcium level in the microatmosphere of the presumed myoblasts must be right for the commencement and synchronization of the fusion process (Shainberg, 1969). Calcium intervenes in the preparation of the plasmatic membrane for fusion, according to experimental results (Couch and Strittmatter, 1983). Contact then takes place between the plasmatic membranes of the neighbouring myoblasts. The «specialized unions» between neighbouring myoblasts strengthen the fusion process in the places where the specialized membrane merge between neighbouring myoblasts (Kelly and Zacks, 1969; Shimada, 1971). The calcium ion regulates the contacts

Fig. 3. Stage 22. Golgi complex (Gol) with dictyosomes and Golgi vesicles (gv) at the side of a faded membrane (MD) in the fusion between clear myoblasts x 12,000


MD
Gol
MD
MD
g
vg
vg
1 µ

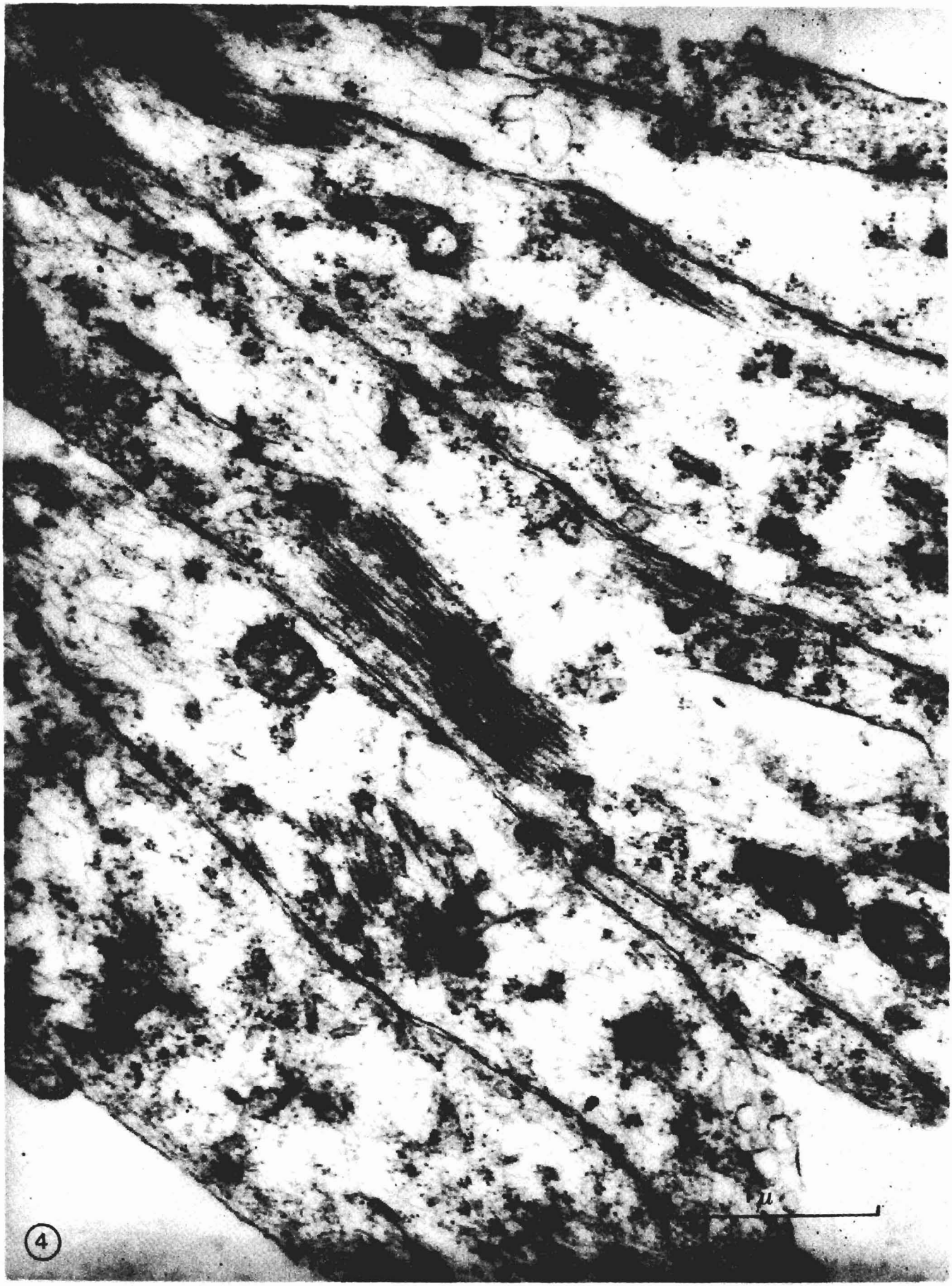

Fig. 4. Stage 23. Cross section of several myoblasts in areas not yet affected by fusion. x 12,000

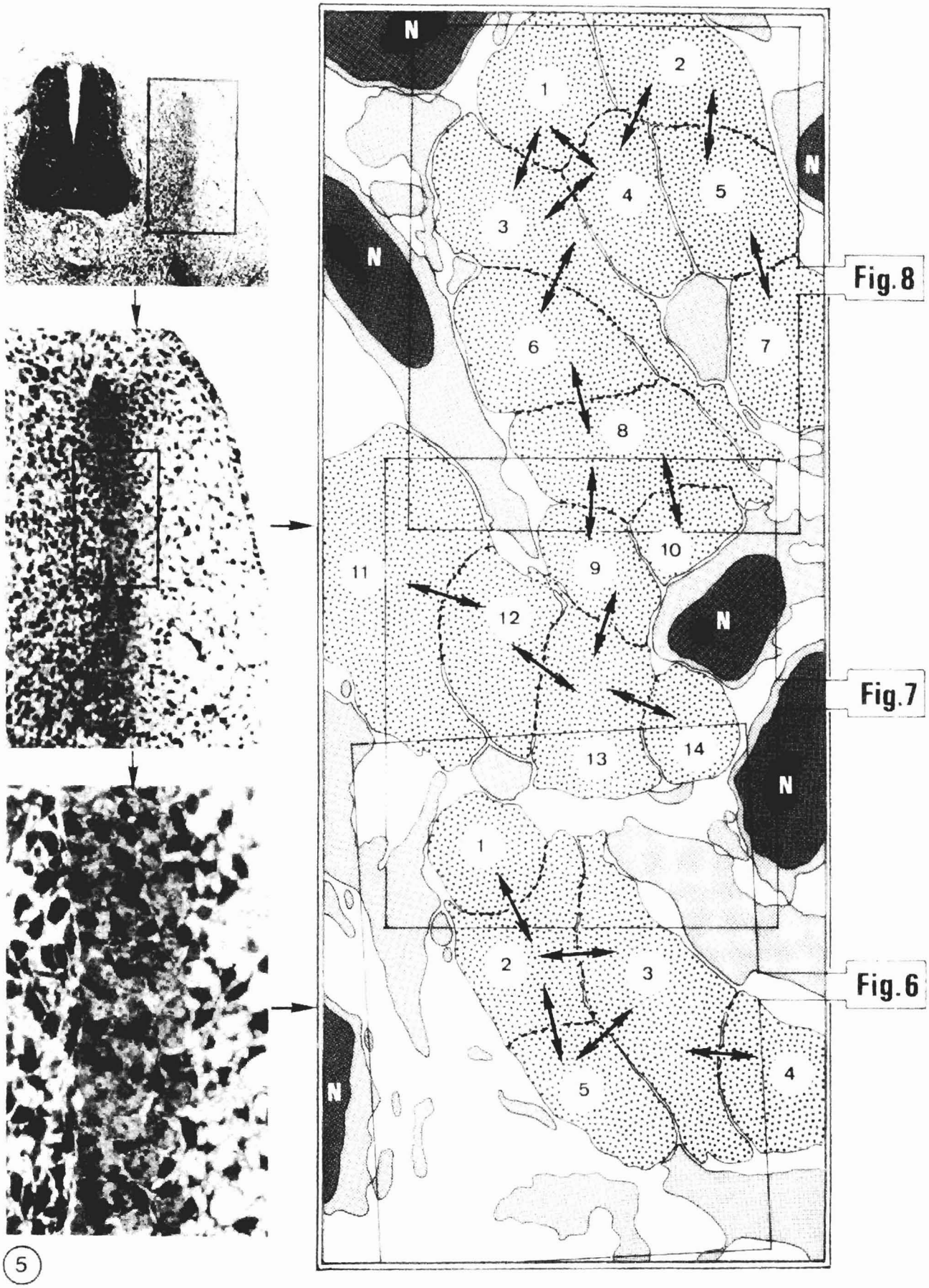
N
1
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Fig. 8
Fig. 7
Fig. 6
5

Fig. 5. Cross section of brachial myotome. Stage 24, and drawing (from carbon copy of electron microscope mural) of the square, where groups of 5 and 14 myoblasts can be observed, mergin (double arrow= fusion points). The square shows the following figures (Figs. 6-8).

between the myoblasts, since it is known that a certain level should be reached for cellular adhesion. A process of recognition of the cellular surface must exist (Caplan et al., 1988). This recognition is accompanied by the myoblasts leaving the cell cycle (Holtzer et al., 1975; Nameroff and Munar, 1976). There may be certain factors (probably hormonal in nature) which act upon the surface of the myoblast and regulate the fusion and other aspects of its metabolism (Moss and Strohman 1976). Some authors have detected the formation of «close unions», occlusive or pentalaminar (Kelly and Zacks, 1969; Samosudova et al., 1988), and the formation of «gap junctions» prior to fusion (Shimada, 1971; Rash and Fambrough, 1973; Bischoff, 1978; Couch and Strittmater, 1983), and fusions of the membrane with a «gap» of 20 nm (Gamble et al., 1978). It is known that the regulation of the communicating junctions function is carried out by the effect of calcium, when the diameter of the pores of the plasmatic membrane is regulated for the diffusion of ions. It is known that the calcium channel blockers suppress the fusion of myoblasts (David et al., 1981). Certain substances, such as concanavalin A, have been shown to block fusion (Den et al., 1975; Knudsen and Horwitz, 1978). Calcium intervenes in the enzymatic process which terminates with the hydrolysis of certain membrane proteins (Couch and Strittmatter, 1983).

The calcium ion intervenes in the de-coupling of the Na^+/K^+ pump in active transportation. In experimental terms, it has been proven that the reduction of calcium in primary cultures of birds, calves and rats blocks the fusion of the myoblasts (Shainberg et al., 1969, 1971; Yaffe, 1971) with low calcium levels, fusion is suppressed (Vertel and Fischman, 1973) but the proliferation and alignment of the myoblast is still permitted (Couch and Strittmatter, 1983). If the concentration of calcium in the culture medium is modified, the commencement and duration of the fusion process can be controlled (Yaffe, 1968; Richler and Yaffe, 1970; Shaimberg et al., 1971). On the contrary, when calcium chloride is added to the culture medium, the myoblasts tend to merge (Moss and Strohman, 1976). Thin cytoplasmic bridges have been seen to form, of around 15 Å in diameter between adjacent myoblasts (Kalderon, 1980), which could be the first morphological aspect observed prior to fusion.

As a result of mutual recognition, the myoblasts merge and align themselves in groups (Nameroff and Munar, 1976), which after they leave the mitotic cell cycle are necessary for fusion (Nameroff and Munar, 1976). Although this alignment is not a determining factor in the fusion process, it is necessary and has been observed *«in vitro»* (Knudsen and Horwitz, 1977, 1978). In experimental terms, it is known that the reduction of calcium suppresses the alignment of the myoblasts and fusion (Shainberg et al., 1969, 1971; Yaffe, 1971; Nameroff and Munar, 1976), but does not interfere in the general metabolism of the presumed myoblast or in cellular division, in the case of re-entry into the mitosis cycle (Shainberg et al., 1969, 1971; Yaffe, 1971). Fusion between fusiform myoblasts takes place at the aligned ends (Caplan et al., 1988) and the lateral walls, at least in the myotome observed by us.

B.- Ultrastructural morphological process in the fusion of myoblasts

The myoblasts which are «fusion-competent» start to become a «syncitium», when their membrane boundaries partly disintegrate, undergoing a process which we suggest is divided into seven stages, if we observe carefully the images shown under the electron microscope, which have been speeded up by the effects of calcitonin.

Stage 1

Membrane rupture stage. The plasmatic membranes of both myoblasts start to disintegrate in several places opposite each other and symmetrical to each other (Figs. 1, 2) (Dessouky and Hibbs, 1965; Przybylski and Blumberg, 1966; Robertson et al., 1990).

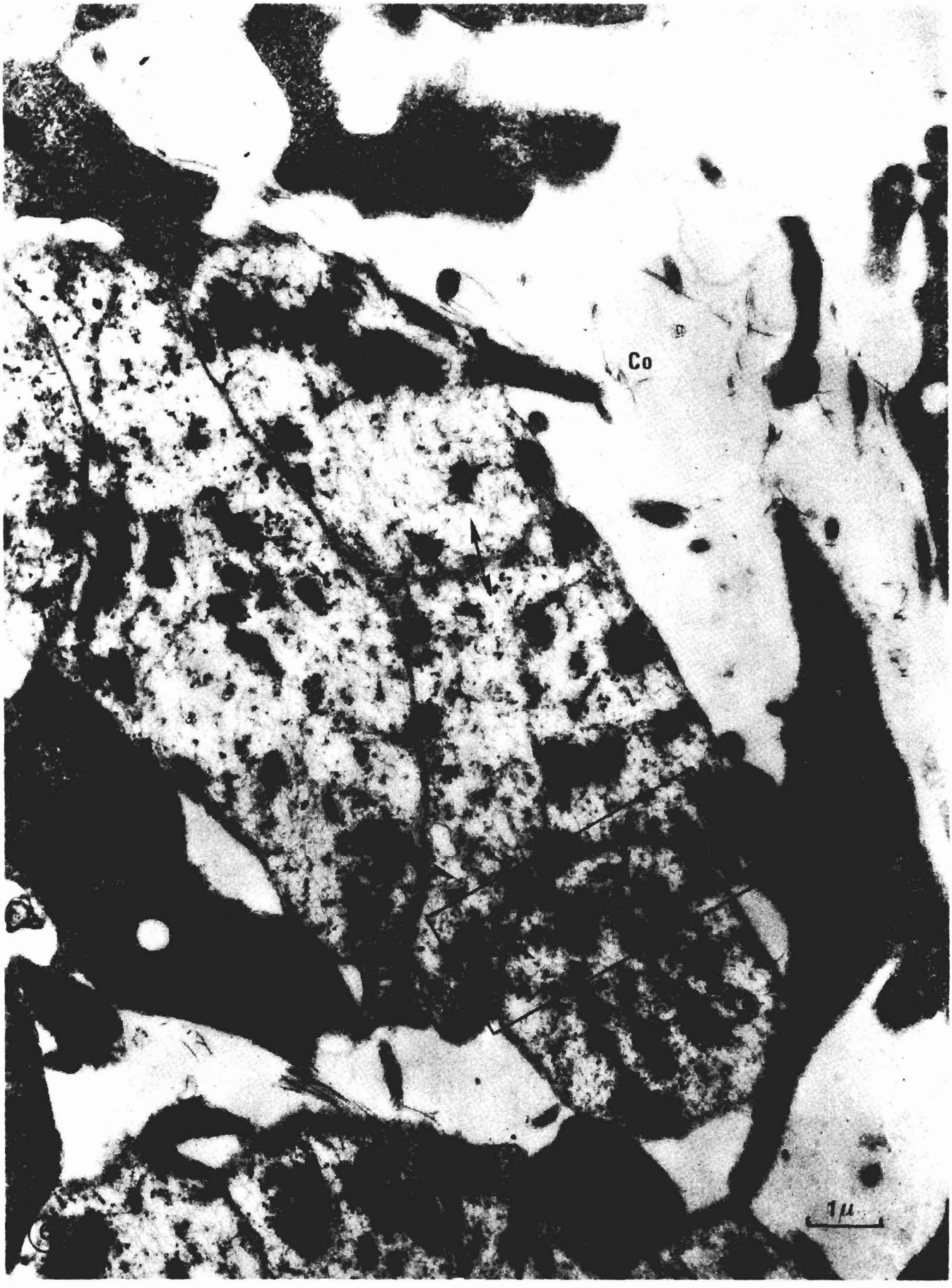
Stage 2

Double, closed, elongated lamina stage. Union of the broken ends of the membranes due to welding of the membrane of one myoblast with that of a neighbouring myoblast (Figs. 1, 2). The ends of the other external lamina (extracytoplasmic) and those of internal lamina (cytoplasmic), become welded to those of both myoblasts respectively, since phospholipid membranes are able to spontaneously seal themselves in order to give rise to closed structures, as occurs in the endocytic process (Figs. 1, 2) (Darnell et al., 1988).

Stage 3

Small ringed lamina aligned lengthwise stage. Fragmentation of the «double closed lamina» due to successive ruptures and welding, as in the two previous stages (Figs. 1, 2).

Fig. 6. Stage 24 (calcitonin). Several myoblasts merging (light and dark) (double arrow= fusion). Collagen fibres (Co). One myoblast attached to another in fusion process. It corresponds to square of mural in Fig. 5. The square is magnified in Fig. 2e. x 4,400


Co
1μ

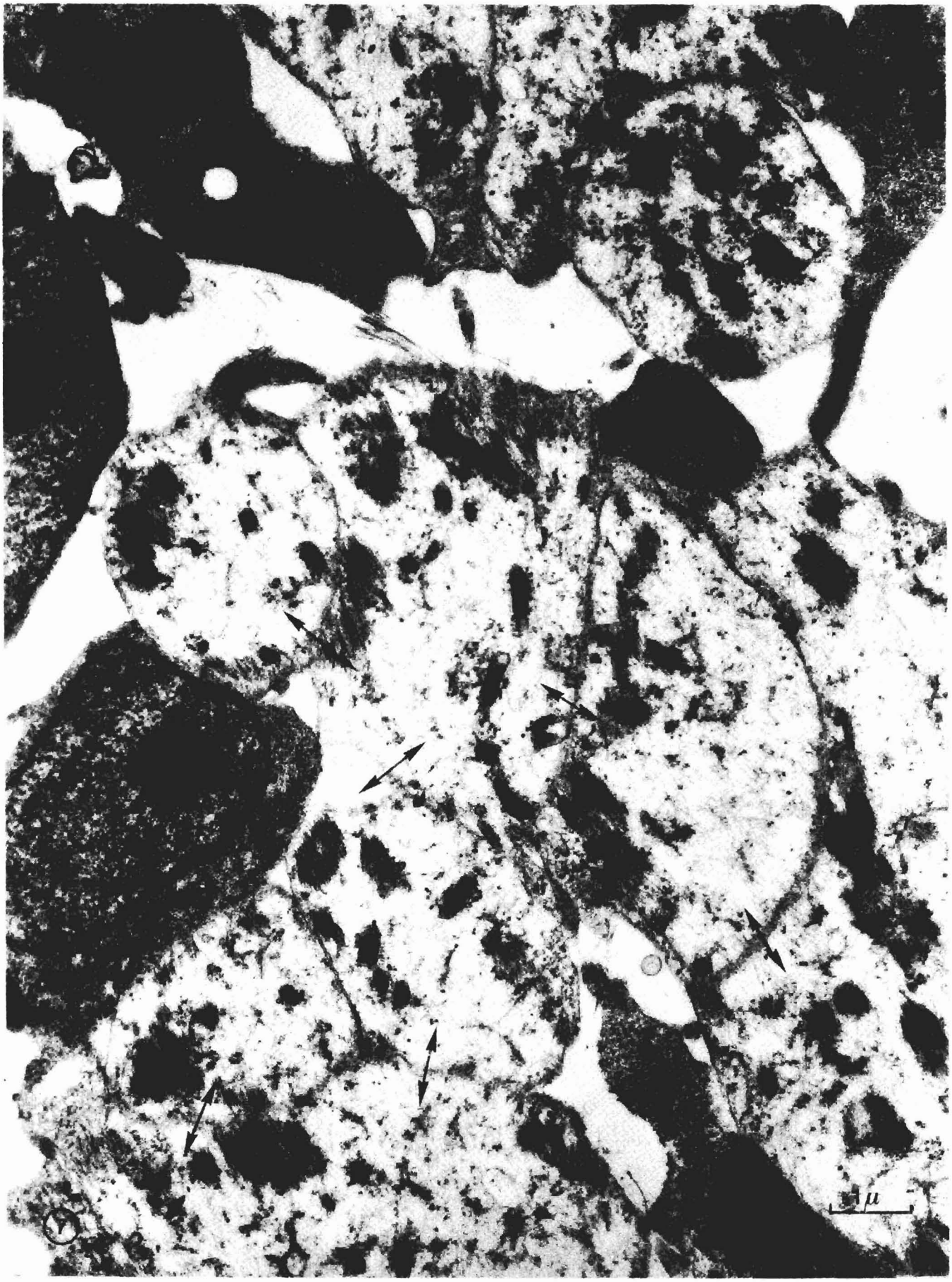


Fig. 7. Stage 24 (calcitonin). Several myoblasts merging (double arrow= fusion). Dark peripheral myoblasts. It corresponds to the square of the mural in Fig. 5. x 4,400

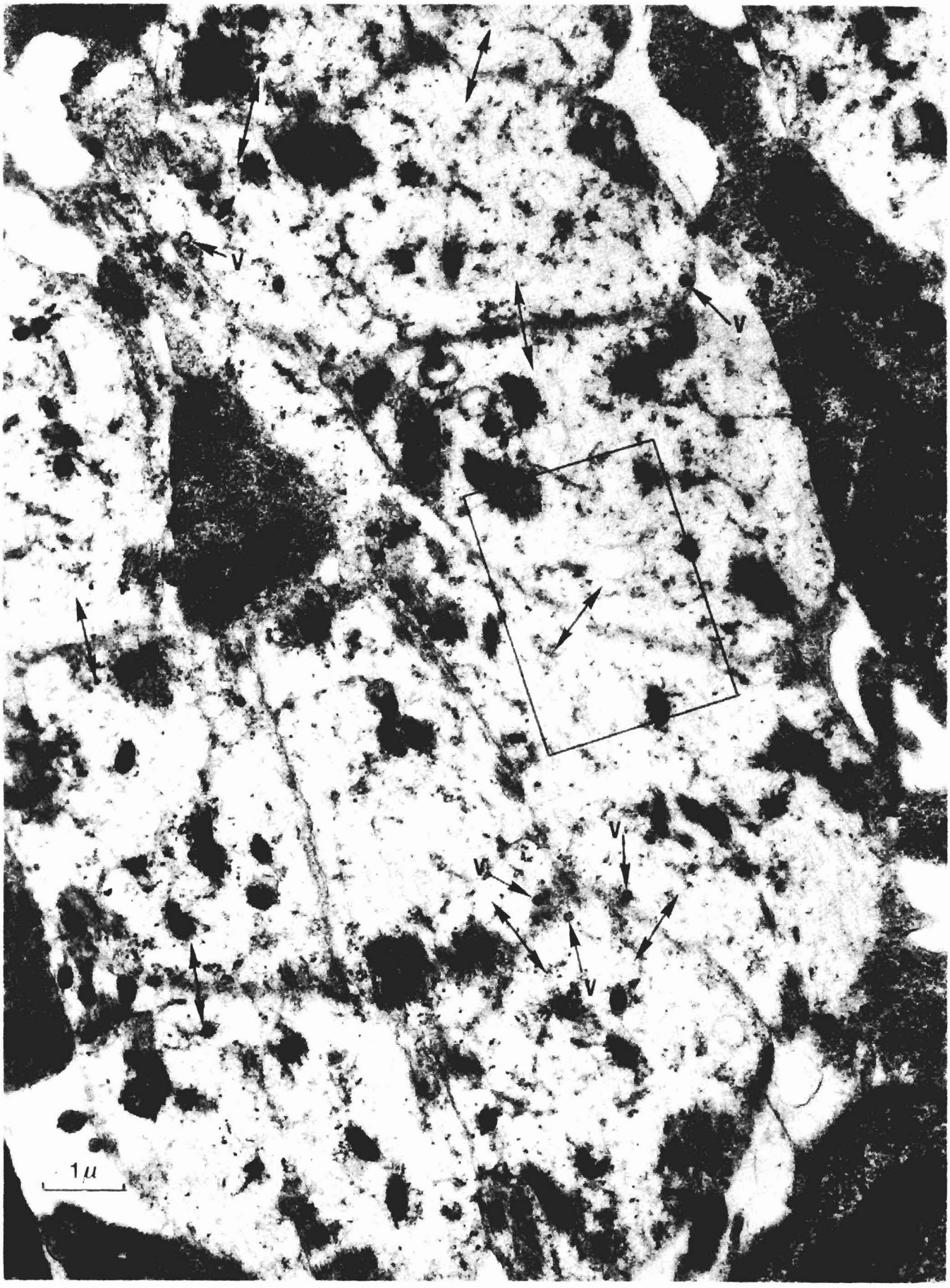


Fig. 8. Stage 24 (calcitonin). Several myoblasts merging (double arrow= fusion). Large vesicle (V). It corresponds to mural in Fig. 5. The square is magnified in the following figure (9). x 4,400

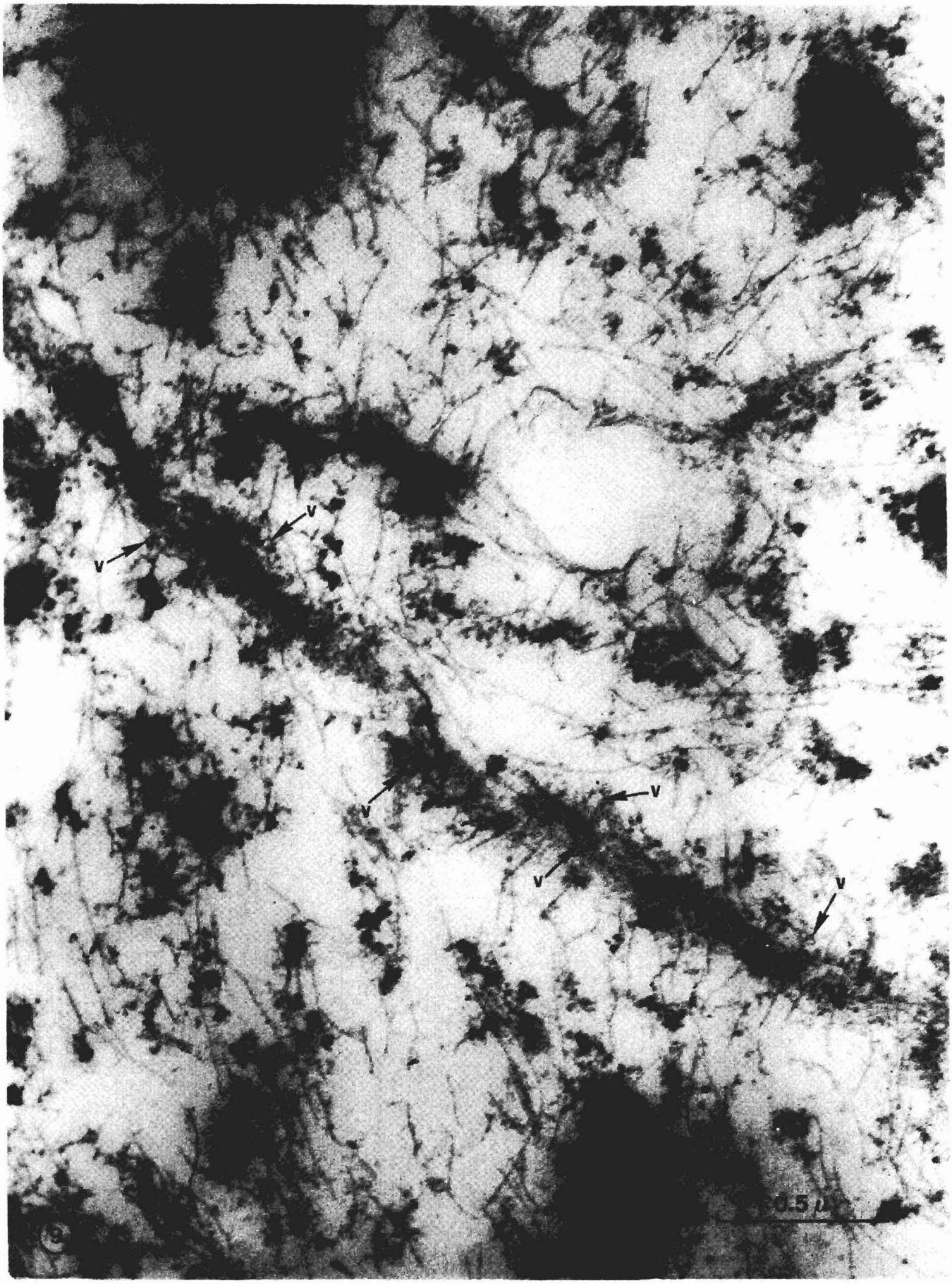

v
v
v
v
v
v
0.5 μ

Fig. 9. Stage 24 (calcitonin). Faded area between two myoblasts, in accordance with the square of the previous figure (8), amorphous material (ma); large vesicles (V); small vesicles (v). x 20,000

Stage 4

Large disperse vesicle stage. Fragmentation of the «small ringed lamina» due to successive ruptures and welding (Fig. 2a,c) (Hay, 1963; Shafiq, 1963; Dessouky and Hibbs, 1965; Przybylski and Blumberg, 1966).

Stage 5

Imprecise boundaries with amorphous material in diffuse area stage. Emigration of large vesicles, fragmentation of the same and the appearance of small aligned vesicles among faded amorphous material (Fig. 9). The large vesicles disappear or become part of the dictyosomes of the Golgi complex (Fig. 3). The number of vesicles decreases during development (Dessouki and Hibbs, 1965). The phospholipid bilayer proteins become destructed and separate from the lipid molecules leaving disintegrated membrane debris (see «technical artifacts» in Lipton and Konigsberg, 1972). The debris contributes to the formation of the «spherical vesicles» or liposomes that originate in the mechanical dispersion of phospholipids (Wakelam, 1985; Darnell et al., 1988). These would be the small vesicles found between the faded amorphous material of non-structural dispersed proteins (Fig. 9).

Stage 6

Cytoplasmic fusion stage. This is the commencement of a true syncitium by which wide areas of passage and union are formed between two myoblasts, with a union of cytoplasms. Cellular continuity. There are two nuclei and one single cytoplasm (Hay, 1963; Shafiq, 1963; Przybylski and Blumberg, 1966; Fischman, 1967).

Stage 7

Prefunctional syncitial stage. The synthesis of myofilaments occurs and the initiation of sarcomerogenesis in the cytoplasm leading to the fusion of both myofilaments (Fig. 8). 50 Å and 100 Å filaments can be observed (Przybylski and Blumberg, 1966; Fischman, 1967).

In our case for the fusion of myoblasts in the myotome of the chick embryo, these seven stages take place between several myoblasts grouped into bundles of from 10-14, in which the process of differentiation of actin and myosin myofilaments has already commenced, and the sarcomerogenesis process has started (H.H. stage 22), Dessouky and Hibbs (1965) did not observe this cytodifferentiation when fusion begins in H.H. stages 18-19. Apart from this, the first indication of the formation of the myotome appears at around H.H. stage 13 at the dorso-medial side of the somite in the chick embryo, according to Przybylski and Blumberg (1966), who observe mononucleate myoblasts up to H.H. stage 23. The fusion of myoblasts is observed by these authors by chance in myotome cells, and the multinucleate process is initiated at H.H. stages 20-25. It appears that all the grouped myoblasts are destined to merge, and generate an enlogated multinucleate myoblastic cell (Fischman, 1967). It is rare to observe union complexes (or desmosomes) between the myotome myoblasts at H.H. stage 15-16 (Przybylski and Blumberg, 1966) and stages 22, 23 and 24 according to our studies. Neither is mitosis observed in these myotome myoblasts at H.H. stages 12-14 according to Przybylski and Blumberg (1966) and in stages 22, 23 and 24, according to our studies. The myoblasts of the myotome become elongated in a fusiform fashion without the synthesis of DNA occurring, as shown by Stockdale and Holtzer (1961) with tritiated timidine, up to H.H. stage 18-19. To favour the fusion process more effectively, several invaginations and interdigitations are produced (pseudopodial processes) in the plasmatic membrane between two neighbouring myoblasts or cytoplasmic flaps which tend to surround or partly become attached to another myoblast, at whose point of contact there are areas of faded membranes (Fischman, 1967; Kelly and Zaks, 1969; Gamble et al., 1978; Campion et al., 1981; Samosudova et al., 1988).

In conclusion, with regard to the question of «when» and «where» fusion takes place, we believe and suggest that the mechanism described in seven stages always takes place both in the myogenesis of the embryonic muscle tissue (myotome) (a), as well as in that of the adult muscle tissue (differentiation of muscles *«in situ»*) (b), and in both cases this is preceded by the hormonal and biochemical preparation described above.

a) In the myotome, the myoblasts merge laterally at their ends, to produce a thicker, larger cell (which is still not a myotube), which serves to carry out the first flexion movements on the notochord, which are the most primitive movements performed by the embryonic body. Ondulating movements which the embryo makes in the amniotic fluid are due to the successive contraction of the different myotomes in the cranial-flow direction.

b) In the development of the muscles *«in situ»*, the fusion of myoblasts takes place mainly at the ends (several joined *«in chain»*) which gives rise to extremely elongated pseudo-cylindrical myocells with several nuclei or myotubes from which the multinucleate syncitial skeletal muscle fibre is formed (Przybylski and Blumberg, 1966). The growth of the skeletal muscle fibre depends on the continuous recruiting of nuclei from a population of mononucleate myoblasts (Caplan et al., 1988). For this to occur, the myoblasts form groups of 3-10 myoblasts where the centrally-located ones are

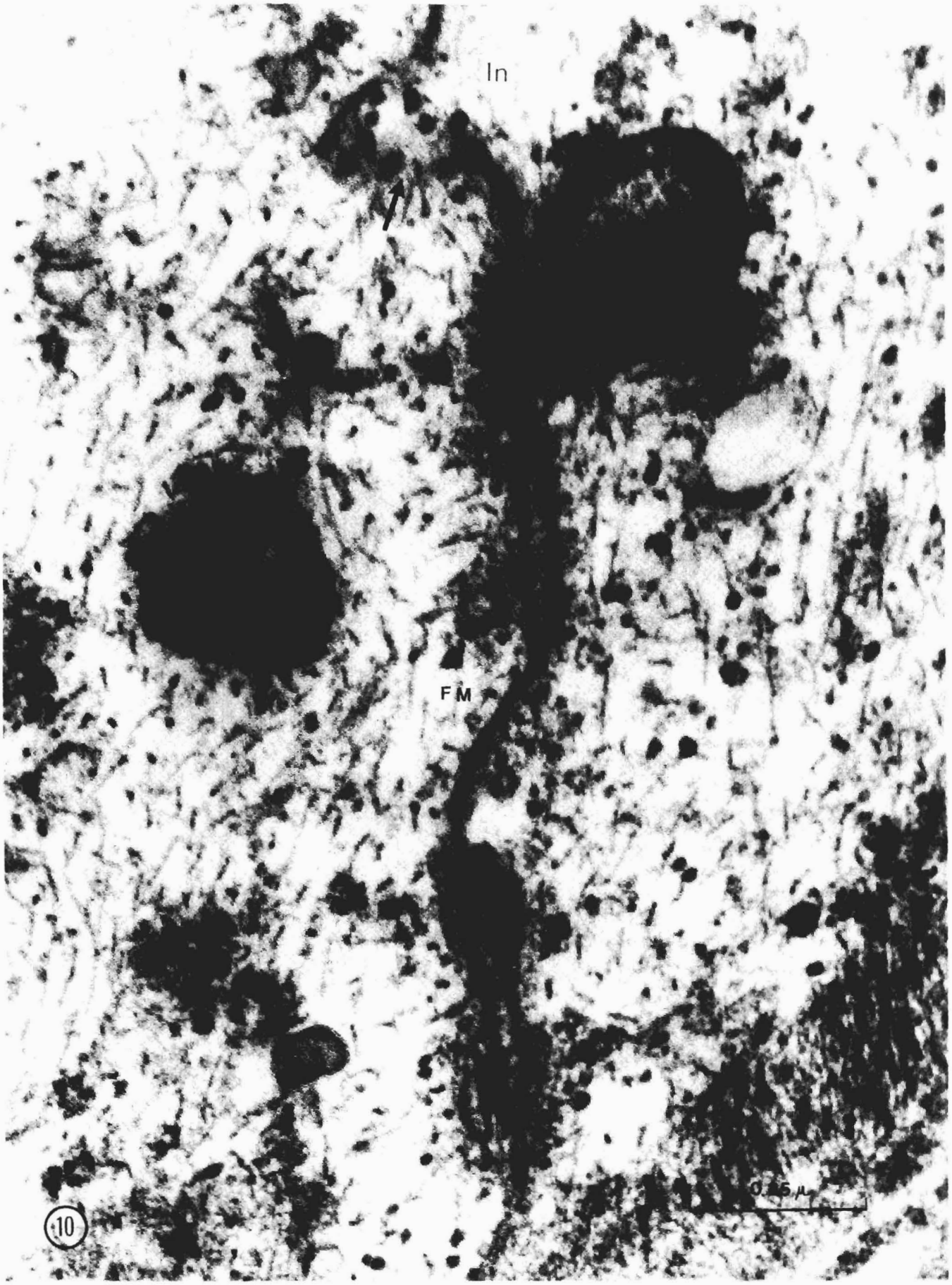


Fig. 10. Stage 24. Membrane fusion (FM) between two light myoblasts. Cytoplasm fusion (arrow) in invaginations (In). x 31,500

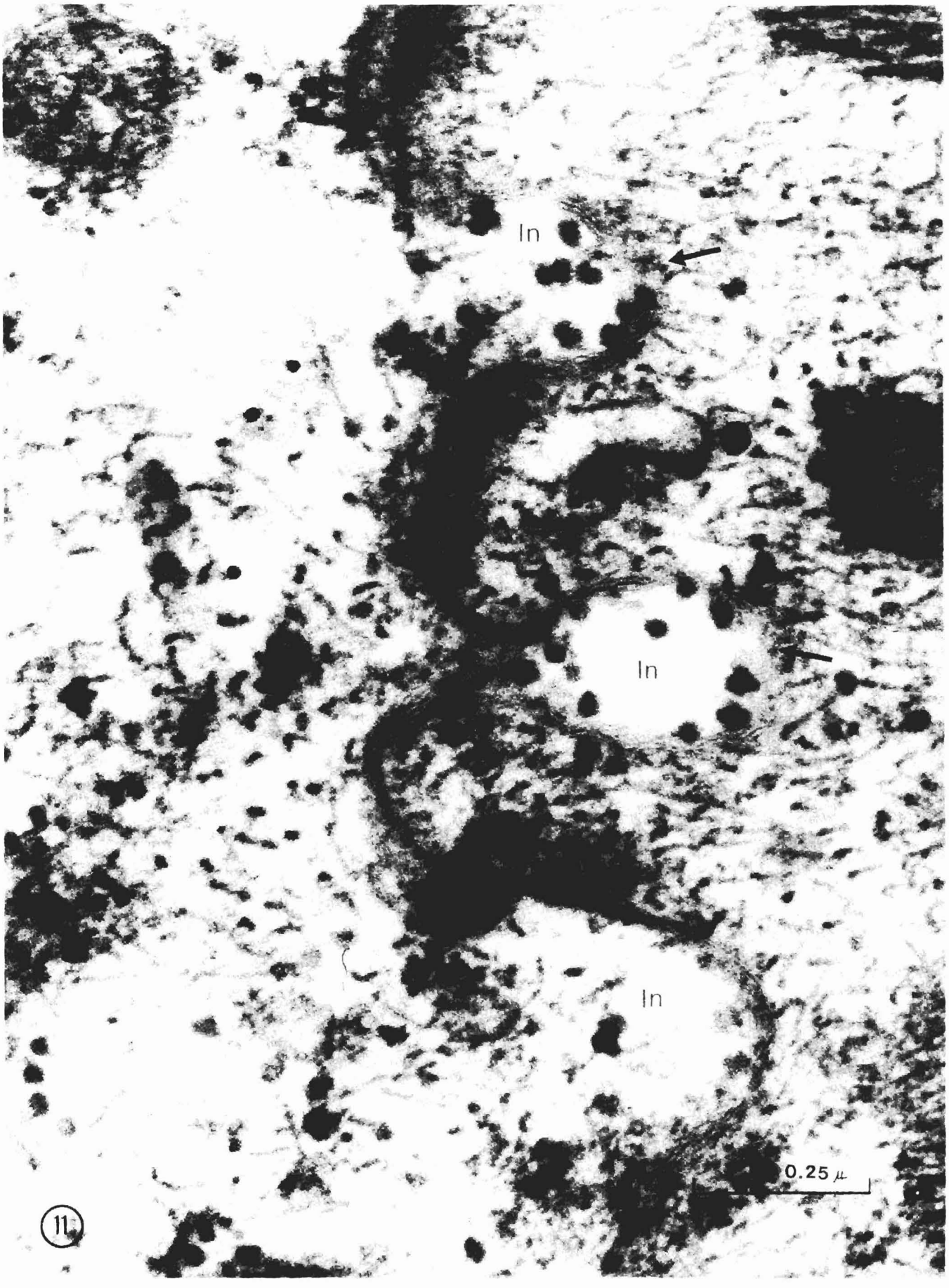


Fig. 11. Stage 24. Interdigitation and invagination (In) between two light myoblasts. Cytoplasm fusion (arrow) x 31,500

greater in volume and have less basophilia and more micro-filaments, and those located peripherically are more basophilic and darker, since they contain many ribosomes (Fischman, 1967). In this grouping of myoblasts, half-way to fusion, the plasmatic membrane may only be visible in the periphery of the group (Fischman, 1967). The groups of myoblasts appear surrounded by a basal membrane which indicates its common intention to merge. Then the myotubes can increase their number of nuclei by fusion (Kelly and Zacks, 1969) and incorporation of myoblasts located laterally to the myotube or at the ends (inside the basal membrane) (Caplan, 1987) at the expense of the peripheral basophilic myoblasts (satellite cells) (Przybylski and Blumberg, 1966). The regeneration of the skeletal muscle fibres is verified by these satellite cells, located inside the basal membrane, where these primitive myoblasts may produce mitosis (Kelly and Zacks, 1969) permitting one myoblast to merge with the muscle fibre (regeneration in the case of traumatisms), and another myoblast to remain as a separate mother satellite cell (Betz et al., 1966; Moss and Lebond, 1971; Gamble et al., 1978). This process makes the muscle fibres grow in length and thickness (Przybylski and Blumberg, 1966; Bodemer, 1970). The myoblast incorporated into the myotube or muscle fibre does not divide again (Shimada et al., 1967; Campion et al., 1981). The fusion of mononucleate myoblasts (satellite cells) with the myotubes may be halted, if the synthesis of DNA is suppressed with nitrogenated mustard (Konigsberg et al., 1960) and with 5-bromodeoxy-uridine, with reversible supresses fusion, but not mitosis or the synthesis of DNA (Stockdale et al., 1964).

After having said the above, we may ask ourselves «why» biology makes this seemingly senseless effort of dividing cells only to merge them again afterwards. Our suggestion as an explanation for this is that the purpose («why» or «for what reason») lies in the fact that the myoblasts may align themselves and then merge at the ends, originating an elongated fibrocell which will better fulfil the function of becoming shortened, since it is better able to reduce its length. If the cell were to simply multiply its nuclei, a rounded or ovoid cytoplasm would result with a much smaller diameter than the elongated multinucleate fibrocell originated by the fusion mechanism we have described above.

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References

Bardem C.R. (1990). The development of the musculature of the body wall of the pig, including its histogenesis and its relations to the myotomes and to the skeletal and nervous apparatus. John Hopkins Rep. 9, 367-399.

Bassleer R. (1962). Etude de L'angmentation du nimbre de moyaux des bourgeons musculaires cultivés *in vitro*. Observations sur le vivent, dosages cytophotometriques et histoautoradiografiques. Z. Entwicklunggsgesch. 123, 184-205.

Bergman R.A. (1962). Observations on the morphogenesis of rat skeletal muscle. John Hopkins Hosp. Bull. 11, 187-201.

Betz E.H., Firket H. and Reznik M. (1966). Some aspects of muscle regeneration. Int. Rev. Cytol. 19, 203-227.

Bintliff S. and Walker B.E. (1960). Radioautographic study of skeletal muscle regeneration. Am. J. Anat. 106, 233-245.

Bischoff R. and Holtzer H. (1969). Mitosis and the processes of differentiation of myogenic cells *in vitro*. J. Cell Biol. 41, 188-200.

Bischoff R. (1978). Myoblast fusion. In: Membrane fusion. Poste G. and Nicolson G.L. (eds). Elsevier. Amsterdam. pp 127-179.

Bloom W. and Fawcett D.W. (1964). Tratado de Histología. Ed. Labor. Buenos Aires. 5ª Ed. pp 246-248.

Bloom W. and Fawcett D.W. (1978). Tratado de Histología. Ed. Labor. Barcelona. 7ª Ed. pp 330-331.

Bodemer C.W. (1970). Embriología moderna. Ed. Interamericana. México. pp 274-279.

Boyd J.D. (1960). Development of striated muscle. In: Structure and function of muscle (Bourne). Vol. 1. Acad. Press. New York.

Buell D.N. and Fahey J.L. (1969). Limited periods of gene expression in immunoglobulin - synthesizing cells. Science 164, 1524-1525.

Buell D.N., Sox H.C. and Fahey J.L. (1971). Immunoglobulin production in proliferating lymphoid cells. In: Developmental aspects of the cell cycle. Cameron I.L., Padilla G.M. and Zimmerman A.M. (eds). Academic Press. New York. pp 279-296.

Buckley P.A. and Konigsberg I.R. (1974). Myogenic fusion and the duration of the postmitotic gap (G1). Dev. Biol. 37, 193-212.

Campion D.R., Fowler S.P., Hausman G.I. and Reagan J.O. (1981). Ultrastructural analysis of skeletal muscle development in the fetal pig. Acta Anat. 110, 277-284.

Capers C.R. (1960). Multinucleation of skeletal muscle *in vitro*. J. Biophys. Biochem. Cytol. 7, 559-566.

Caplan A., Carlson B., Fanlkner J., Fischman D.A. and Garret W.J. (1988). Skeletal muscle. In: Injury and repair of the musculo-squeletal shoft tissues. Woo S.L.-Y. and Buckwalter J. (eds). Park Ridge, I.L. American Academy of Orthopedic Surgeons. pp 213-291.

Chevremont M. (1940). Le muscle squelletique cultive *in vitro*. Transformation d'element musculaires en macrophagues. Arch. Biol. 51, 313-333.

Couch C.B. and Strittmatter W.J. (1983). Rat myoblast fusion requires metalloendoprotease activity. Cell 32, 257-265.

Darnell J., Lodish H. and Baltimore D. (1988). Biología celular y molecular. Ed. Labor. Barcelona.

David J.D., See W.M. and Higginbotham C.A. (1981). Fusion of chick embryo skeletal myoblasts: role of calcium influx preceding membrane union. Dev. Biol. 82, 292-307.

Den H., Malinzak D.A., Keating H.J. and Rosenberg A. (1975). Influence of concanavalin A, wheat germ agglutinin and soybean agglutinin in the fusion of myoblasts *in vitro*. J. Cell Biol. 67, 826-834.

Dessouky D.A. and Hibbs R.G. (1965). An electron microscope study of the development of the somatic muscle of chick embryo. Am. J. Anat. 116, 523-566.

Eycleshymer A.C. (1904). The cytoplasmic and nucleus changes in the striated muscle cell of Necturus. Am. J. Anat. 3, 285-310.

Firket H. (1958). Recherches sur le synthése des acides desoxiribonucleiques et la preparation á la mitose dans des cellules cultivées *in vitro* (Etude cytophotométrique et autoradiographique). Arch. Biol. 69, 1-16.

Fischman D.A. (1967). An electron microscope study of myofibril formation in embryonic chick skeletal muscle. J. Cell Biol. 32, 557-575.

Gamble H.J., Fenton J. and Allsop G. (1978). Electron microscope observations on human fetal striated muscle. J. Anat. 126, 567-589.

Godman G.C. (1957). The regeneration and redifferentiation of mammalian striated muscle. J. Morphol. 100, 27-81.

Godman G.C. (1955). The effect of colchicine on striated muscle in tissue culture. Exp. Cell Res. 8, 488-499.

Ham A.W. and Cormack D.H. (1983). Tratado de Histología. Ed. Interamericana. México. 8ª Ed. pp 627-629.

Hamburger V. and Hamilton H.L.(1951). A serie of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92.

Hamilton W.J., Boyd J.D. and Mossman H.W. (1964). Embriología Humana. Ed. Interamericana. Buenos Aires. 3ª Ed. pp 450-452.

Hay E.D. (1961). Fine structure of differentiating muscle in development myotomes of *Amblyostoma opacum* larvae. Anat. Rec. 139, 236.

Hay E.D. (1963). The fine structure of differentiating muscle in the salamander tail Z. Zelforsch. 59, 6-34.

Heidenhain M. (1911). M. Handbuch der Anatomie des Menschen. Plasma und Zelle. L.2. Gustav Fischer-Verlag. Jena.

Herrmann H. (1963). Quantitative studies of protein synthesis in some embrional tissue. In: Cytodifferentiation and macromolecular synthesis. Locke M. (ed). Academic Press. Inc. New York.

Heuson-Stiennon J.A. (1965). Morfogenése de la cellule musculaire striée etudiée en microscope électronique. I. Formation des structures fibrillaires. J. Microsc. 4, 657-678.

Holtzer H. (1959). Some further uses of antibodies for analyzing the structure and development of muscle. Exp. Cell Res. (Suppl.) 7, 234-243.

Holtzer H., Abbott J. and Lash J. (1958). On the formation of multinucleated myotubes. Anat. Rec. 131, 567.

Holtzer H., Croop J., Dienstman S.R., Ishikawa H. and Somlyo A.P. (1975). Effects of cytochalasin-B and colcemide on myogenic cultures. Proc. Natl. Acad. Sci. USA. 72, 513-517.

Kalderon N. (1980). Muscle cell fusion. In: Membrane-membrane interactions. Gilula N.B. (ed). Raven Press. New York. pp 99-118.

Kelly A.M. and Zacks S.I. (1969). The histogenesis of rat intercostal muscle. J. Cell Biol. 42, 135-153.

Knudsen K.A. and Horwitz A.F. (1977). Tandem events in myoblast fusion. Dev. Biol. 58, 328-338.

Knudsen K.A. and Horwitz A.Z. (1978). Differential inhibition of myoblast fusion. Dev. Biol. 66, 294-307.

Konigsberg I.R. (1965). Aspects of cytodifferentiation of skeletal muscle. In: Organogenesis. De Haan R.L. and Ursprung H. (eds). Holt, Rinchart and Winston. New York.

Konigsberg I.R. (1971). Diffusion-mediated control of myoblast fusion. Dev. Biol. 26, 133-152.

Konigsberg I.R., McElvain N., Tootle M., Herrmann H. (1960). The dissociability of deoxy-ribonucleic acid synthesis from the development of multinuclearity of muscle cells in culture. J. Biophys. Biochem. Cytol. 8, 333-343.

Lash J.W., Holtzer H. and Swift H. (1957). Regeneration of mature skeletal muscle. Anat Rec. 128, 679-693.

Lipton B.L. and Konigsberg I.R. (1972). A fine structural analysis of the fusion of myogenic cells. J. Cell Biol. 53, 348-364.

Marchock A. and Herrmann H. (1967). Studies of muscle development. I. Changes in cell proliferation. Dev. Biol. 15, 129-155.

Miller J.B. (1992). Myoblast diversity in skeletal myogenesis: how much and to what end? Cell 69, 1-3.

Moss F.P. and Leblond C.P. (1971). Satellite cells as the source of nuclei in muscles of growing rat. Anat. Rec. 170, 421-436.

Moss P.S. and Strohman R.C. (1976). Myosin synthesis by fusion-arrested chick embryo myoblast in cell culture. Dev. Biol. 48, 431-437.

Nameroff M. and Munar E. (1976). Inhibiton of cellular differentiation by phospholipase C: II separation of fusion and recognition among myogenic cells. Dev. Biol. 49, 288-293.

Naville A. (1922). Histogenese et regeneration du muscle chez les Anoures. Arch. Biol. 32, 37-171.

Pogogeff I.A. and Murray M.R. (1946). From and behavior of adult mammalian skeletal muscle *in vitro*. Anat. Rec. 95, 321-336.

Price H.M., Howes E.L., Blumberg J.M. (1964). Ultrastructural alterations in skeletal muscle fibers injured by cold. II. Cells of the sarcolemmal tube: observations on «discontinuous» regeneration and myofibril formation. Lab. Invest. 13, 1279-1302.

Przybylski R.J. and Blumberg J.M. (1966). Ultrastructural aspects of myogenesis in the chick. Lab. Invest. 15, 837-861.

Rash J.E. and Fambrough D. (1973). Ultrastructural and electrophisiological correlates of cell coupling and cytoplasmic fusion during myogenesis *«in vitro»*. Dev. Biol. 30, 166-186.

Remak R. (1845). Uber die Entwiklung der Muskel Primitivbundel. Frorieps Neue Notizen. 35, 305-308.

Reynolds E.S. (1963). The use of lead citrate at high pH as an electron - opaque stain in electron microscopy. J. Cell Biol. 17, 208-213.

Richler C. and Yaffe D. (1970). The *in vitro* cultivation and differentiation capacities of myogenic cell lines. Dev. Biol. 23, 1-22.

Robertson T.A., Grounds M.D., Mitchell C.A. and Papadimitriou J.M. (1990). Fusion betwen myogenic cells *in vivo*: an ultrastructural study in regenerating murine skeletal muscle. J. Struct. Biol. 105, 170-182.

Samosudova N.V., Shungskaia V.E. and Larin I.S. (1988). Osobennosti ul'trastruktury piatisloinogo kontakta i ego rol' v sliianii mioblastov. Tsitologiia 30, 1073-1077.

Shafiq S.A. (1963). Electron microscope studies on the indirect flight muscles of *Drosophila melanogaster*. II. Differentiation of myofibrils. J. Cell Biol. 17, 363-373.

Shainberg A., Yagil G. and Yaffe D. (1969). Control of myogenesis *in vitro* by Ca concentration in nutritional medium. Exp. Cell Res. 58, 163-167.

Shainberg A., Yagil G. and Yaffe D. (1971). Alterations of enzymatic activities during muscle differentiation *in vitro*. Dev. Biol. 25, 1-29.

Shimada Y. (1971). Electron microscopy observations on the fusion of chick myoblasts *in vitro*. J. Cell Biol. 48, 128-142.

Shimada Y., Fischman D.A., Moscona A.A. (1967). The fine structure of embryonic chick skeletal muscle cells differentiated *in vitro*. J. Cell Biol. 35, 445-453.

Stockdale F. and Holtzer H. (1961). DNA synthesis and myogenesis. Exp. Cell Res. 24, 508-520.

Stockdale F., Okazaki K., Nameroff M. and Holtzer H. (1964). 5-Bromo-deoxiuridine: effect on myogenesis *in vitro*. Science 146, 533-535.

Strehler B.L., Konigsberg I.R. and Kelley J.E. (1963). Ploidy of myotube nuclei developing *in vitro* as determined with a recording double beam microspectrophotometer. Exp. Cell Res. 32, 232-241.

Van Deurs B. (1975). The use of tannic acid-glutaraldehyde fixative to visualize gap and tight junctions. J. Ultrastruct. Res. 50, 185-192.

Vertel B.M. and Fischman D.A. (1976). Myosin accumulation in mono-nucleated cells of chick muscle cultures. Dev. Biol. 48, 438-446.

Wakelam M.J.D. (1985). The fusion of myoblasts.. Biochem. J. 228, 1-12.

Weed I.G. (1936). Cytological studies of developing muscle with special reference to myofibrils mitochondria, Golgi apparatus and nuclei. Z. Zellforsch. 25, 516-540.

Yaffe D. (1968). Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA. 61, 477-483.

Yaffe D. (1971). Developmental changes preceding cell fusion during muscle differentiation *in vitro*. Exp. Cell Res. 66, 33-48.

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