SOME OBSERVATIONS ON THE FINE STRUCTURE AND METABOLIC ACTIVITY OF NORMAL AND GLYCERINATED VENTRICULAR MUSCLE OF TOAD

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ABSTRACT

Fine structure, enzyme activity, and transmembrane potentials of normal and glycerinated ventricular muscle of the toad were studied. For electron microscopy, osmium tetroxide and Araldite were used. Plasma membranes are firmly attached to Z bands. Both the T system and sarcoplasmic reticulum are poorly developed. Small bodies of medium density may be lysosomes derived from the Golgi zone. Denser bodies may be catecholamine granules. Fine tubules of unknown significance, about 200 A in diameter and of considerable length, lie in conspicuous, although infrequent bundles. Glycogen and mitochondria are abundant. After weeks of extraction in 50 per cent buffered glycerol, most organelles were still present, and much of the gross damage was probably due to osmotic destruction of membranes weakened by extraction. Many mitochondria were well preserved. Plasma and nuclear membranes had diffuse outlines and tended to be broken. Considerable activity remained of the enzymes succinic dehydrogenase, cytochrome oxidase, and phosphorylase after the extraction, but decreased with prolonged soaking. The normal transmembrane potential was about 95 mv; in extracted muscle after 6 weeks it was about 35 mv. The view that glycerinated muscle is a simple system of actin and myosin is clearly wrong. The activity of other organelles still present must affect the actions of many drugs and ions experimentally added.

INTRODUCTION

In 1949, Szent-Györgyi introduced the use of muscles soaked in glycerol for contraction-relaxation studies. Since then, glycerol-extracted muscles have been used in a variety of experiments designed to elucidate the events involved in contraction and relaxation (7, 11, 13, 18, 51). Generally, it has been assumed that the changes induced in the contractile activity of glycerinated muscles by varying the composition of the fluid bathing them have been due to a direct effect on the contractile proteins themselves. It would appear, however, that insufficient attention has sometimes been given to the structure and composition of the preparations, so that the interpretation of results presents some difficulties and uncertainties. Thus, if glycerol-extracted muscle retained other structures in addition to the contractile proteins, then the polyphosphate-induced relaxation of glycerol-treated muscle reported by Bowen and Laki (1960) might reflect the action of the polyphosphate on a cellular component other than the actomyosin system; the ouabain-increased, ATP-induced contraction of glycerol-extracted cardiac muscle (Lee, 1961) may not indicate a direct ac-

tion of the glycoside on the actomyosin contractile system; and the action of Mg^{++} on glycerinated muscles may not necessarily be due to the effect of these ions on the contractile proteins, as concluded by Watanabe *et al.* (1963).

This paper compares the morphology, enzyme activity, and transmembrane potentials of normal and glycerol-extracted ventricular muscle of the toad. The results indicate that glycerinated cardiac muscle retains structures in addition to the contractile proteins, and that at least some of the mitochondrial and sarcoplasmic enzymes remain active, even after prolonged treatment with glycerol. To determine whether or not these observations can be applied to glycerol-extracted skeletal muscle requires further study.

MATERIALS AND METHODS

Only healthy, active, freshly caught toads (*Bufo marinus*) were used.

Preparation of Glycerol-Treated Muscle

The beating hearts of pithed toads were rapidly excised, and strips of ventricular muscle approximately 2 cm long and 3 mm wide were immediately immersed in ice cold 50 per cent glycerol (v/v) containing 10 mM phosphate buffer (pH 7.0), and then tied to pre-cooled glass slides so that the strips were straight but unstretched. The slides and attached muscles were stored in the 50 per cent buffered glycerol at 0°C for 72 hours, the glycerol solution being renewed daily. The muscle strips, still attached to the glass slides, were then placed in fresh, cold glycerol solution and stored at −15°C for varying lengths of time, ranging from 3 to 300 days, until used.

In this paper, the time referred to as duration of glycerol treatment is that time which elapsed after completion of the initial 72 hours' immersion in glycerol at 0°C.

Electron Microscope Studies

For studies of normal myocardium, beating ventricular muscle was excised from freshly pithed toads and fixed for 2 hours in ice cold 2.5 per cent osmium tetroxide containing either a chromate (Dalton, 1955) or *s*-collidine (Bennett and Luft, 1959) buffer.

Portions of the same glycerol-extracted strips that were used for the enzyme studies were fixed in the same way. In the first attempts, some glycerol remained in the tissue after dehydration. The result was an extremely soft block of Araldite. In the next attempts, the tissue was washed for 1 hour, either with phosphate buffer before fixation, or in water after fixation. It was found that too rapid addition of either buffer or fixative to the glycerol-saturated tissue caused osmotic damage. The best results came from a very slow replacement of glycerol by fixative, as follows. Small pieces of tissue were placed in 1 ml of the 50 per cent glycerol mixture at −5°C. Over a period of 2 hours, the temperature was allowed to rise to about 5°C. During this time the glycerol mixture was replaced, a drop at a time with thorough mixing, by cold fixative. The tissue was then placed in fresh cold fixative in a roller-tube for a further 2 hours.

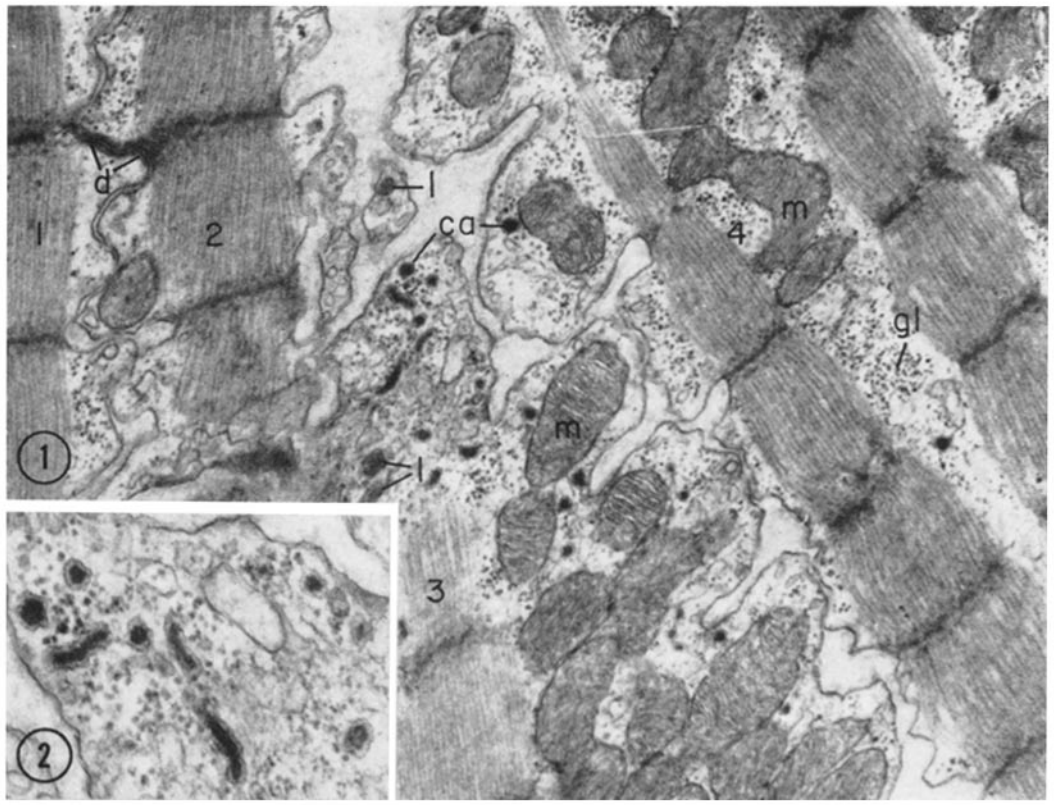
All specimens were dehydrated rapidly in acetone for about 15 minutes, and embedded in Araldite according to the method of Luft (1961), except that propylene oxide was not used.

Unsupported sections, about 1,000 A thick, cut with glass knives in a Cambridge Huxley-pattern microtome, and stained with lead salts by the Karnovsky method A (1961), were examined in a Siemens Elmiskop I.

FIGURE 1 Normal toad ventricle, chromate buffer. Portions of 4 contracted muscle fibres (*1* to *4*) showing attachments of Z bands to the corrugated plasma membranes. These may be grazing slices from large fibres, but they are probably from small ones. Fibre *2* contains a single myofibril; fibre *4* contains a shell of fibrils around a cytoplasmic core. Note the numerous glycogen particles (*gl*), the elongated mitochondria (*m*), the desmosome (*d*) attached to the Z lines, and the small bodies of high density (? catecholamines) (*ca*) and of medium density (? lysosomes) (*l*). The high density bodies are perhaps 10 times more numerous in this field than in most areas, and some are unusually elongated. × 14,000.

FIGURE 2 An enlargement of the centre of Fig. 1. The ? catecholamine granules have a conspicuous membrane envelope. × 29,000.

FIGURE 3 Normal ventricle, *s*-collidine buffer. Portions of 4 small muscle fibres in transverse section, showing the very close interrelationships found particularly in the trabeculae carneae. The plasma membranes are either about 200 A or about 800 to 1000 A apart. In the latter case, basement membrane (*bm*) fills the gap and *caveolae intracellulares* (*c*) are more numerous. × 40,000.



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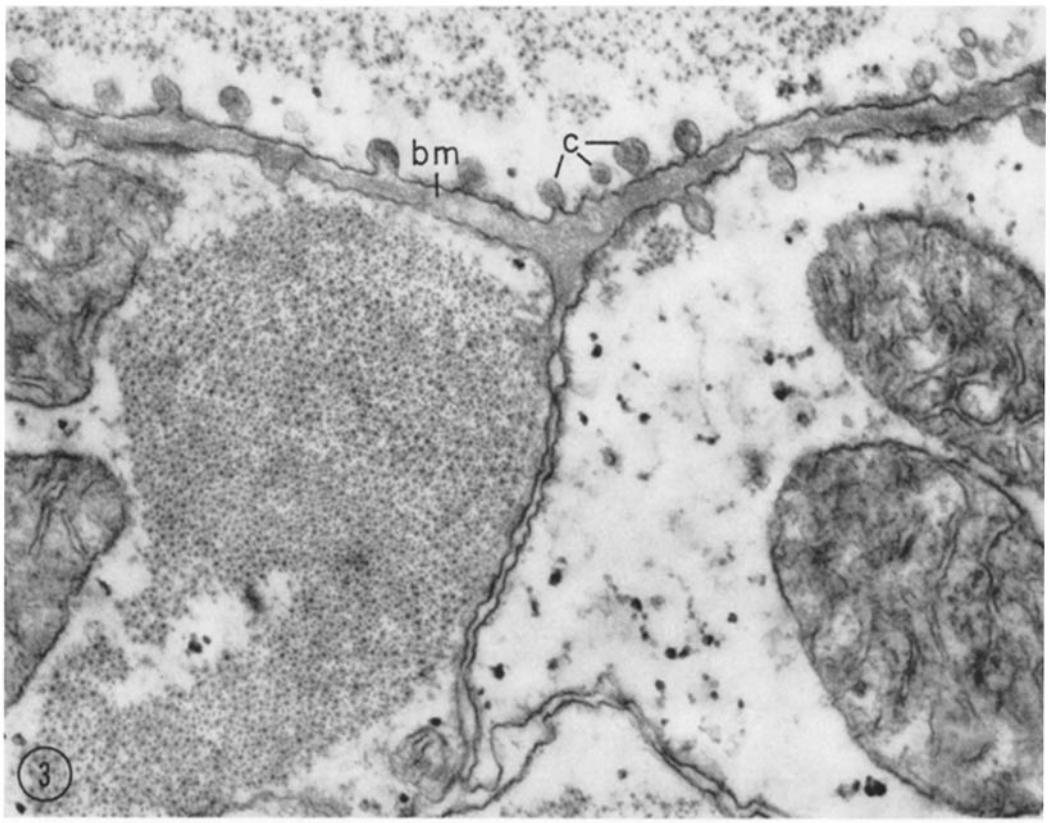

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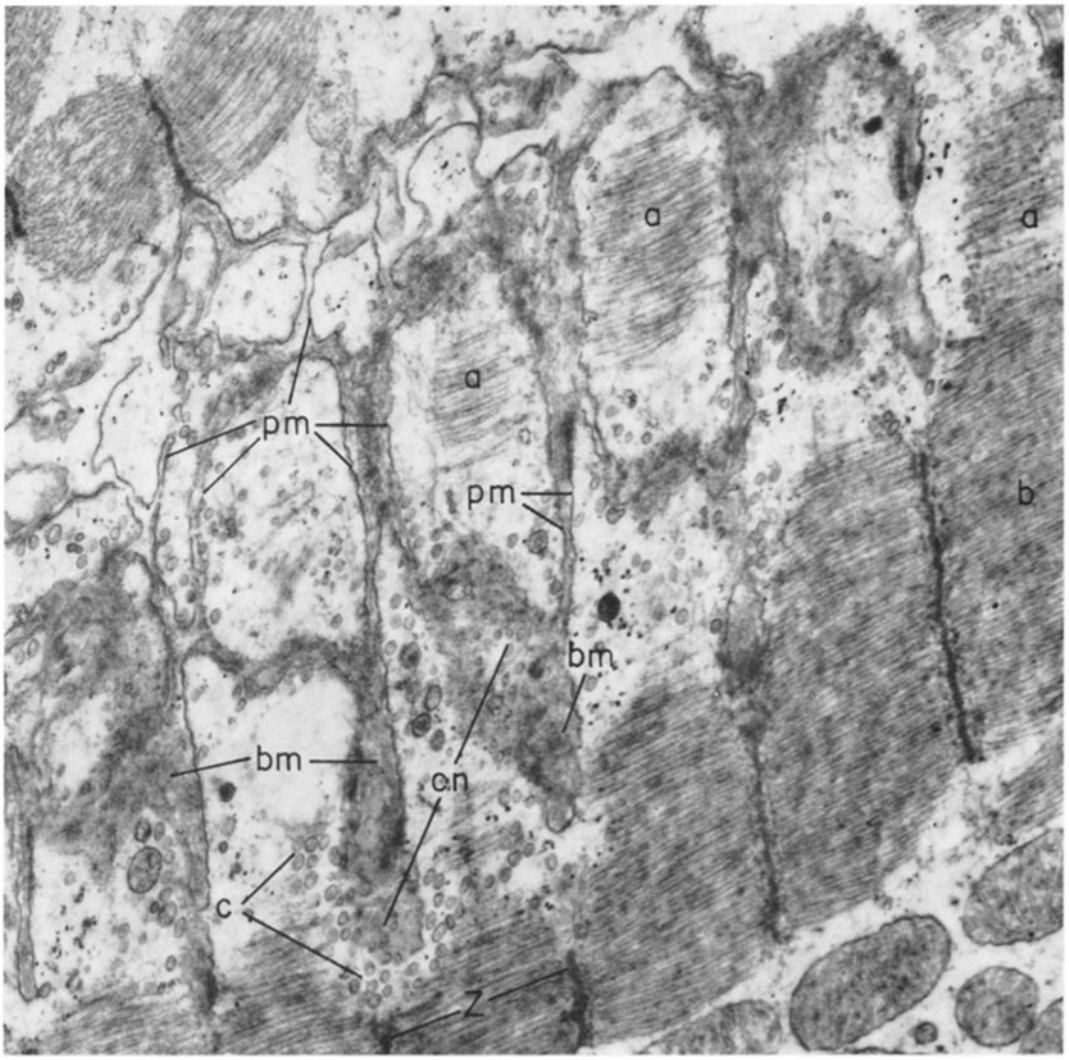


FIGURE 4 Normal ventricle, *s*-collidine buffer. A grazing slice from the surface of a large fibre shows that the apparently tubular invaginations of the plasma membrane (*pm*) are, in fact, circumferential corrugations of the surface. The broad processes of dense material (*bm*) are a mixture of basement membrane, tangentially cut plasma membrane, and some Z band material, in the depths of the folds. This is proved where the plane of section leaves the Z bands (*Z*) of the myofibril and enters, firstly, the broad zone containing deeper parts of *caveolae* (*c*), and then the plasma membrane and basement membrane zone containing also the small necks of *caveolae* (*cn*). Profiles of large tubes do not appear deep to the myofibrils. The right edge of the figure shows that the myofibril is very broad and that the filaments at *a* and *b* are portions of one fibril. The lower right corner shows the non-fibrillar core of the fibre devoid of large tubes. × 20,000.

Procedure for the Determination of Specific Enzymes Including Succinic Dehydrogenase, Cytochrome Oxidase, and Phosphorylase

Following their removal from the 50 per cent glycerol solution described above, strips of muscle were immersed in 15 per cent glycerol (v/v) at 0°C for 60 minutes, after which they were either homogenized at 0°C or pulverized with a pre-cooled crushing device (Nayler and Wright, 1963). In either case, the presence or absence of succinic dehydrogenase and cytochrome oxidase activity in the glyc-

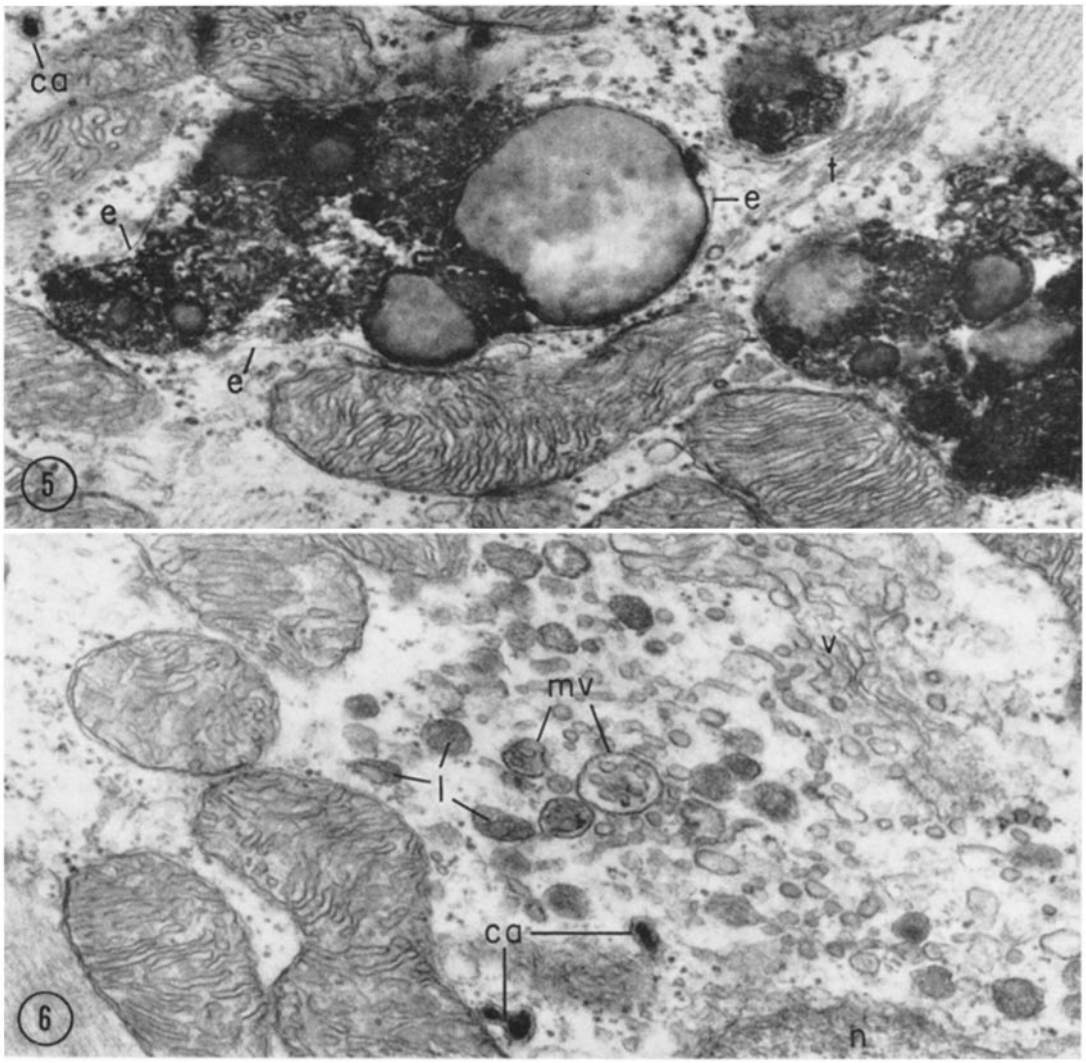


FIGURE 5 Normal ventricle, chromate buffer. Lipofuscin or lipochrome granules with their delicate membranous envelopes (*e*). Compare the latter with the heavier membrane surrounding the ? catecholamine granule (*ca*). Some poorly preserved fine tubules are seen at *t*. × 35,000.

FIGURE 6 Normal ventricle, chromate buffer. A Golgi zone near a nucleus (*n*). There are Golgi vesicles (*v*), multivesicular bodies (*mv*), and bodies of medium density (? lysosomes) (*l*). The smaller multivesicular body has a background density like that of the dense bodies; this is possibly a transition stage, and was usually more common in other Golgi zones. The shaved surface of a mitochondrion lies between the two ? catecholamine granules (*ca*). The large dense granules commonly found in mitochondria are absent from these toad mitochondria. × 35,000.

erinated muscle was demonstrated by the standard manometric technique described by Umbreit, Burris, and Stauffer (1957). Incubations were performed at 25°C, and all reagents were of A.R. (Analytical Reagent) grade. Phosphorylase activity was estimated according to the method of Cori and Illingworth (1956), the inorganic phosphate being determined by the method of Lowry and Lopez (1946). Freshly excised, non-glycerinated, ventricular muscle was similarly assayed for the enzymes succinic dehydrogenase, cytochrome oxidase, and phosphorylase.

Measurement of Transmembrane Resting Potentials

Transmembrane resting potentials were measured by the standard microelectrode technique of Ling and Gerard (1949), using strips of freshly excised, glycerol-extracted muscle suspended isometrically under a constant resting tension, and bathed for at least 30 minutes with Ringer's solution containing 1.3 mM Ca^{++}, as described by Nayler and Emery (1962).

Glass microelectrodes were filled with 3 M KCl and had a resistance of 30 to 50 megohms; the indifferent electrode was silver-silver chloride. In each case, resting potentials were recorded from at least four impalements, and the observed variations fell within the calculated experimental limits of accuracy (error in measurement of resting potential = ± 2 mv). The validity of the measuring and recording apparatus was checked regularly by inserting a 100 mv signal between the microelectrode and earth.

OBSERVATIONS

Normal Muscle Fine-Structure

Grimley and Edwards (1960) correctly likened the heart muscle of the toad to that of the turtle (Fawcett and Selby, 1958). The muscle cells are relatively narrow, the intercalated discs are generally in the form of small and isolated steps confined to single myofibrils, the sarcoplasm is abundant in the larger fibres and contains much glycogen, and, in contrast with its elaborate and repeating pattern in mammalian cardiac muscle (Porter and Palade, 1957), the endoplasmic reticulum is poorly developed.

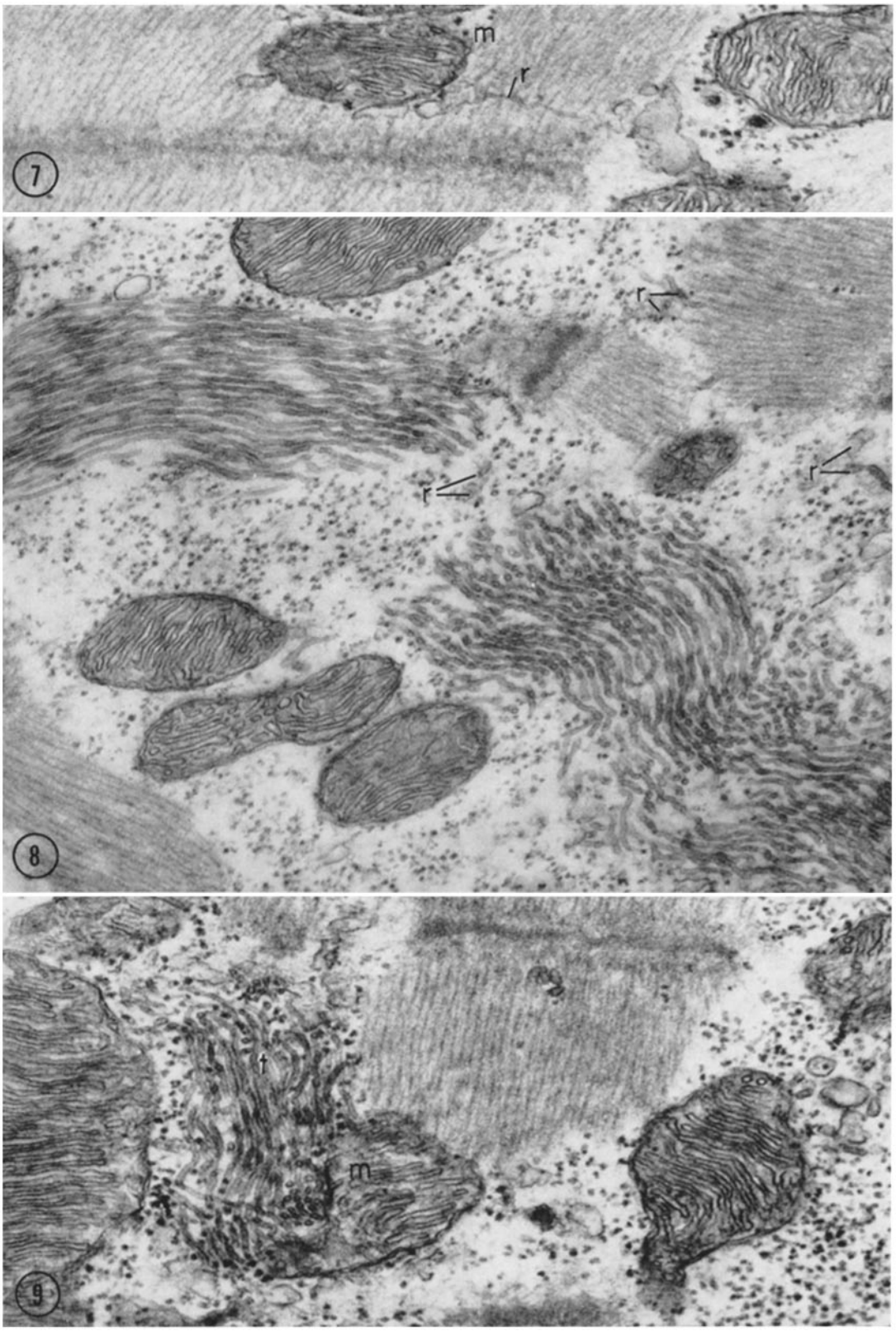
The basic structure and arrangement of the muscle fibres are, in general, similar to those of other animals (Porter and Palade, 1957; Battig and Low, 1961; Stenger and Spiro, 1961). Striated branching myofibrils, either singly or in narrow parallel groups, are interspersed with closely packed mitochondria. In many small fibres the myofibrils tend to lie at the periphery, whereas clumps of crowded mitochrondria occupy a central cytoplasmic core of the fibre (Figs. 1 and 4). Many even smaller fibres, perhaps branches, contain only one myofibril. The nuclei lie in the central core. Various organelles are concentrated about the poles of the nuclei: there is a small Golgi apparatus with a peripheral zone containing dense bodies (Poche, 1958; Battig and Low, 1961) and a few multivesicular bodies (Fig. 6). Outside this is a zone of randomly arranged endoplasmic reticulum. Occasionally, there is a centriole in the plane of section near a nucleus.

We have found a number of examples of bundles of long fine tubules in the muscle fibres, generally near the nucleus (Figs. 8 and 9). The tubules are loosely packed and usually in parallel. Each is

FIGURE 7 Normal ventricle, chromate buffer. An obliquely cut, contracted myofibril, penetrated by a portion of sarcoplasmic reticulum (*r*) linking collections of sarcoplasm containing mitochondria close to the Z line. The mitochondria at *m* are lying in a shallow groove in the fibril: the restricted field makes them appear buried. Most of the fragments of reticulum recognizable in this series had vague outlines and obscure relationships. × 35,000.

FIGURE 8 Normal ventricle, chromate buffer. A large bundle of tubules of unknown function. The tubules show both fine and coarse undulations. The former causes fine beading, particularly when the plane of section grazes the undulations. The coarse undulations sweep the bundles in and out of the plane of section, and give rise to transversely cut profiles. A few fragments of reticulum (*r*) seem to be associated with Z or I band regions. × 35,000.

FIGURE 9 Normal ventricle, chromate buffer. A bundle of fine tubules (*t*) passing round a mitochondrion (*m*). Both are entering the plane of section at the same place, so that the tubules overlie the obliquely cut edge of the mitochondrion. The relationships are confused, but there is probably no continuity between the two. Towards the upper left corner, some of the tubules are tending to turn horizontally and to the left. This orients them with the general direction of the zone occupied by some very ill defined fragments of reticulum lying in the sarcoplasm to the left of the obliterated I band of the contracted myofibril. × 35,000.


m
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7
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about 200 A in diameter after lead stain and contrasty development. Most of the tubules are smooth-walled, but some are slightly corrugated. The bundles sweep through the plane of section, and their origins and significance are unknown. At times some of these tubes appear to pass into a mitochondrion, but this is almost certainly a spurious appearance due to the mitochondrion lying over the point at which the tubules leave the plane of section (Fig. 9). Thus, the obliquely cut external mitochondrial membranes are not resolved, so that there seems to be no barrier between the tubules and the cristae.

Lysosomes, fat droplets, and smaller bodies of both medium and high density (Figs. 1, 4, and 10) are scattered sparsely through the cytoplasm.

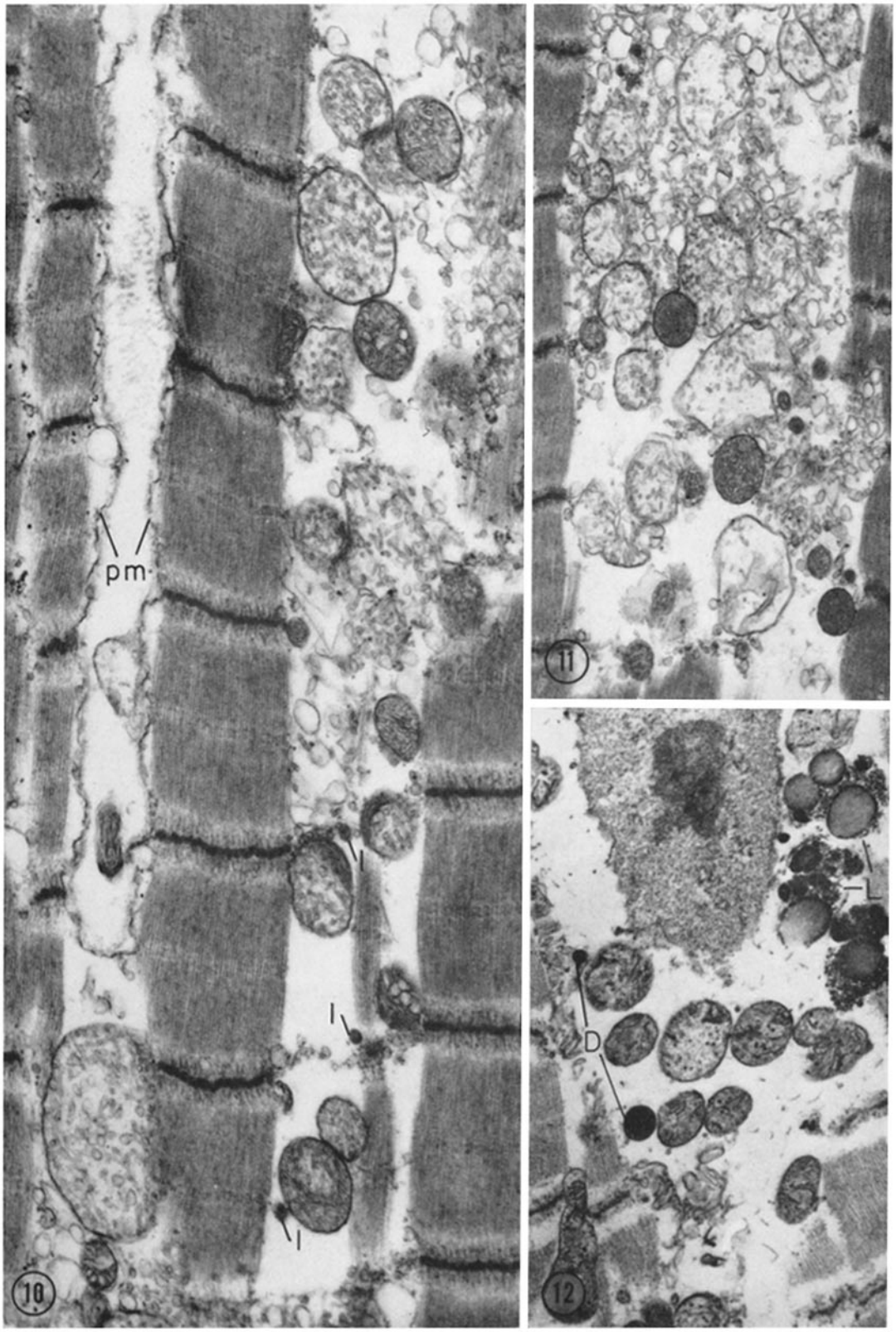
Some lysosomes (Fig. 5) have developed into typical lipochrome or lipofuscin granules or cytolysomes (de Duve, 1959; Novikoff and Essner, 1962; Björkerud, 1963). The scattered small bodies of medium density (Figs. 1 and 10) are oval and bounded by a single membrane, and most are completely filled with dense material. They range up to 2,500 A in diameter. They appear to be identical with the dense bodies around the Golgi zone, where there is, apparently, no division between the obvious dense bodies and vesicles of similar size and shape that contain smaller vesicles about 500 A in diameter lying in a material of medium density (Fig. 6). The small, very dense bodies (Figs. 1 and 2) are spheroidal or rod-shaped, the shape perhaps depending on the plane of section, and because the dense contents are retracted from the membrane they have a more prominent membranous envelope than the other less dense bodies. They resemble those found in cyclostome cardiac muscle cells by Bloom (1962) who suggested that they contained catecholamine, and they are most dense after a chromate-buffered fixative followed by a lead stain. The density of the granules in some vesicles of autonomic axons, currently thought to contain norepinephrine, is also enhanced by chromate buffer and lead stain (Merrillees, unpublished).

Apart from that in the perinuclear zone, the endoplasmic reticulum is inconspicuous. Vesicles are scattered deep to the plasma membrane and

FIGURE 10 Relaxed ventricle extracted in glycerol for 3 days, chromate buffer. The specimen was washed for 1 hour in toad Ringer's solution in an attempt to remove all glycerol before fixation. The glycerol was completely removed, but was replaced too rapidly by water: many mitochondria are exploded. The surviving mitochondria show different degrees of swelling. Segments of many erupted cristae cause confusion. At the lower right, lines of vesicles between neighbouring Z bands suggest the presence of a transverse system. Along the extreme left margin, a few similar vesicles are associated with the I bands between two myofibrils. The plasma and basement membranes (*pm*) are damaged but almost intact, and are still attached to the Z lines. No caveolae are recognizable. The small bodies of medium density (*l*) (? lysosomes) are preserved, but the ? catecholamine granules are not recognizable, and there are few glycogen-like particles in this area. Neighbouring areas contained much more glycogen (Fig. 14). × 16,000.

FIGURE 11 A neighbouring part of the same section as Fig. 10. Severe mitochondrial damage is seen: a few mitochondria survive, but they are unusually rounded, suggesting some internal pressure. Some damage is appearing in the I bands at top right; this may be a shrinkage artefact; it is common after longer glycerol extraction. × 11,000.

FIGURE 12 Ventricle glycerol-extracted for 4 weeks, chromate buffer. Portion of the same specimen as in Figs. 10 and 11, but left in glycerol for 4 weeks longer. Fixed by very slow replacement of glycerol solution by fixative over a period of 2 hours, starting at −5°C. Fewer mitochondria are exploded, although most structures have become more fragile with the longer extraction. I bands are ruptured. The nuclear membrane is broken, but the nucleus and nucleolus are otherwise fairly well preserved. Lipofuscin granules (*L*) appear normal except for damaged membranes. Much of the visible damage to the various membranes probably occurs during fixation. Dense droplets (*D*), resembling lipid, are scattered through the cytoplasm; similar but smaller ones lie in the mitochondria. This suggests a breakdown of lipoprotein material. × 12,000.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between the myofibrils, and, occasionally, a small rounded vesicle lies within a myofibril in the Z band or near the A–I junction. However, we have not recognized any longitudinal vesicular network regularly related to the sarcomeres. Some vesicles and perhaps fine tubules are sometimes associated with the sarcomeres (Figs. 7 to 9), but either they are inconstant in any one plane of section, or our methods of preservation were inadequate. In the great majority of cases, the tubular images are so vague that one suspects either loss of structures or an artificial production of vesicles from a normally non-vesicular material. There seem to be portions of fine (200 A) transverse tubules in the zone between the Z bands of neighbouring myofibrils (Fig. 8), but these also are inconstant or inadequately preserved.

The sarcolemma makes frequent direct contacts with a local expansion of the periphery of the underlying Z bands (Figs. 1, 4, and 10). In contracted muscle, many of these contacts are made by invaginations of the plasma membrane, apparently in the form of deep and narrow clefts or tubes, usually filled with basement membrane, but sometimes the latter is excluded from the bottoms of the clefts. The tubular appearance is quite misleading. Grazing longitudinal sections show that these clefts pass for considerable distances around the circumference of the fibre at each Z band (Fig. 4). The clefts are seen only in strongly contracted muscle; they are not permanent structures, but merely deep corrugations produced by the contraction of the muscle in the fixative, exaggerated perhaps by swelling of the muscle cells in hypotonic fixative. The appearance of the folds reveals that the attachment of the sarcolemma to the Z line is strong. There is no transverse tubular system of large tubular invaginations of the sarcolemma such as that seen in many animals (Lindner, 1957; Simpson and Oertelis, 1962; Nelson and Benson, 1963). Sections of large tubules lined by basement membrane are not found between the deeper myofibrils in toad cardiac muscle.

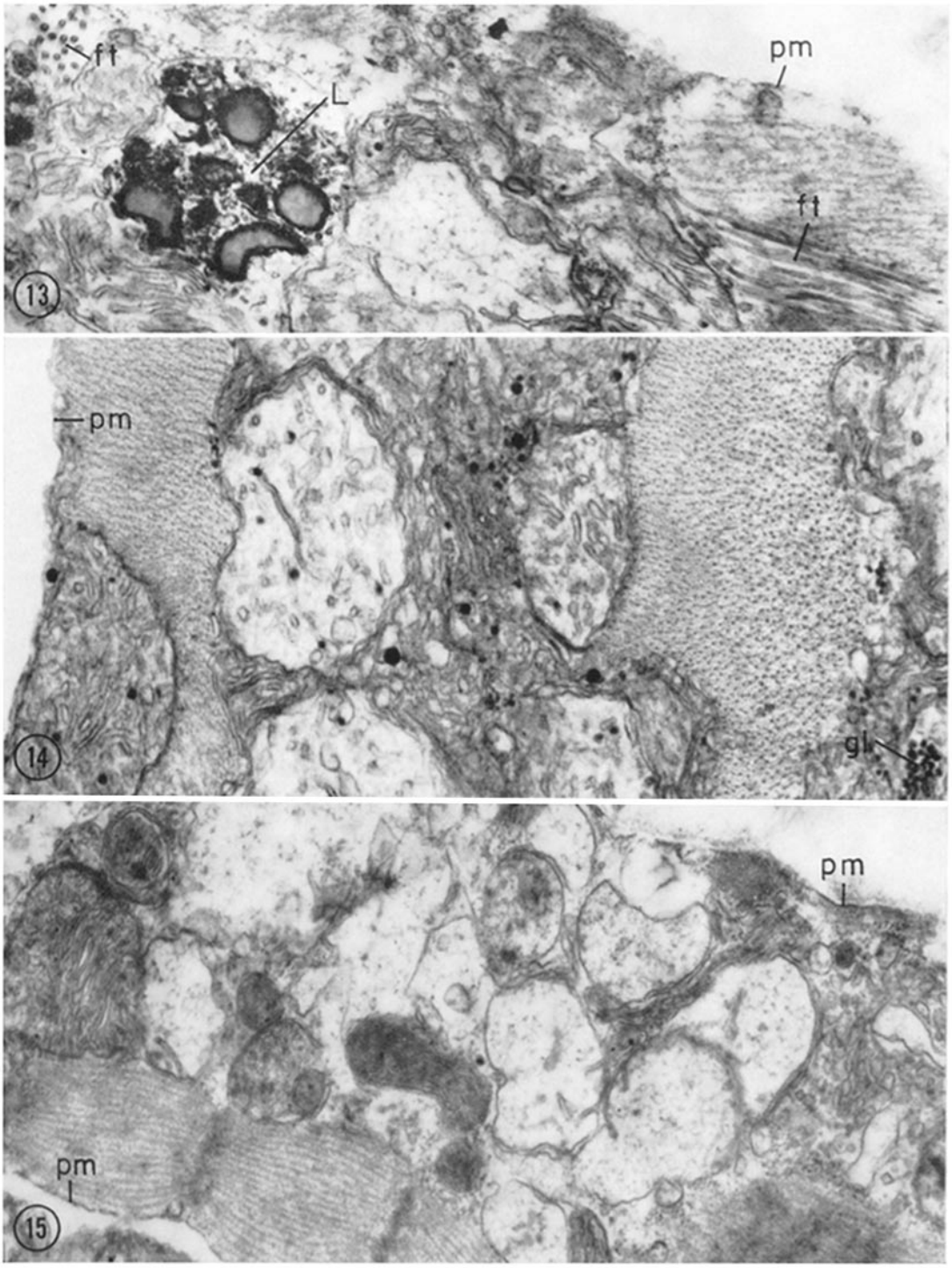
In both transverse and longitudinal sections, long lengths of neighbouring plasma membranes are either about 200 A or about 800 A apart. *Caveolae intracellulares* are more numerous along the more widely spaced membranes (Fig. 3). Desmosomes and intercalated discs are as described by Grimley and Edwards (1960). The preservation of this material is inadequate for a useful comparison with Huxley's (1957) description of the myofilaments in striated muscle.

The mitochondria are typical of cardiac muscle. They tend to lie in groups under the sarcolemma, or in the sarcoplasmic core when there is one. In the larger fibres they lie in single rows between neighbouring myofibrils. In sections their profiles are irregular in size and shape, but closely resemble each other in texture. They contain no granules. The cristae lie in a background of considerable density. Very rarely a mitochondrion is swollen or is involved in the growth of a lysosome.

FIGURE 13 Ventricle glycerol-extracted for 3 days, *s*-collidine buffer. There is gross damage due to unsuitable techniques. No attempt was made to remove all glycerol: this resulted in an extremely soft block. No attempt was made to prevent osmotic damage: the mitochondria are severely affected. Lipofuscin granules (*L*) and fine tubules (*ft*) are preserved. The plasma membrane (*pm*) is damaged. × 30,000.

FIGURE 14 Ventricle glycerol-extracted for 3 days, chromate buffer. Portion of the same specimens as in Figs. 10 and 11. Dense droplets are seen in mitochondrial areas. This suggests some mitochondrial damage due to other than osmotic causes, which was not great in this particular area. The density of the droplets suggests a lipid material. These droplets were seen only occasionally. Myofilaments and glycogen granules (*gl*) are well preserved. The plasma membrane (*pm*) is intact but pale. × 35,000.

FIGURE 15 Ventricle glycerol-extracted for 10 months, chromate buffer. There is osmotic damage because the glycerol was replaced by tap water before fixation, and the replacement period, 15 minutes, was too short. Some remaining glycerol caused a soft block. Yet, many mitochondria are well preserved. The myofibril is contracted; this was unusual in extracted specimens. The two plasma membranes (*pm*) are still recognizable. After long extraction, the sections stain poorly, with general low contrast. × 24,000.


ft
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Nothing in their appearance suggests that there is more than one type of mitochondrion in a normal preparation.

The glycogen particles are scattered irregularly through the sarcoplasm, in high concentration in some areas. Sometimes small accumulations are wedged between myofilaments in the I band, buried deeply in the myofibrils.

TABLE I

Succinic Dehydrogenase Activity in Freshly Excised and Glycerol-Treated Ventricular Muscle

	Succinic dehydrogenase activity (in μlO_2/10 min.*) 25°C		
	Prep. No.	A	B
Freshly excised muscle	1	8.2	17.0
	2	9.1	18.5
	3	10.0	22.0
	4	9.2	21.3
After 3 weeks' immersion in glycerol solution	5	7.8	15.0
	6	7.0	15.5
	7	6.8	14.2
	8	7.4	15.2
After 6 weeks' immersion in glycerol solution	9	6.3	12.9
	10	6.0	12.2
	11	5.5	14.0
	12	6.8	12.3
After 10 months' immersion in glycerol solution	13	4.4	7.9
	14	3.2	7.0
	15	1.8	4.1
	16	4.1	7.3

In which A and B represent two different tissue dilutions (Umbreit *et al.*, 1957).
* Microliters of oxygen in 10 minutes.

Glycerol-Extracted Muscle

More obvious damage can be caused to the muscle by osmosis during replacement of the glycerol than is caused by the extraction itself. Membranes and other structures are more fragile after extraction, but if great care is taken during preparation there is reasonably good preservation, and much of the remaining damage seen in these plates could probably be avoided with technical refinements. The most obvious change in all extracted specimens was a loss of sharpness in the images of membranes. These structures seemed swollen, having a powdery or finely granular appearance, with diffuse outlines rather than sharply defined edges. This appearance together with a less than normal staining reaction led to fuzzy low-contrast images.

The muscle in the cold glycerol was relaxed, and, if kept cold, usually remained relaxed in the fixative. The plasma membranes remained smooth and free of clefts. The extracted membranes are evidently more delicate than normal because there were frequently many small breaks. Many, if not all, of these probably occurred during preparation, because the more gently the tissue was handled, the fewer there were. Tissue in which the glycerol was replaced slowly by fixative during a period of 2 hours, with a rise in temperature from −5°C to 5°C over the same time, had almost intact plasma and basement membranes although the membranes lacked sharpness. There were no *caveolae intracellulares* in extracted membranes, but vesicles of the endoplasmic reticulum persisted.

The nuclear membranes were seriously weakened, and many were ruptured, even after careful handling (Fig. 12). Well preserved nuclei appeared to have almost normal density. Nucleoli were little affected.

The myofilaments were usually unaffected, but sometimes, after several weeks of extraction, the sarcomeres were ruptured through each I band, between the N line and the A–I junction (Fig. 12). This suggested a severe shrinkage artefact, or a weakening of the actin filaments.

Desmosomes and intercalated discs were intact in all specimens. With the exception of the catecholamine-like granules, all major sarcoplasmic structures seemed to be preserved, although their positions had been disturbed by osmotic tensions. Thus, the glycogen, lysosomes, small bodies of medium density, mitochondria, and reticulum were preserved, although the glycogen was considerably diminished in some areas. The reticulum and mitochondria suffered most from osmosis, and when mitochondria had ruptured, injecting cristae and sheets of membrane between the myofibrils, it became impossible to differentiate reticulum from cristae (Fig. 11). The bundles of tubules of unknown function were well preserved (Fig. 13).

Even after 10 months in glycerol many of the mitochondria were unchanged except for the loss of sharpness that affected all membrane-images

(Fig. 15). But in all specimens, the mitochondrial membranes were better preserved than all other membranes, with the possible exception of the bundles of fine tubules. The mitochondria were damaged more by osmosis than extraction. With increasing care, more mitochondria appeared normal. There were, however, two forms of mitochondrial change that were probably directly due to extraction damage rather than to osmosis; firstly, the cristae tended to be broken into short segments, and secondly, some mitochondria, either whole or ruptured, contained dark droplets resembling lipid (Fig. 14). The droplets appeared inconstantly, confined to small areas of tissue. Mitochondrial swelling and rupture were probably due to osmosis, but the effects were very variable. Apparently normal mitochondria lay amongst slightly or grossly swollen or ruptured ones. In one 3-day specimen (Figs. 10 and 11), which was washed for 1 hour with toad Ringer solution before fixation, many mitochondria had ruptured explosively, injecting vesicles derived from cristae throughout the cytoplasm. A portion of this same specimen remained in glycerol for a further 4 weeks, and then the cold glycerol was replaced by cold fixative over a period of 2 hours (Fig. 12). The mitochondria are far less affected than in the 3-day portion.

TABLE II

	Cytochrome oxidase activity (in μlO_2/10 min.* at 25°C)		
	Prep. No.	A	B
Freshly excised muscle	1	36.0	66.6
	2	34.6	68.0
	3	35.5	68.8
	4	37.0	72.3
After 3 weeks' immersion in glycerol solution	5	37.2	69.0
	6	31.3	58.8
	7	35.2	59.1
	8	32.0	55.5
After 6 weeks' immersion in glycerol solution	9	26.6	50.6
	10	28.4	54.2
	11	28.8	53.8
	12	35.0	58.6
After 10 months' immersion in glycerol solution	13	16.8	40.1
	14	22.1	43.2
	15	20.5	39.6
	16	19.8	40.0

In which A and B represent two different tissue dilutions.
* See footnote to Table I.

The Demonstration of Active Enzymes in Freshly Excised and Glycerol-Treated Muscle

The enzymes succinic dehydrogenase, cytochrome oxidase, and phosphorylase were found to be present and active in both the freshly excised and glycerol-treated muscles. The activities varied according to the duration of immersion in the glycerol solution.

TABLE III

Phosphorylase Activity in Freshly Excised and Glycerol-Treated Ventricular Muscle

	Phosphorylase activity, at 25°C (in μM PO_4^- liberated/10 mg wet wt)	
	Prep. No.	μM PO_4
Freshly excised muscle	1	0.244
	2	0.199
	3	0.196
	4	0.126
	5	0.190
	6	0.250
After 3 weeks' immersion in glycerol solution	7	0.150
	8	0.234
	9	0.231
	10	0.165
	11	0.208
After 6 weeks' immersion in glycerol solution	12	0.122
	13	0.168
	14	0.201
	15	0.115
After 10 months' immersion in glycerol solution	16	0.105
	17	0.086
	18	0.074
	19	0.098

Succinic dehydrogenase activity found in the freshly excised and glycerol-treated muscles is displayed in Table I, the activity being expressed in terms of microliters of oxygen used during 10 minutes' incubation at 25°C. The cytochrome oxidase activity in the same series of muscles is similarly displayed in Table II, in which the activity of the enzyme is again expressed in terms

of microliters of oxygen used during 10 minutes' incubation at 25°C.

Succinic dehydrogenase and cytochrome oxidase are both respiratory enzymes normally located in the mitochondria (Perry, 1956). The enzyme phosphorylase, however, which catalyses the reaction: glycogen + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate, is probably of sarcomplasmic origin (Perry, 1956). From the results displayed in Table III, it can be seen that phosphorylase was present in both the freshly excised and glycerol-treated muscles, some activity remaining even after 10 months' immersion in the glycerol solution. This enzyme is known to exist in two forms (Green and Cori, 1943; Rall, Wosilait, and Sutherland, 1956; Belford and Feinleib, 1959) the *a* form, which is active in the absence of adenosine-5-phosphate, and the *b* form which requires this nucleotide for activity. After 3 and 6 weeks' immersion in glycerol, the percentage of phosphorylase present in the *a* form was greater than that in the freshly excised muscle.

TABLE IV

Transmembrane Resting Potentials of Freshly Excised and Glycerol-Treated Ventricular Muscle

	Transmembrane resting potentials (in mv) at 25°C	
	Prep. No.	mv*
Freshly excised muscle	1	95
	2	93
	3	92
	4	94
	5	95
	6	92
After 3 weeks' immersion in glycerol solution	7	44
	8	40
	9	42
	10	40
	11	37
After 6 weeks' immersion in glycerol solution.	12	35
	13	35
	14	30
	15	30
	16	33

* Standard Error ± 2 mv.

Transmembrane Resting Potentials

The transmembrane resting potentials recorded from freshly excised and glycerol-extracted strips of ventricular muscle are summarized in Table IV. In each case, the resting potential was measured after the muscle had soaked for at least 30 minutes in Ringer's solution containing 1.3 mM Ca^{++}.

DISCUSSION

The results of this investigation show, firstly, that the general structure of ventricular muscle of toad is similar to that of other animals (2, 20, 23, 33, 47, 50); secondly, that glycerol-extracted ventricular muscle of toad retains many structures in addition to the actomyosin system; thirdly, that considerable quantities of sarcoplasmic and mitochondrial enzymes remain active in the glycerol-treated muscle, even after long periods of glycerol immersion; and fourthly, that a transmembrane resting potential persists in the glycerol-treated muscle, which, if interpreted in accordance with the generally accepted ionic hypothesis of cellular electrical activity (Hodgkin, 1951), implies that some functional activity remains in the plasma membrane.

Whereas much of the normal muscle structure requires no discussion here, the bundles of tubules, the small bodies of medium and high density, and the sarcoplasmic reticulum do.

The function of the bundles of fine tubules is unknown. The tubules are similar to those that spread along the sarcomere from the intermediary elements of the "triad" during the contraction wave in the salamander tadpole tail (Franzini and Porter, 1962). These were also seen in rat skeletal muscle undergoing denervation atrophy (Pellegrino and Franzini, 1963). However, the bundles seen in those studies were never very large, and were closely associated with a sarcomere. When they did increase in number in denervated muscle, they tended to anastomose into a compact labyrinthine structure. Anastomosis by means of numerous cross-bridges was also a feature in the bundles of parallel fine tubules forming the "crystalloids" in the interstitial cells of the opossum testis described by Christensen and Fawcett (1961): the bridges held the tubules in an arrangement of square packing. There is no evidence that the tubules in toad heart muscle anastomose, but there is some evidence that they

may have some association with a transverse tubule system (Fig. 9), although in this respect the micrographs leave much to be desired.

The occurrence of several structures in the same region is not evidence that one is derived from the other; however, the presence of small bodies of medium density in the peripheral region of the Golgi zone (Fig. 6) tends to support the claim of Novikoff *et al.* (1962) that the lysosome is derived from Golgi vesicles. The lysosome is rich in acid phosphatase, and this enzyme was found by Beckett and Bourne (1958), using light microscopy, in perinuclear granules in the myocardial and skeletal muscle cells of goat and man. Similar perinuclear dense bodies were reported in electron microscope studies of cardiac muscle of rat and man by Poche (1958), and of man by Battig and Low (1961). The small lysosomes, illustrated by Pellegrino and Franzini (1963) in skeletal muscle undergoing denervation atrophy, resemble many of the small bodies of medium density in normal toad heart. Pellegrino and Franzini point out that lysosomes have never been detected by electron microscopy in normal muscle. They refer, of course, to the lysosome in a stage before it has become involved in a recognisable lipofuscin or lipochrome granule. These small bodies require further investigation.

The small, very dense, encapsulated granules, resembling those in the cyclostome heart (Bloom, 1962), may contain catecholamines. Östlund (1954) reported finding about 1 μg per gm of catecholamine in amphibian heart, 75 to 90 per cent being epinephrine. Nayler (1958, unpublished) found from 1.25 to 1.6 μg per gm of catecholamine in the ventricular muscle of *Bufo marinus*, about 80 per cent being epinephrine. In mammalian cardiac muscle[1] there is less epinephrine than norepinephrine (Paasonen and Krayer, 1958).

Both longitudinal and transverse elements of the sarcoplasmic reticulum are poorly developed. It has been suggested that the longitudinal system serves relaxation (Muscatello *et al.*, 1961); and that it may be of minor importance and, thus, poorly developed, in cardiac muscle, which is slowly and passively stretched as the ventricle fills (Porter, 1961).

The transverse tubular or T system is generally supposed to convey the impulse from the sarcolemma to deeper myofibrils (Andersson-Cedergren, 1959). We have found no large-tubule system of sarcolemmal invaginations, but we are not certain that the preservation in our tissue was adequate to show accurately the extent of a fine transverse tubule system if one exists. We found suggestive fragments of tubules and small vesicles associated with Z and I bands from time to time. Our plates were chosen to show some of these and, therefore, give an exaggerated idea of their frequency in our specimens. However, it is difficult to accept the idea that some Z membranes in a fibril are favoured with an impulse-conducting system whilst others are not. Of course, many of the muscle fibres are too narrow to have deep myofibrils requiring a special communicating system.

There is remarkable structural preservation in the glycerol-extracted muscle, even after many weeks. Apart from the loss of the possible catecholamine granules, the major structures are preserved, although the plasma and nuclear membranes are often damaged, and, after weeks of treatment, the I bands are often broken between the N line and the A–I junction. Evidently an uncoupling in some components makes them delicate and prone to mechanical injury. Other structures, however, appear little affected. Lysosomes, mitochondria, and the bundles of fine tubules of unknown function are particularly well preserved.

This preservation contrasts with Bergman's (1958) findings on glycerol-extracted sartorius of frog. He reported the disappearance of mitochondria, glycogen, lipid, and the longitudinal component of the reticulum; after 3 days of treat-

[1] Palade (*Anat. Rec.* **139**, 262, 1961) has described a granule, about 1,000 to 2,500 A in diameter, present in large numbers in the atrial muscle cells of the rat and rare in ventricular muscle. The granules appeared to derive from the Golgi apparatus and almost disappeared from the muscle of animals treated with reserpine.

In the mammalian heart most of the catecholamine is norepinephrine and is in much higher concentration in the atrium than in the ventricle. Palade's description of the appearance of these granules in the Golgi zone is very similar to that of our granules of medium density (lysosome?). It will be necessary to isolate these granules in order to study their chemistry. A full account on the atrial granules by J. Jamieson and G. E. Palade is in press in *The Journal of Cell Biology*.

ment very little remained other than myofibrils, the transverse component of the reticulum, and a damaged plasma membrane; but he did not have the advantage of epoxy resins: polymerization damage and the less effective support of methacrylate, added to osmotic damage, probably helped to cause the loss of structures in his study. Until an examination of skeletal muscle has been repeated, using the present techniques, our observations should be used cautiously in predicting the effect of glycerol on skeletal muscle.

The present demonstration of active enzymes and many mitochondria in glycerol-extracted muscle verifies Perry's prediction of 1956. When discussing the use of glycerol-extracted muscle, he argued that after glycerol treatment "the fiber must still contain appreciable amounts of sarcoplasmic enzymes" and that the enzymatic activity of sarcosomes "should be taken into consideration in assessing the total enzymatic activity of these fibers." Wilson *et al.* (1959) noted that respiratory enzymes were present in glycerol-extracted skeletal muscles, and they stressed the need for knowledge of the structure of these preparations before data obtained from their use could be interpreted. Despite the continued lack of this information, many preparations of glycerol-extracted muscle are still used (13, 18, 19, 28, 35) and the results of these recent, as well as of earlier, experiments (6, 11, 53) are still interpreted in terms of the contractile proteins alone.

The well preserved lysosomes and small bodies of medium density suggest that the lysosomal enzymes also remain active.

It is odd that neighbouring mitochondria should react so differently. Almost certainly, simple osmosis caused most of the mitochondrial swelling in this study, because the more the care taken with tonicity, the less the mitochondrial swelling found. Some mitochondria seem to be less sensitive to osmosis, but we do not know why; they may be "leaky" or they may be relatively impermeable to glycerol.

Other experiments (Nayler and Wright, 1963) have shown that isolated hearts of toad respond to cold by increasing the percentage of the *a* form of the enzyme phosphorylase. Thus, the higher percentage of the *a* form in the glycerol-extracted muscles of the present series may reflect the response to cold rather than to glycerol.

The presence of transmembrane resting potentials of the same order of magnitude as those found in both potassium chloride–depolarised cardiac muscle cells (Hoffman and Cranefield, 1960) and in cells bathed with the chelating agent ethylenediaminetetraacetic acid (Nayler and Emery, 1962) raises several questions: Does the contractile behaviour after glycerol extraction resemble that of preparations depolarized by potassium chloride? Or does glycerol chelate the calcium ions normally found in the plasma membrane and thus disturb the membrane's function?

Unfortunately, the preservation of the plasma membranes in the electron microscope studies was too poor to help in the question of potentials. The *caveolae intracellulares* had disappeared and there were many small and large breaks in the vaguely outlined membranes. The breaks were probably preparative exaggerations of more subtle changes that had existed before fixation, and, rather than dispute the role of the plasma membrane in the maintenance of the "transmembrane" potential, it would be better to regard the breaks as gross artefacts. In the general course of electron microscope work, we have often found that the plasma membranes are fragile after manipulations involving delayed fixation, oxygen lack, etc., and that the effects depend very much upon subsequent methods of preparation.

The results of the present study indicate that there are a number of points in glycerinated cardiac muscle at which drugs and ions can act, and that the interpretation of their activities simply in terms of the contractile proteins alone becomes untenable.

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