Virchows Arch. Abt. A Path. Anat. 346, 361—373 (1969)

Ultrastructural Lesions of the Myocardial Cell in Coxsackie B_4 Virus Infected Mice*

R. S. Sohal and G. E. Burch

Department of Medicine of Tulane University School of Medicine, and Charity Hospital of Louisiana, New Orleans

Received August 22, 1968

Ultrastrukturelle Veränderungen an Muskelfasern des Myokards nach Infektion von Mäusen mit Coxsackie B_4-Virus

Zusammenfassung. Beschreibung der elektronenoptischen Befunde in Herzmuskelfasern nach Infektion von Mäusesäuglingen mit Coxsackie B_4-Virus. Die Veränderungen bestehen im wesentlichen in einer Lockerung des Zellgefüges, Bildung von Komplexen aus membranösen Bläschen, Reduktion der Golgi-Körper, Kernschrumpfungen, Verdichtungen des Kernchromatins und Zunahme der Ribosomen. Zu diesen spezifischen Veränderungen kommen noch unspezifische Veränderungen, die bei jeder Zellschädigung beobachtet werden.

Summary. This study describes aspects of the specific changes which occur in the myocardial cells of Coxsackie B_4 virus infected suckling mice. These changes consisted of cellular shedding, membrane-vesicle complex formation, disappearance of Golgi body, nuclear shrinkage and densification of chromatin and an increase in ribosomal population. These changes are in addition to the nonspecific changes which probably result as a nonspecific cellular response to injury.

Coxsackie B virus group is known to cause myocarditis (Rabin *et al.*, 1964; Sun *et al.*, 1967), valvulitis and endocarditis in experimental animals (Burch *et al.*, 1966; Sun *et al.*, 1967) and in man (Burch *et al.*, 1967, 1968). Localization of Coxsackie B virus antigen in the damaged myocardial cells by immunofluorescent technics (Rabin *et al.*, 1964; Sun *et al.*, 1967) indicates that the myocardium, epicardium, mural endocardium and valves are target tissues for these viruses. Although a great deal of information is available on the cytopathic effects of enterovirus on the tissue culture cells, similar *in vivo* studies are incomplete (Godman, 1966). The purpose of this report is to describe the ultrastructural lesions of the myocyte produced *in vivo* by Coxsackie B_4 virus in the myocardium of young suckling mice.

Material and Methods

Coxackie B_4 virus used in these experiments was originally recovered by Kibrick and Benirschke (1958) from a 10-day-old infant who died with encephalo-hepato-myocarditis. The virus was obtained as monkey kidney culture strain (MK) and prepared for inoculation in rhesus monkey kidney cultures. Control fluid from MK culture free of virus was also obtained. Virus and control fluid were stored at −65° C.

* Supported by grants from the U.S. Public Health Service, the Rudolph Matas Memorial Fund for the Kate Prewitt Hess Laboratory and the Rowell A. Billups Fund for Research in Heart Disease.

Twenty-eight suckling five-day-old mice of a random strain of HaM/ICR breed were inoculated intraperitoneally with 0.1 ml of fluid containing 10^3 $TCID_{50}$. A second group of twently-eight animals of the same age and stock were injected with 0.1 ml of virus-free fluid from monkey kidney culture and a third group of animals was not inoculated.

Microbiology. Hearts of two animals from inoculated and uninoculated groups sacrificed at 24 hours, 3, 6, 10 and 21 days were immediately washed in Hanks solution supplemented with penicillin and neomycin and ground in 1 ml of the same solution. The suspension was centrifuged at 1,000 RPM for 10 minutes. The supernatant was saved and stored at −65° C until the cultures were performed. 0.1 ml of the fluid was inoculated into each of 4 Rhesus monkey kidney (MK) cell tubes for every specimen. The tubes were examined daily for cytopathic effect (CP) and after one week the fluid was pooled and inoculated into new MK tubes. When definite CP effect was detected, the culture was considered positive. CP effect was produced in the initial cultures of specimens of the Coxsackie B_4 inoculated mice sacrificed at 24 hours and 3 days and in the second passage of those sacrificed at 24 hours, 3, 6 and 10 days. No change was observed in the specimens of the animals sacrificed on the twenty-first day after inoculation or of the controls. Coxsackie B_4 virus was identified in the positive cultures.

Neutralizing antibodies were determined in the sera of experimental and control mice sacrificed at 24 hours, 3, 6, 10 and 21 days. Coxsackie B_4 virus adapted to KB cells, 10^2 $TCID_{50}$, and diluted serum were kept at 37° C for one hour, planted on stationary KB cultures and incubated for 48 hours at 35° C. Antibody titers were expressed as the initial dilution of serum inhibiting virus effect. Sera of the Coxsackie B_4 virus infected mice sacrificed on sixth, tenth and twenty-first days had neutralizing antibodies to a dilution of 1:16, whereas sera of the control mice did not show any antibodies.

Electron Microscopy. For electron microscopy, six animals from each group sacrificed at third, sixth and tenth day were used.

Small pieces of the ventricular myocardium were fixed in 4% phosphate buffered (Millonig, 1962) glutaraldehyde for 2 hours at 4° C, washed in phosphate buffer and refixed in 1% osmium tetroxide for 1 hour. Subsequent to the glutaraldehyde fixation and buffer wash but prior to the osmium fixation, some tissue blocks were subjected to glycogen digestion by incubation in 0.5% alpha-amylase in Millonig's buffer, pH 7, for 1—2 hours at 37° C (Coimbra and Leblond, 1966). Control tissues were kept in buffer solution without alpha-amylase. The tissues were dehydrated in an ascending series of ethanol and embedded in Maraglas. Thin sections were cut with an LKB "Ultrotome" microtome, stained with uranyl acetate and lead citrate. A Siemens "Elmiskop I" electron microscope was used for observations.

Observations

The normal ultrastructure of the mouse myocardial cell is similar to that described by Moore and Ruska (1957) for other mammalian hearts. The myofiber is limited laterally by a sarcolemma. At intervals plasma membranes of the two adjacent cells invaginate to form an intercalated disc. Myofibrils present the usual striated appearance. Mitochondria lie in the perinuclear region and between the myofibrils. Components of sarcoplasmic reticulum and glycogen particles are distributed between and within the myofibrils (Fig. 1).

Because there was considerable variation in the degree of myocardial cell damage within all the groups of infected animals and even within the same animal, an account of the temporal sequence of the cellular changes was not attempted. However, maximum damage was observed on the sixth day after inoculation. Myocardial damage by the virus was focal. Myocardial cells frequently showed the formation of blunt pseudopodia-like processes which extended from the surface of cells into the interstitial spaces (Figs. 2, 3). Some of these processes lacked a cytoplasmic continuity with the myofiber. These cytoplasmic

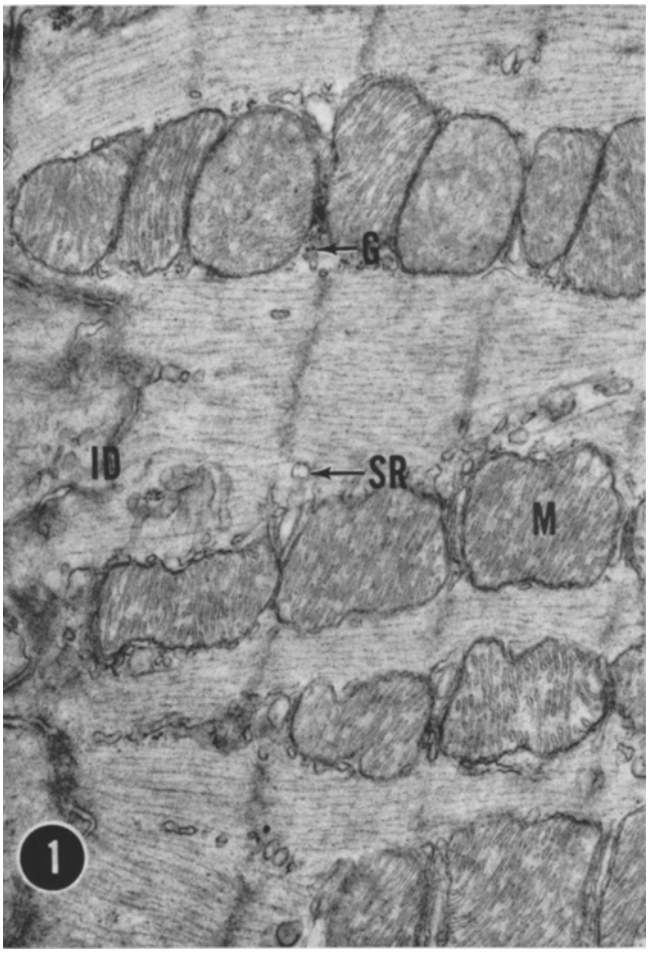

Fig. 1. A longitudinal section through a myofiber of the heart of a normal mouse. Myofibrils present the usual striated appearance. Mitochondria (*M*), sarcoplasmic reticulum (*SR*) and glycogen particles (*G*) occupy the interfibrillar spaces. An intercalated disc (*ID*) is shown. ×35,000

masses contained ribosomes and densely stained particles approximately 250 Å in diameter.

Mitochondria remained relatively unaltered or showed a minor distention of their cristae in severely damaged areas. A few mitochondria appeared to be transforming into dense myelin-like structures. Usually, Golgi structures were not identified in the damaged cells. In the perinuclear region as well as near the periphery of the myofiber, membrane vesicular complexes characterized by aggregates of vesicular and membranous structures were present (Fig. 3).

In necrotic cells dense particles approximately 250 Å in diameter and a few mitochondria were the most predominant remaining structures (Fig. 4). These particles differed from ribosomes and glycogen in their size, intense stainability

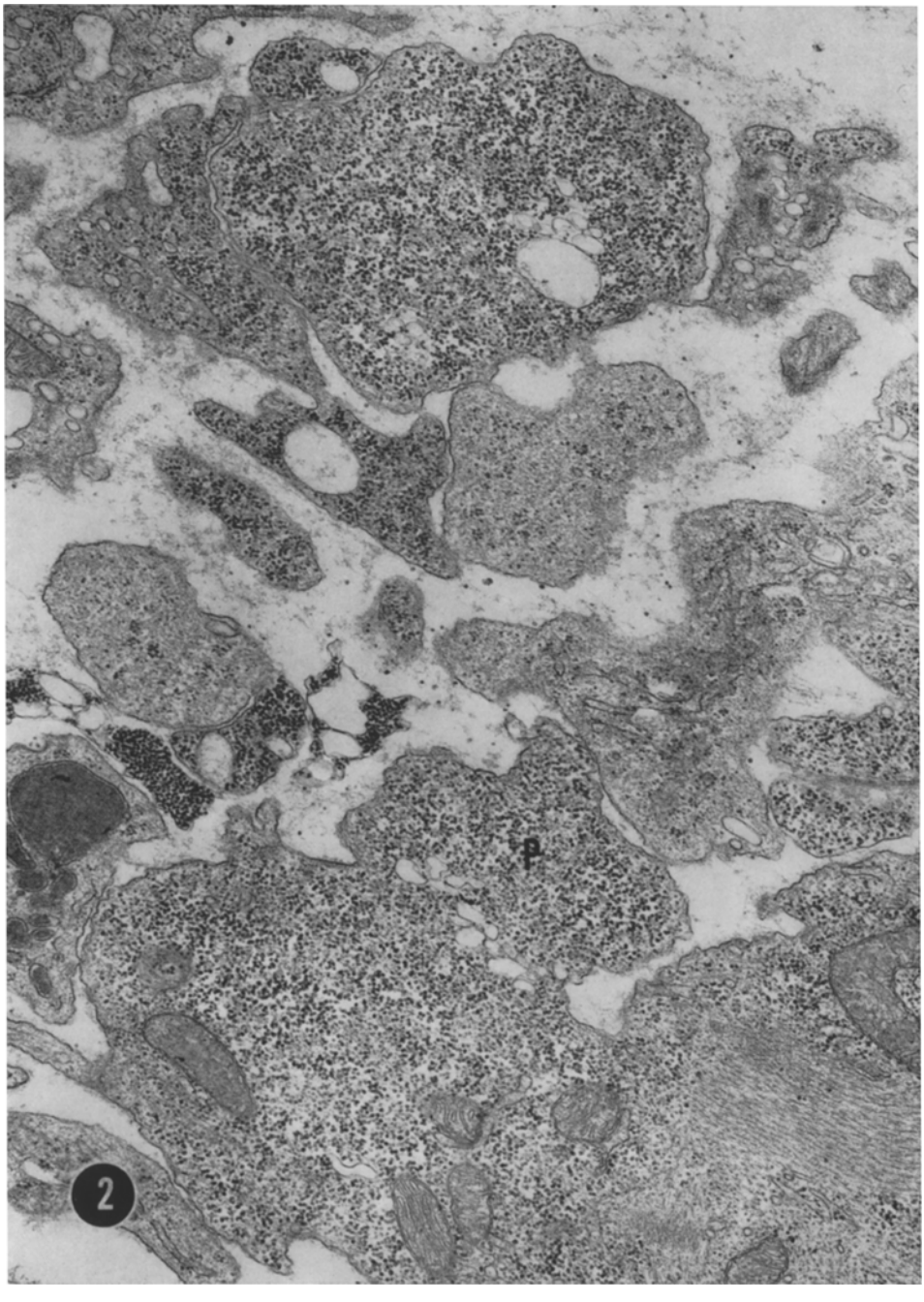

Fig. 2. Peripheral region of a myofiber from the heart of a mouse infected with Coxsackie B_4 virus. Blunt pseudopod-like processes (*P*) heavily populated with granular material extend from the periphery of the cell. Cytoplasmic masses containing similar granules but without any continuity with the myofiber are seen within the interstitial spaces. ×27,000

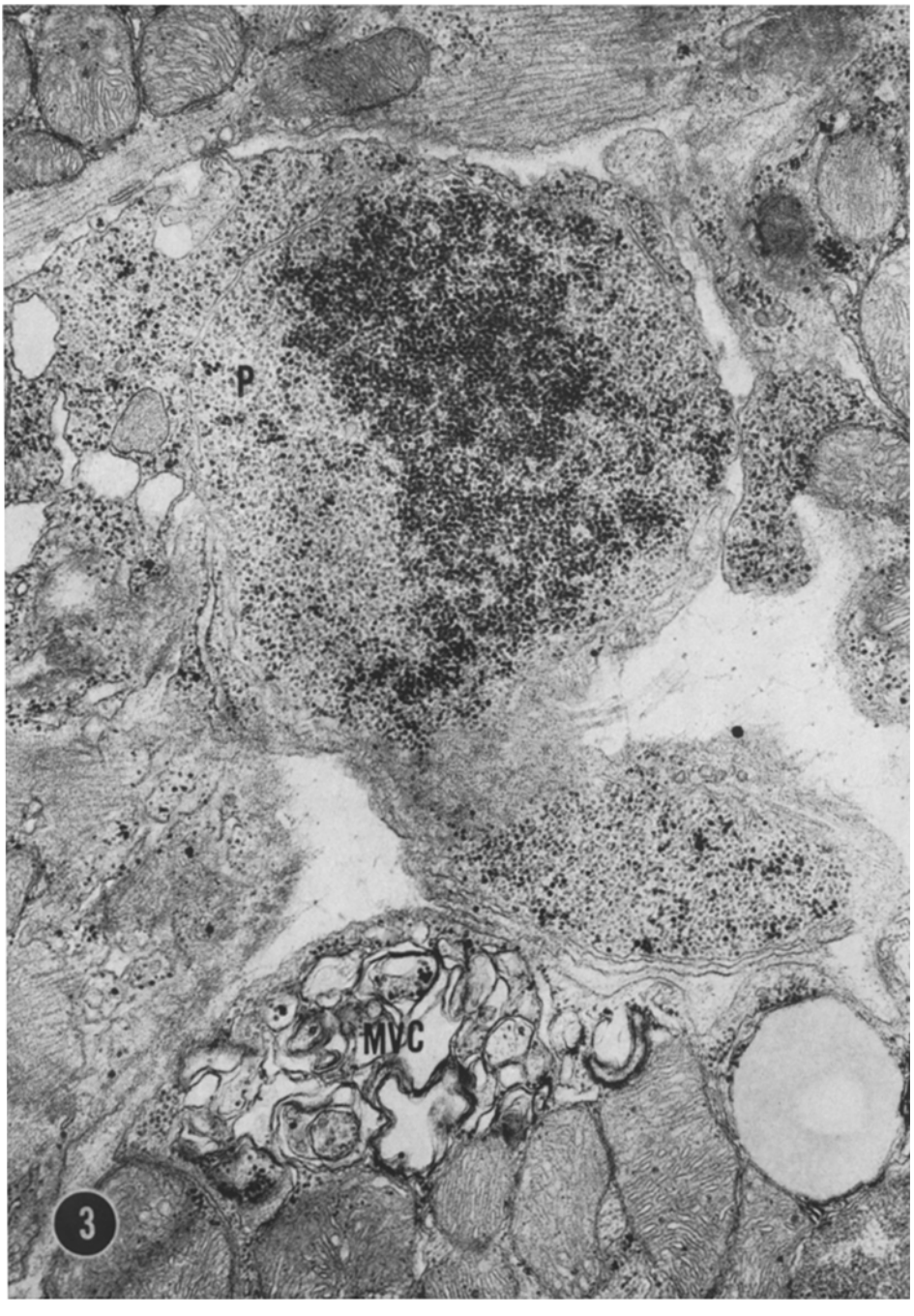

Fig. 3. A pseudopod-like process (*P*) extending from the periphery of a myocyte of an infected animal containing numerous dense particles approximately 250 Å in diameter. A membrane-vesicle complex (*MVC*) is found in the peripheral region of a myocardial cell. ×35,000

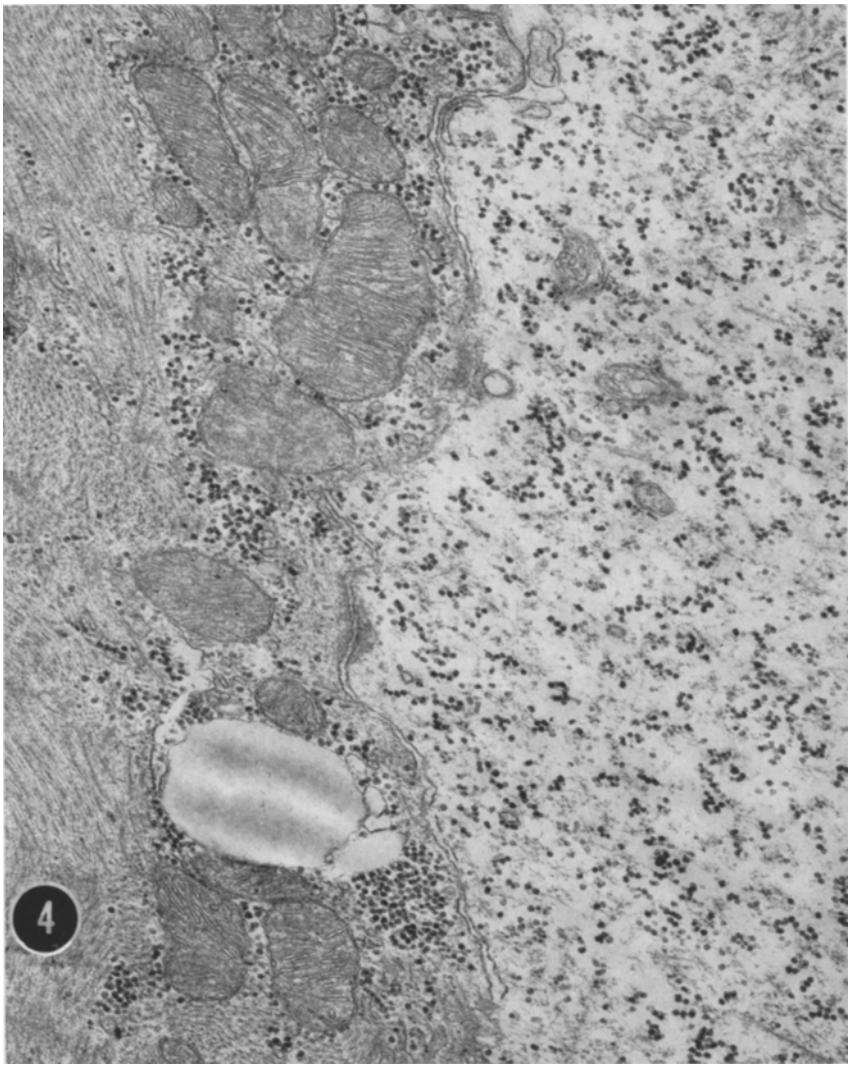

Fig. 4. Myocardium of an infected mouse showing a degenerated myocardial cell adjacent to a relatively intact cell. The necrotic cell contains dense particles approximately 250 Å in diameter and a few abnormal mitochondria. ×32,000

and regular contour. Furthermore, unlike glycogen particles, these particles remained undigested by incubation of glutaraldehyde fixed tissues in 0.5% alpha amylase (Figs. 5, 6). The size and shape of these particles are compatible with Coxsackie B virus particles. However, regular viral arrays as have been observed in Coxsackie infected culture cells were absent.

The contractile elements showed very prominent changes. In some areas myofilaments were fragmented into small, coarse filaments without any particular orientation. Electron dense contraction bands were numerous (Fig. 7). In necrotic areas myofibrils had undergone a total dissolution. The nuclear envelope

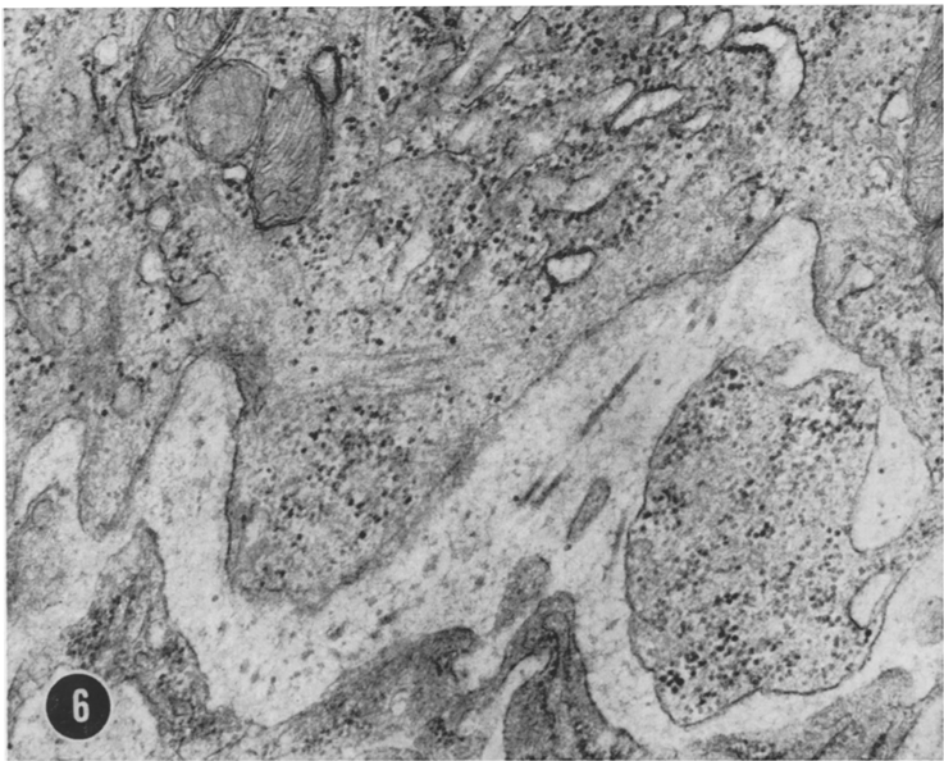

Fig. 5. Myocardium of a normal mouse incubated in alpha-amylase. Any cytoplasmic particles which might be considered as glycogen were digested. ×31,000

Fig. 6. Myocardium of an infected mouse incubated in alpha-amylase. The characteristic particles approximately 250 Å in diameter are scattered throughout the cytoplasm and remain undigested by the enzyme. ×50,000

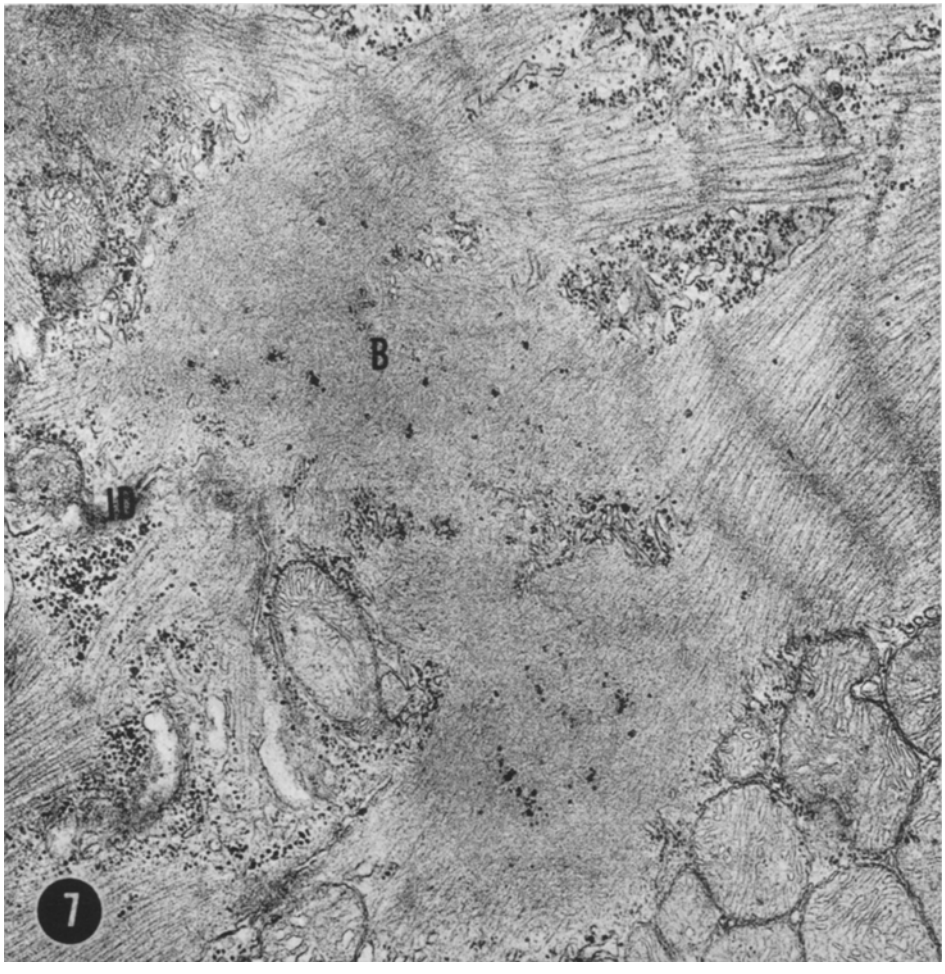

Fig. 7. Myocardium of an infected mouse showing a dense contraction band (*B*) in the proximity of an intercalated disc (*ID*). ×21,000

and the tubules of sarcoplasmic reticulum were moderately dilated in some regions. There were many membrane-bound autophagic structures containing mitochondria, myofilaments, ribosomes and large lipid droplets in the myocardial sarcoplasm (Fig. 8). Both the morphologically altered and unaltered cytoplasmic components were observed within the confine of these structures. Most of the cells showed a marked increase in the ribosomal particles (Fig. 9).

Nuclei were very irregular in outline and their chromatin showed extreme densification (Fig. 10). The dense chromatin was mainly distributed in the peripheral region of the nuclei. Some regions of some intercalated discs were dilated and membranous structures were observed within these intercellular regions (Fig. 11).

The control groups did not show any significant alterations of the myocardial cell.

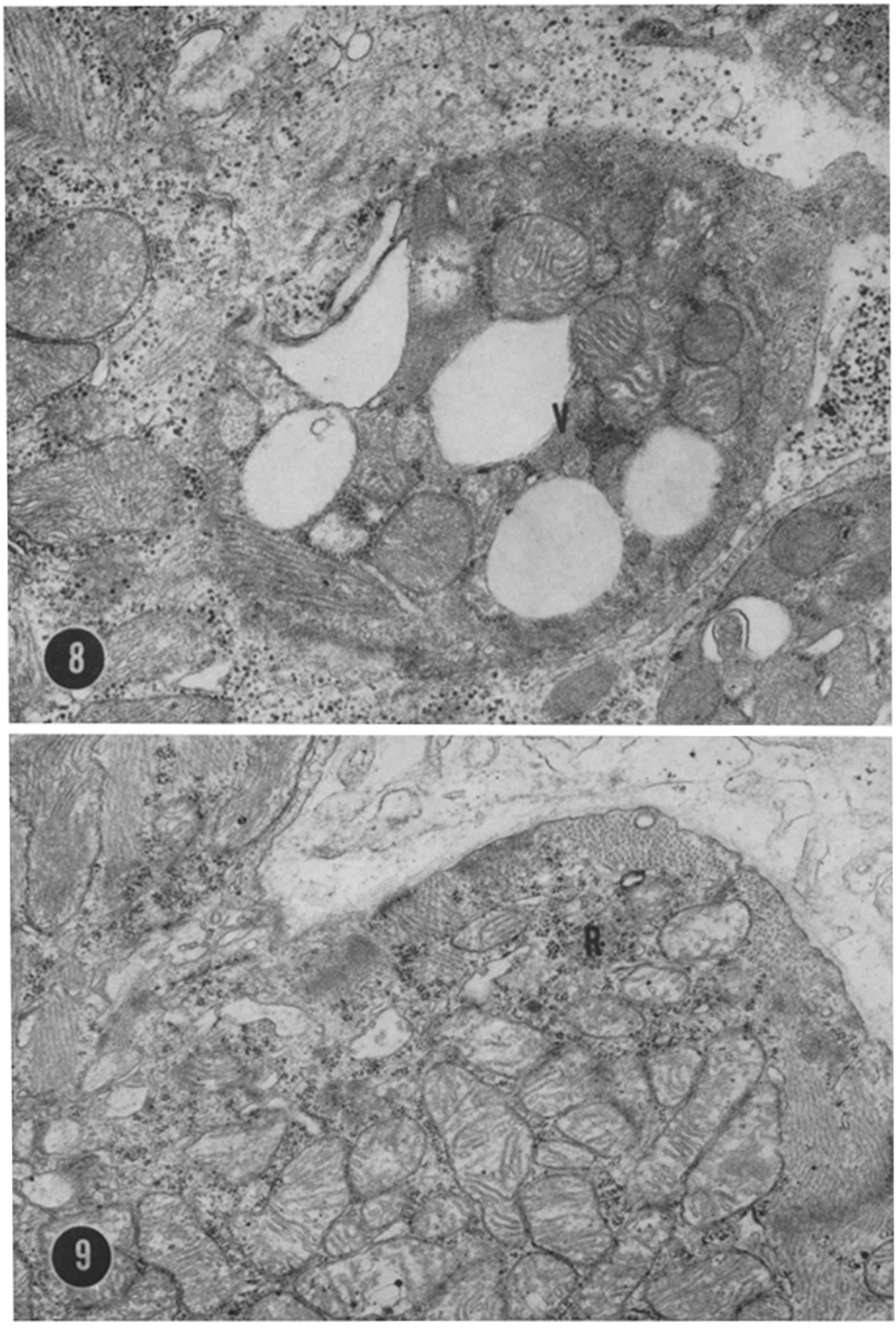

Fig. 8. Myocardium of an infected mouse showing autophagic vacuoles (*V*). ×29,000

Fig. 9. Myocyte of an infected mouse showing a rich distribution of clusters of ribosomes (*R*). ×33,000

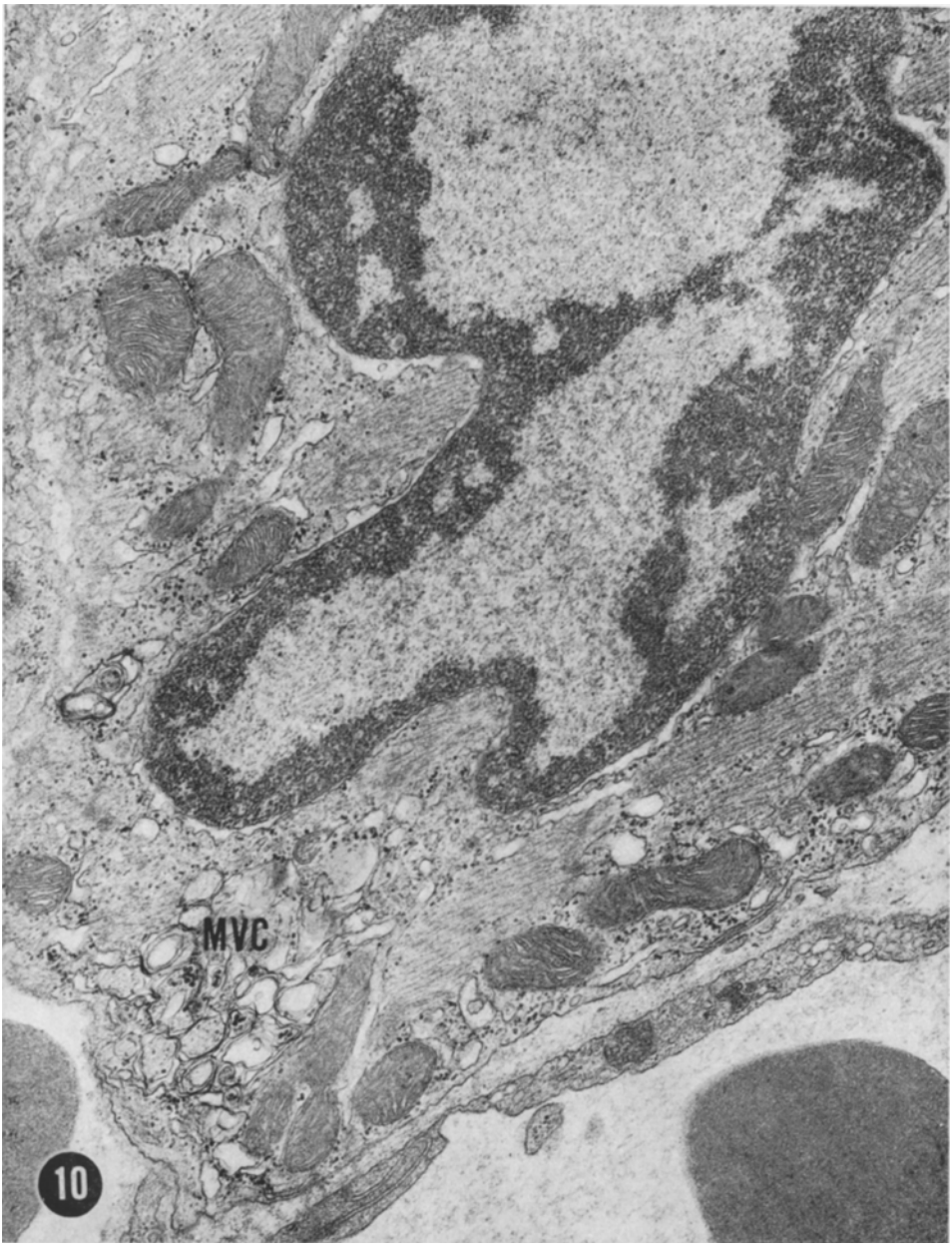

Fig. 10. Myofiber from an infected mouse showing a nucleus with an irregular contour. Dense chromatin is distributed along the nuclear periphery. Fine tubular structures are seen in the nucleoplasm. The nuclear envelope is slightly dilated. A membrane-vesicle complex (*MVC*) is found in the perinuclear zone. ×30,000

Discussion

Cytopathic changes which occur in enterovirus infected tissue culture cells involve nuclear shrinkage, margination and compaction of chromatin, cytoplasmic shedding, ribosomal increase, Golgi body destruction, membrane-vesicle

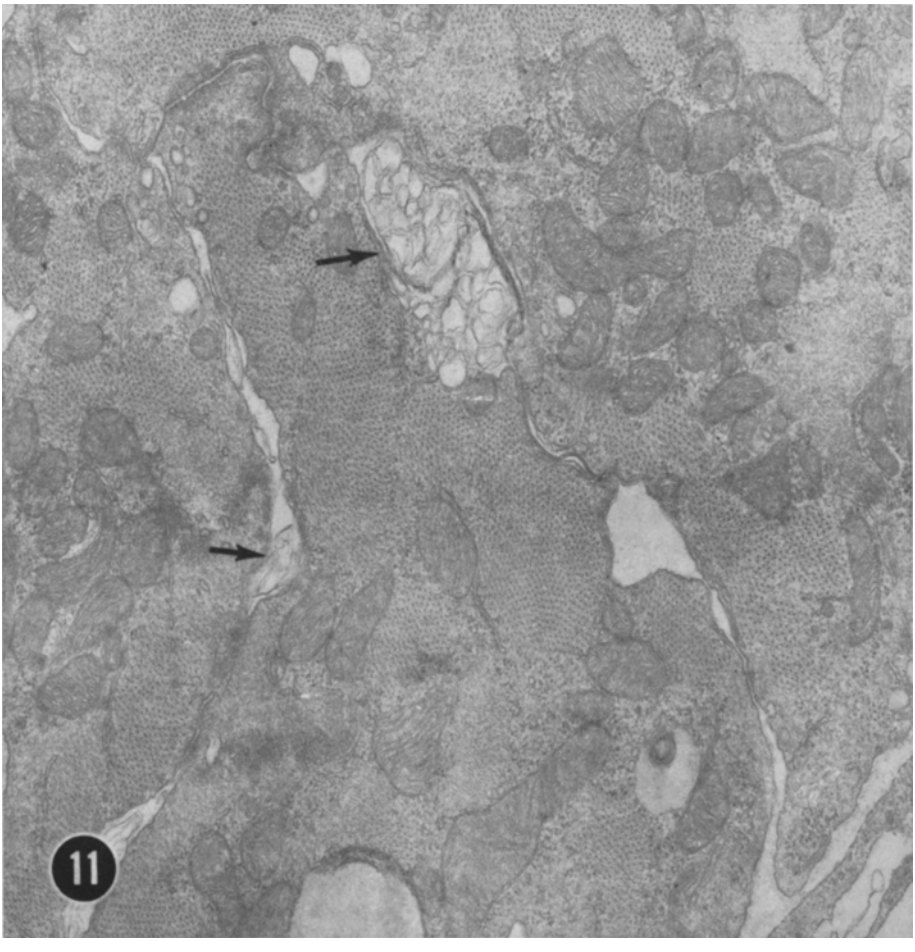

Fig. 11. Intercalated disc of an infected mouse. Membranous vesicles (arrows) are located in the intercellular space of the intercalated disc. ×22,000

complex formation and appearance of virus particles (Godman, 1966). From the present study it is apparent that some of the ultrastructural changes of the myocardial cell, e.g. cytoplasmic shedding in the form of pseudopod formation and detachment, formation of membrane-vesicle complexes, Golgi body destruction, shrinkage of nuclei as indicated by their irregular contour, densification and margination of chromatin and an increase in ribosomes, are similar to those which occur in infected tissue culture cells. However, certain changes such as dilation of the components of sarcoplasmic reticulum, distention of mitochondrial cristae and formation of contraction bands are known to occur under various hypoxic and anoxic conditions (Bryant *et al.*, 1958; Herdson *et al.*, 1965; Arcos *et al.*, 1968) and thus should be considered as possibly a nonspecific myocardial cell response to anoxic injury.

The presence of numerous dense rounded particles approximately 250 Å in diameter in the myocardial cells of infected mice is of particular interest. The

morphologic appearance of these particles is somewhat consistent with that of Coxsackie virus particles (Stuart and Fogh, 1961). Since no regular viral arrays were observed, these particles cannot be definitely identified as virus. However, it is known that crystallization of the virus is seen only under conditions where local synthesis of viral assembly is more rapid than the viral dissemination.

Shedding of the cytoplasm presumably containing viral material by the enterovirus infected culture cells is an early menifestation of cytopathology (Godman, 1966). Cellular shedding has been regarded as an exaggeration of the process of exocytosis of foreign bodies by the cell. Similarly, the formation of membrane-vesicle complexes in the perinuclear region is one of the main features of enterovirus infected tissue culture cells (Barski, 1962). Although such complexes are regarded as probably lipid in nature (Godman, 1966), the exact function of these structures is not clearly understood. Dales and Franklin (1962) considered them as nonspecific cellular response to injury. Godman (1966) suggested that they may be involved in sequestration and expulsion of unenveloped viral material or in repair of plasma membrane. Jezéquél and Steiner (1966) considered that the membrane-vesicle complexes are engaged in lytic activity and represent cellular defense response. The presence of autophagic vacuoles in the myocardial cell is another indication of cellular autolysis.

The significance of the regional dilation of the intercalated disc and of the presence of vesicular structures in these regions is unknown. Recent studies of Sohal *et al.* (1968) and Kawamura and Konishi (1967) indicate that under certain pathologic states the intercalated disc undergoes structural alterations. The functional consequences of these alterations remain undetermined.

References

Arcos, J. C., R. S. Sohal, S. C. Sun, M. F. Argus, and G. E. Burch: Changes in ultrastructure and respiratory control in mitochondria of rat heart hypertrophied by exercise. Exp. Molec. Path. **8**, 49—65 (1968).

Barski, G.: The significance of *in vitro* cellular lesions for classification of enteroviruses. Virology **18**, 152—154 (1962).

Bryant, R. E., W. A. Thomas, and R. M. O'Neal: An electron microscopic study of myocardial ischemia in the rat. Circulation Res. **6**, 699—709 (1958).

Burch, G. E., N. P. DePasquale, S. C. Sun, W. J. Mogabgab, and A. R. Hale: Endocarditis in mice infected with Coxsackie virus B_4. Science **151**, 447—448 (1966).

— S. C. Sun, K. C. Chu, R. S. Sohal, and H. L. Colcolough: Interstitial and Coxsackievirus B myocarditis in infants and children. J. Amer. med. Ass. **203**, 1—8 (1968).

— — H. L. Colcolough, R. S. Sohal, and N. P. DePasquale: Coxsackie B viral myocarditis and valvulitis identified in routine autopsy specimens by immunofluorescent techniques. Amer. Heart J. **74**, 13—23 (1967).

Coimbra, A., and C. P. Leblond: Sites of glycogen synthesis in rat liver cells as shown by electron microscope radioautography after administration of glucose-H^3. J. Cell Biol. **30**, 151—175 (1966).

Dales, S., and R. M. Franklin: A comparison of the changes in fine structure of L cells during single cycles of viral multiplication, following their infection with the viruses of Mengo and encephalomyocarditis. J. Cell Biol. **14**, 281—302 (1962).

Godman, G. C.: Cytopathology of enteroviral infection. In: Int. Rev. Exp. Path. (edit. G. W. Ricter and M. A. Epstein), vol. 5, p. 67—110. New York: Academic Press 1966.

Herdson, P. B., H. M. Sommers, and R. B. Jennings: A comparative study of the fine structure of normal and ischemic dog myocardium with special reference to early changes following temporary occlusion of a coronary artery. Amer. J. Path. **46**, 367—386 (1965).

JEZÉQUÉL, A. M., and J. W. STEINER: Some ultrastructural and histochemical aspects of Coxsackie virus-cell interactions. Lab. Invest. **15**, 1055—1083 (1966).

KAWAMURA, K., and T. KONISHI: Ultrastructure of the cell function of the heart with special reference to its functional significance in excitation conduction and to the concept "disease of the intercalated disc". Jap. Circulat. J. **31**, 1533—1543 (1967).

KIBRICK, S., and K. BENIRSCHKE: Severe generalized disease (encephalohepatomyocarditis) occurring in newborn period and due to infection with Coxsackie virus group B. Pediatrics **22**, 857—875 (1958).

MILLONIG, G.: Further observations on a phosphate buffer for osmium solutions in fixation. In: Fifth Internat. Congr. for Electron Microscopy (edit. S. S. BREESE JR.), vol. 2, p. 8. New York: Academic Press 1962.

MOORE, D. H., and H. RUSKA: Electron microscope study of mammalian cardiac muscle cells. J. biophys. biochem. Cytol. **3**, 261—267 (1957).

RABIN, E. R., S. A. HASSAN, A. B. JENSEN, and J. L. MELNICK: Coxsackie virus B_3 myocarditis in mice. An electron microscopic, immunofluorescent and virus-assay study. Amer. J. Path. **44**, 775—797 (1964).

SOHAL, R. S., S. C. SUN, H. L. COLCOLOUGH, and G. E. BURCH: Ultrastructural alterations of the intercalated disc in exercised rat hearts. Lab. Invest. **18**, 49—53 (1968).

STUART, D. L., JR., and J. FOGH: Micromorphology of FL cells infected with polio and Coxsackie viruses. Virology **13**, 177—190 (1961).

SUN, S. C., H. L. COLCOLOUGH, G. E. BURCH, N. P. DEPASQUALE, and R. S. SOHAL: Immunofluorescent study of B_4 Coxsackievirus valvulitis in mice. Proc. Soc. exp. Biol. (N.Y.) **125**, 157—162 (1967).

— R. S. SOHAL, G. E. BURCH, K. C. CHU, and H. L. COLCOLOUGH: Coxsackievirus B_4 pancarditis in cynomolgus monkeys resembling rheumatic heart lesions. Brit. J. exp. Path. **48**, 655—661 (1967).

Dr. G. E. BURCH
Department of Medicine
Tulane University School of Medicine
1430 Tulane Avenue
New Orleans, Louisiana 70112 (USA)