PATHOGENESIS OF COXSACKIE VIRUS INFECTION

MULTIPLICATION OF VIRUS AND EVOLUTION OF THE MUSCLE LESION IN MICE*

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PLATE 12

Infection of newborn mice with Coxsackie, or C, viruses is anatomically manifested by the appearance of acute widespread degenerative lesions of the striated muscle, focal myocardial necrosis, generalized fat necrosis, encephalomyelitis, hepatitis, and pancreatitis (1–4). Thus, the Coxsackie group of viruses cause a systemic disease involving many diverse organ systems of which, in our experience, the skeletal muscle is the tissue most constantly affected.

The Connecticut-5 strain is one which is highly injurious to the central nervous system, fat bodies, liver, and pancreas of suckling mice. Of the antigenic types at present grouped together as the Coxsackie viruses, it is apparently least destructive to striated muscle. Because of the relative mildness of its muscular effects, the Conn.-5 virus was selected for detailed study, in order to follow the evolution of the muscular lesions. In addition to following the histological development of the lesion, the distribution of virus in various tissues was determined quantitatively by titration of such tissues at frequent intervals after infection.

Material and Methods

Virus and Mice.—The Conn.-5 strain was isolated from the stools of a patient with aseptic meningitis in New Haven, in 1948 (5). In mice (Bagg Swiss) not over 24 hours old, titers of 10^{-6} were obtained consistently. In mice 4 days old, the titer was generally less by one to two logs. Because the survival rate in 4 to 5 day old mice was higher than in the newborn, the older mice were selected in order to follow some of the lesions into the recovery phase. The virus was used in its eighth mouse passage as a $10^{-3.5}$ concentration of infected brains (about 10 ID_{50}), with the mice each receiving 0.02 ml. intraabdominally. The same concentration of normal brains from mice 5 to 8 days old (the age at which infected mice were harvested for virus) was inoculated into 40 control mice. None of the control mice developed signs of illness. However, they were sacrificed for histologic examination at intervals similar to those used for the experimental animals. None showed any pathologic changes.

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Plan of Experiment.—Eighteen litters comprising 150 mice were inoculated. Starting 1 day after inoculation, 5 to 13 mice were selected for sacrifice. On any one day, no more than one mouse was taken from a single litter. Mice without signs were taken on days 1 through 8. On the 5th day, mice began to develop signs (weakness, incoordination, spasticity, tremors, paralysis) and some of these were sacrificed on the first day of illness on days 5 through 8. Some mice were not sacrificed, in order to permit the disease to progress. Most of these

TABLE I

Effect of Intraabdominal Inoculation of the Conn.-5 Strain and Plan of Harvest of Mice for Study

	Day after inoculation of virus													
	1	2	3	4	5	6	7	8	9	10	11	12	14	18
	Percentage of mice with first signs of illness or death each day													
Mice with first signs of illness			0	0	4	4	4	20	10	6	0			
Dead mice			0	0	0	3	3	10	5	4	0			
	No. of mice sacrificed each day													
Mice with signs of illness														
Sacrificed for histologic study					2	2	2	3*	2*	2‡	2§	1‖	1**	
Sacrificed for virus study								2	3				2‡‡	1§§
Mice without signs of illness														
Sacrificed for histologic study	2	2	2	2	2	2	2	2						
Sacrificed for virus study	3	3	3	3	2	5	4	4						

* One mouse inoculated intracerebrally.

‡ 1st day of paralysis on 9th day. Sacrificed 1 day later.

§ 1st day of paralysis on 10th day. Sacrificed 1 day later.

‖ 1st day of paralysis on 10th day. Sacrificed 2 days later.

** 1st day of paralysis on 10th day. Sacrificed 4 days later.

‡‡ 1st day of paralysis on 9th day. Sacrificed 5 days later.

§§ 1st day of paralysis on 10th day. Sacrificed 8 days later.

died; however, a few survived and these were sacrificed several days later. The plan of harvest is shown in Table I.

Collection and Preparation of Tissues for Histological Study and for Subinoculation.—On a particular day, mice selected for sacrifice were bled to death from the heart, and the blood stored at −20°C. for subsequent testing for virus (or antibody). 2 to 5 mice were then autopsied and fixed in Zenker's or other fixatives and 2 to 7 mice frozen whole. The latter mice were later thawed, and tissues removed as aseptically as possible using separate instruments for each tissue. The following tissues were taken for virus determinations in this order: brain, heart, liver, intestinal tract from the pylorus to the lower sigmoid, and "muscle." The last

tissue called "muscle" consisted of the carcass which remained after all the organs, head, feet, and skin had been removed. No attempt was made to remove the cartilage, fat, bone, and spinal cord. The intestinal tract was cut open in part and after the contents were removed, the walls were washed in 6 changes of physiological saline. While this did not separate completely the contents from the wall, the contents were free of intestinal tissue and the walls contained only small amounts of intestinal contents.

Tissues and intestinal contents were ground with 1 per cent of heated horse serum to make a 10 per cent suspension. The suspensions of blood, brain, heart, liver, and muscle were each spun at 1500 R.P.M. for 10 minutes to sediment large particles. The intestinal wall and the contents were spun at 18,000 R.P.M. for 20 minutes for removal of bacteria as well, previous work having shown that the virus does not sediment at this speed (3). In diluting the tissues for titration, 1 per cent horse serum was used. For the 10^{-2} concentration of tissue, 0.1 ml. of a solution containing 10 mg. of streptomycin and 2,000 units of penicillin was added to each 0.9 ml. of the diluent. If the result of the virus titration of the tissue was less than 10^{-2}, then 0.1 ml. of the penicillin-streptomycin solution was added to each ml. of the 10^{-1} tissue suspension in a repeat test.

To determine the virus end-points, serial tenfold dilutions were made and two boxes of mice, each containing 8 newborn, were used at each dilution. Each mouse was injected with 0.02 ml. subcutaneously in the back between the shoulders where the skin is loose. Mice were observed for 14 days and no death during the first 2 days was regarded as due to the virus. Aliquots of the 10^{-1} tissue suspensions were frozen and retitrated if end-points were not reached or if there were a high proportion of non-specific deaths in the crucial boxes.

For histologic study paraffin sections were cut at 6 to 8 micra and stained with hematoxylin-eosin-azure, Mallory's phosphotungstic acid-hematoxylin, or iron-hematoxylin, Millar's stain, and Masson's trichrome stain.

RESULTS

Distribution of Virus in Tissues during the Incubation Period and Following the Onset of Disease

The results of titrations carried out on the tissues of 35 mice are presented in Table II. Of these, 27 mice without signs of illness were selected for harvest on days 1 to 8, 5 on the 1st day of paralysis, and 3 on the 6th to 9th day of paralysis.

On the day following the inoculation, virus could not be detected in any of the tissues. This is in keeping with studies with other viruses in which the virus cannot be detected during the early period of incubation. However, by the 2nd day multiplication was well under way in all tissues examined except the brain. The titers of the blood, heart, liver, intestinal wall, and muscle and also of the intestinal contents were all at approximately the same level, $10^{-3.4}$ to $10^{-4.5}$.

Blood.—(Text-fig. 1.) The titer on the 2nd day was $10^{-3.4}$ and remained at this level through the 5th day, $10^{-3.5}$ to $10^{-4.0}$, after which it fell to $10^{-1.5}$ on the 6th day. In the mice without signs it could not be found on the 7th and 8th days. However, in the animals *paralyzed* on the 7th and 8th days viremia was present to a level of $10^{-2.5}$ and $10^{-1.0}$ respectively. No virus was present in the blood of animals sacrificed 6 and 9 days following the onset of paralysis.

Neutralizing antibody tests (3) were carried out on all the lysed whole blood samples

listed in Table II. Starting dilutions were 1:20. All were negative except for the two samples from mice sacrificed 6 and 9 days after onset of paralysis. Against 100 ID_{50} of virus, these sera had titers of 1:40 and 1:80 respectively. The failure to detect either virus or antibody in the 7th and 8th day specimens of the mice without overt disease may have been due to the low concentration of antibodies, some of which may have been used to bind virus spilling into the blood as a result of the breakdown of infected cells.

Brain.—(Text-fig. 1.) Of the tissues studied, only the brain had no detectable virus on the 2nd day after inoculation. On the 3rd day, however, virus was present, titer $10^{-3.2}$, and this increased to $10^{-5.0}$ on the 4th day. In the mice without signs, this level was maintained through the 8th day. In the mice with paralysis, the titer on the 1st day of paralysis was $10^{-6.5}$, the maximum level found for this tissue. On the 6th day of paralysis, the titer fell to

TABLE II

Titer of Virus in Selected Tissues of Mice with and without Signs of Disease

	Blood	Brain	Heart	Liver	Intestinal contents	Intestinal wall	Muscle
Mice without signs							
Day 1	0	0	0	0	0	0	0
2	3.4	0	3.5	4.5	3.5	3.8	4.5
3	4.3	3.2	5.2	4.0	3.5	4.0	5.8
4	3.5	5.0	5.5	5.5	5.0	4.5	7.0
5	4.0	4.4	5.1	5.8	5.7	3.8	6.0
6	1.5	4.5	5.0	5.0	5.5	5.5	6.0
7	0	5.5	5.0	5.2	6.2	5.5	6.2
8	0	5.5	4.3	6.0	5.8	4.1	6.3
Mice with paralysis							
Day 7, 1st day of paralysis	2.5	6.5	4.0	5.0	5.5	6.0	6.8
8, 1st " " "	1.0	6.5	5.3	6.5	6.0	6.2	6.8
14, 6th " " "	0	2.5	1.5	3.0	2.0	2.0	3.8
18, 9th " " "	0	1.2	0	0	0	0	1.0

0 indicates no infectivity detected at 10^{-1} concentration of tissue. Titers are recorded as reciprocals of the log of the ID_{50} end-point dilution.

$10^{-2.5}$, and on the 9th day to $10^{-1.2}$; on the latter day virus could be found only in the brain and muscle.

Heart.—(Text-fig. 2.) The titer on the 2nd day was $10^{-3.5}$ and increased to $10^{-5.2}$ on the 3rd day. This level was maintained through the 8th day. On the 1st day of paralysis the level did not change, but it fell to zero 9 days later.

Liver.—(Text-fig. 2.) The titer was $10^{-4.5}$ on the 2nd day and was not over $10^{-6.0}$ during the subsequent 6 days in the mice without signs. In the paralyzed animals, the titers on the 1st day of disease were $10^{-5.0}$ and $10^{-6.5}$, on the 6th day $10^{-3.0}$, and zero on the 9th day.

Intestines.—(Text-fig. 2.) There was no sharp difference between virus levels of the contents or of the wall itself. The titers were $10^{-3.5}$ and $10^{-3.8}$ respectively on the 2nd day and varied between those levels and $10^{-6.2}$ through the 8th day regardless of whether the mice were paralyzed (1st day of disease) or not. The level fell to $10^{-2.0}$ for both contents and the wall on the 6th day of paralysis and to zero on the 9th day.

Muscle.—(Text-fig. 1.) As mentioned earlier, the "muscle" contained fat, bone, and spinal cord as well as muscle. Its titer of virus was $10^{-4.5}$ on the 2nd day which progressively in-

creased to $10^{-7.0}$ on the 4th day. Between the 5th and 8th days the titer was $10^{-6.0}$ to $10^{-6.8}$ falling to $10^{-3.8}$ on the 6th day of disease, and being still present at $10^{-1.0}$ on the 9th day. While the levels in muscle were not much higher than in other tissues they were consistently so.

To summarize the effect of intraabdominal inoculation, virus appeared to multiply in all the tissues studied starting 1 day later in the brain than in the blood, heart, liver, intestines, and muscle. The level of virus in blood fell off
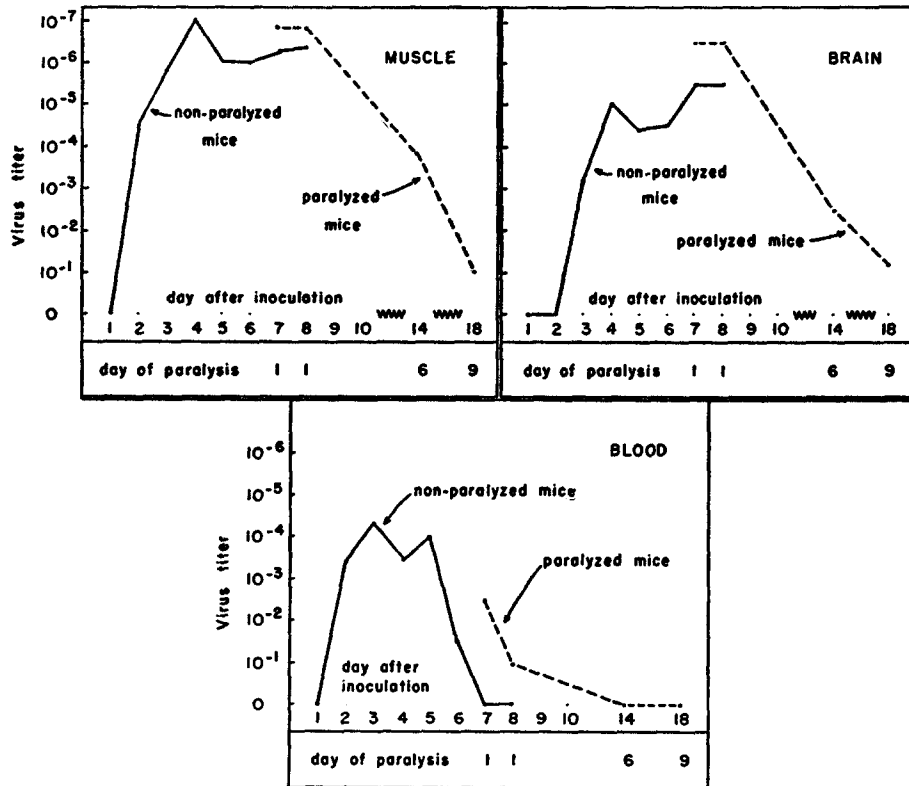

TEXT-FIG. 1. Titer of virus in muscle, brain, and blood of mice during the incubation period and following the onset of disease.

sharply after the 5th day, while it was maintained in the other tissues through the 8th day. In paralyzed animals, the highest levels of virus were present in the muscle and in the brain. Also, it was in these tissues alone that virus persisted in paralyzed animals through the 9th day of disease.

Incidence of Lesions

For histologic study of the evolution of the lesions due to the Conn.-5 strain, 34 mice inoculated when 4 to 5 days old were available. Significant myositis was discovered in 16 (47 per cent) in non-serial sections of limbs and trunk, and

fat necrosis in 16 (47 per cent). 18 of the animals had developed paralysis, and of this number 11 (61 per cent) were found to have central nervous system lesions, 14 (77 per cent) to have muscle lesions, and 15 (83 per cent) to have lesions in the fat. Three instances of myocardial necrosis were found in the paralyzed group. Liver and pancreas of 7 paralyzed mice of this group dis-
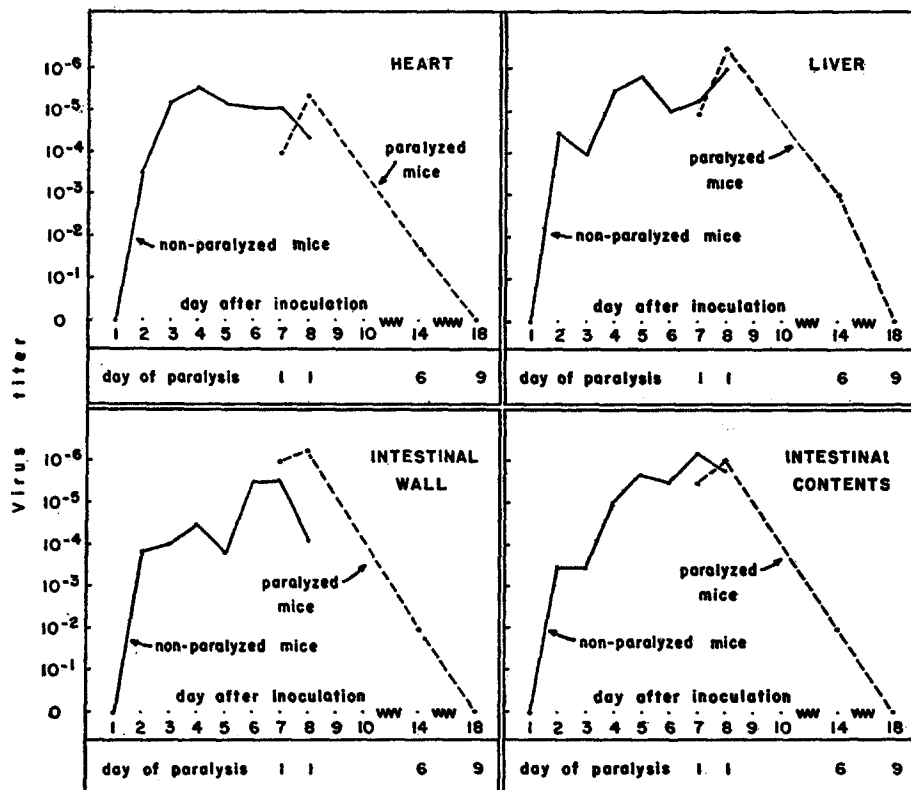
TEXT-Fig. 2. Titer of virus in heart, liver, intestinal wall, and intestinal contents during the incubation period and following the onset of disease.

closed no microscopic lesion; gross changes were not seen in these organs in any case.

About 50 newborn mice, not more than 24 hours old, were also inoculated for histologic study. These were all sacrificed on the 1st day of paralysis after incubation periods of 4 to 6 days. In general, the histologic incidence of myositis in this group was from 70 to 90 per cent, while central nervous system lesions were found in about 25 per cent, fat necrosis in 41 per cent, hepatitis in 50 per cent, pancreatitis in 61 per cent, and myocardial necrosis in 29 per cent. At

this time such mice yielded a pattern of virus distribution similar to that presented earlier for mice inoculated on the 4th to 5th day of age and harvested 8 days later on the 1st day of paralysis.

The incidence and distribution of lesions in the group inoculated at 4 to 5 days of age in relation to time elapsing after inoculation are charted in Table III.

Description of Lesions

In general, the lesions in the central nervous system, adipose tissue, liver, pancreas, heart, and lungs were found to be similar in most particulars with those described by Dalldorf (2) and by Pappenheimer et al. (4).

Encephalomyelitis.—Only an acute picture was seen in the material of this series. The early lesion consists of necrosis of neurones in cortex, hippocampus, midbrain, and particu-

TABLE III

Incidence and Distribution of Lesions in 4 to 5 Day Old Mice after Inoculation with Conn.-5 Virus

Time after inoculation	No. of mice	No. paralyzed	Myositis	CNS lesions	Fat necrosis
days					
1–4	8	0	1/8* 13%	?	0/8 0%
5–8	8	0	2/8 25%	?	1/8 13%
	9	9	5/9 56%	3/9 33%	7/9 78%
9–14	9	9	8/9 89%	8/9 89%	8/8 100%

* 1/8 indicates that of 8 mice studied, 1 showed myositis.

larly medulla, with hyperemia, edema, and perivascular round cell infiltrations. Areas of necrosis, ultimately becoming rarefied, occur in the grey matter. Leptomeningitis has been found in several instances.

Fat Necrosis.—The generalized necrosis of fat is a lesion which, to our present knowledge, is unique to the Coxsackie group of viruses, including those recently described (4, 6). The lesions underwent no consistent change in appearance during the period of observation, except for early calcification, in some cases as soon as the 1st day of signs, and later fibrosis.

Myocarditis.—The cardiac lesions are remarkable for their frankly necrotic character and focal distribution. The anatomic picture is quite different from the "spontaneous" cardiac lesions in older mice reported by Gray (7), in which cellular infiltrations were the outstanding feature. Cardiac lesions have not been found in the present series in the absence of concomitant skeletal muscle disease.

Hepatitis and Pancreatitis.—These remarkable injuries were produced only in "newborn" (1 day old) mice. Our attention was first drawn to these lesions by Pappenheimer (8). They have been described in detail (4); only acute changes appear and death apparently ensues rapidly. The observation that encephalomyelitis does not appear in those animals with pancreatitis or hepatitis is confirmed in our material.

Lungs.—The peculiar emphysema reported previously (4) was present in our material.

Myositis.—The striated muscle lesion generally resembles the so called Zenker's hyaline

degeneration, as was first observed by Dalldorf *et al.* (1, 2) with those Coxsackie viruses studied by these authors. The earliest apparent change is a proliferation of muscle nuclei. Tinctorial changes and swelling of segments of varying numbers of muscle fibers occur, followed by nuclear pyknosis and fragmentation. At the same time the cross-striations disappear and the myofibrils thicken and fuse. These form longitudinal striations which tend to fade and disappear as the degenerating segments become homogeneous refractile hyaline masses. The sarcolemma usually remains intact. The relatively short diseased segment is sharply demarcated from the contiguous intact ends of the muscle fiber of which it is a part. With

Lesions Observed at Daily Interva[l]

Time after inoculation, days	1	2	3	4	5	6			
No. of mice and route of inoculation*	2, IA	2, IA	2, IA	2, IA	4, IA	4, IA			
No. paralyzed	0	0	0	0	2	2			
No. with lesions	0	0	0	1	1	3			
Signs of disease‡	NP	NP	NP	NP	P	NP	P	P	P
Lesions§									
Myositis	0	0	0	+	0	+	0	0	++
Distribution				Focal, limbs		Focal, limb			Focal, limb
Degeneration				Isolated, segmental, earliest stages		Isolated, segmental, early to intermediate			Numerous, s[...] tal, all stag[...]
Reaction				Slight edema and cell infiltration		Slight edema			Moderate ede[...] cell infiltrat[...] Early tube ation
Regeneration				None		None			Proliferation [...] muscle nucl[...]
Myocardial necrosis	0	0	0	+	?	0			0
CNS lesion	0	0	0	?	0	?	+	0	++
Fat necrosis	0	0	0	0	++	0	+++	+++	+++

* IA indicates intraabdominal; IC indicates intracerebral.

‡ NP indicates animal without signs of illness; P indicates paralyzed animal, 1st day of signs.

§ 0 indicates the absence of any pathologic change; ? indicates tissue examined was insufficient for diagnosis.

the occurrence of segmental necrosis, interstitial edema and histiocytic infiltrations make their appearance. The mononuclear phagocytes ultimately affect the débridement of the hyalinized muscle by entering into the sarcolemmic sheaths enclosing the necrotic segments and attacking the hyaline material. At the height of this process the affected segment forms a tube containing many histiocytes, remnants of hyaline substance, and fine regenerating muscle fibers or cells which have begun to grow into the sarcolemmic sheath. The regenerating muscle originates as outgrowths from the interrupted ends or peripheries of still intact muscle fibers, particularly from the ends abutting on the necrotic segments, but occasionally also within the degenerating segments from those nuclei which have escaped lethal injury. It

grows out in the form of fine, thin slips or fibers with basophilic cytoplasm, each with a central row of closely spaced nuclei, whose characteristic structure constitutes a readily identifiable feature of regeneration. These nuclei are elongate, heavily outlined, vesicular, and have 2 large nucleoli one of which is typically rod-like. Growth of the regenerating fibers is guided by the persistent sarcolemmic sheaths. Although restitution of the muscle is evident about a week after paralysis, residual evidences of injury are often present in the form of partly mineralized or hyalinized derivatives of necrotic material. The pathology of myositis produced in mice by the Coxsackie viruses will be more completely detailed in a subsequent

oculation of 4 to 5 Day Old Mice

	8				9	
	IC	4, IA			1, IC	1, IA
	1	2			1	1
	1	3			1	1
P	P	P	NP	P	P	P
++ cal, limb	+++ Generalized in certain limb muscles; widespread in intercostals	+++ Generalized in most limb muscles; focal in epaxial trunk muscles	0	+++ Widespread, limb muscles	++++ Generalized in some limb muscles	++
umerous, segmental, all stages	Extensive, early to late stages	Massive; intermediate to late stages predominate		Very numerous, intermediate stages	Widely scattered; late stages predominate	
oderate edema marked cellular infiltrations. Early tube formation	Moderate edema, scant cellular infiltrations. Early tube formation	Moderate edema and cellular infiltrations. Early tube formation		Moderate edema and cellular infiltrations. Early to advanced tube formation	Severe edema, marked infiltrations. Many tubes, all phases	
roliferation and alignment of muscle nuclei	Early	Moderate early, widespread proliferation ++		Marked, mostly early	Early and intermediate	
	?					?
0	0	0	++	++	++	+++
0	++++	++++	++	+++	+++	++++

communication (9). Observations pertaining to the evolution of the muscle lesion are listed in Tables IV and V, and pertinent photomicrographs are shown in Figs. 1 to 4 of Plate 12.

Alimentary Tract, Kidneys, Suprarenals, Spleen, Bone Marrow, Genitalia.—No morphologic changes were discovered in these tissues. The alimentary tract was notably free from lesions in view of its content of virus.

Temporal Sequences in Development of Myositis

The sequence of events, from the onset of muscle necrosis to early restitution is estimated to take not more than 7 to 9 days. The actual time of onset of the

TABLE V

Extent of Lesions after Onset of Paralysis

|	2nd	2nd	2nd	3rd	3rd	5th
Day of paralysis.........	2nd	2nd	2nd	3rd	3rd	5th
No. inoculated, and route..........	2, IA	2, IA	2, IA	2, IA	2, IA	1, IA
Apparent incubation period, *days*...........	9	10	10	10	10	10
Lesions						
Myositis						
Distribution.........	Wide zones, or entire limb muscles	Variable numbers of fibers in limb and trunk muscles	Massive involvement of selected hind limb muscles	Selected limb muscles and intercostals variably involved	Areate involvement of limb and limb girdle muscles	Small areas in hind limb and trunk muscles
Degeneration.......	Isolated, segmental, all stages from earliest	Isolated, segmental, all stages	Occasional acutely degenerated segments, occasional "remnant masses."	Occasional segments with frank necrosis	Occasional remnant masses	Rare remnant masses
Reaction...........	Marked edema; moderate interstitial infiltration. Extensive tube formation, all phases	Slight edema; localized infiltrations. Marked tube formation	Moderate edema and diffuse interstitial infiltration. Rare advanced forms of tubes	Very slight edema; scant linear infiltrations. Few tubes	Few focal cellular accumulations	None
Regeneration........	Marked activity	Widespread proliferation; early and intermediate phases of regeneration	Affected part consists chiefly of profusion of undifferentiated sarcoblastic slips	Predominantly regenerative picture; young and differentiating fibers	Areas chiefly of regenerating muscle showing all degrees of differentiation	Affected areas chiefly of maturing regenerated fibers
Myocardial necrosis...	0	0	0	+	0	0
CNS lesion..........	+++	0	+++	++++	++++	+++
Fat necrosis........	+++	++	++++	++++	+++	?

muscle lesion in relation to inoculation can be only approximately reckoned, since the overt evidences of disease serve merely as a rough sign of extensive lesions. It is apparent from the lesions found in mice without signs of illness 4, 6, and 7 days after inoculation, that in the absence of central nervous system involvement the manifestations of illness are a function of the number of necrotic muscle fibers, and that, moreover, signs of it may not be recognized until injury is severe. The lesions of all of the group without signs were of focal or very limited distribution. Because the most useful obvious mark of disease was the presence of paralysis, ataxia, or marked weakness, such localized early developing lesions were encountered fortuitously in the healthy appearing animals. It is very evident from the advanced character of the lesions and the reparative phenomena in mice first showing obvious disease on the 9th or 10th day after inoculation, that the actual onset of injury must have occurred at least 1 or 2 days previously.

Degeneration.—Although the changes leading to muscle necrosis occur rapidly, they do not occur uniformly in all locations or in all fibers. It would appear that most of the fibers which are to form the site of injury are attacked within a relatively short space of time, and that the changes which end in complete hyaline necrosis take place in much less than 1 day. However, the injurious process is a continuing one. The number of necrotic fibers is at its peak on the 1st day of paralysis. Yet acute early segmental necrosis in many isolated foci is to be found on the 2nd and 3rd days of paralysis, when the rest of the same muscle shows a predominantly regenerative picture and when débridement of the products of the initial widespread degeneration is advanced or completed.

If it may be assumed that the successive stages of degeneration proceed at a more or less similar rate for all fibers, and that arrest and fixation of the process of degeneration do not occur after it has begun, both of which theses are supported by morphologic evidence, then it must also be assumed that some of the muscle fibers which had hitherto been spared in the more widespread initial injury are subsequently subjected to isolated attack. Thus it comes about in paralyzed animals that very early stages of injury are found in a few scattered segments when the great majority of injured muscle fibers are in advanced or terminal phases of necrosis.

When signs of paralysis first appear, muscle necrosis is always well advanced in one or more muscles. Mice surviving until the 2nd day show scattered fresh necrotic segments in dissimilar stages of evolution, and active phagocytic removal of the products of the widespread severe "first crop" lesions. Mineralized remnants of hyaline material which have escaped phagocytosis are the sole remains of degenerated muscle by the 5th day after onset of paralysis.

Inflammation.—Inflammatory reaction, which first appears concurrently with the early necrotic changes in the muscle, reaches its peak when most of

o———o Acute fiber necrosis

•———• Inflammatory phenomena

△———△ Regeneration of muscle fibers
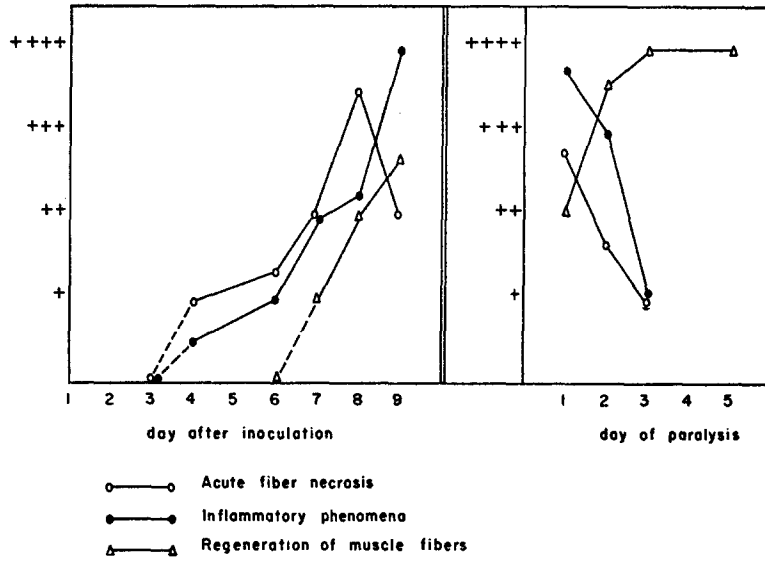
TEXT-FIG. 3. The temporal aspects of degeneration, inflammation, and regeneration are indicated (a) on the left in mice during the incubation period and in those on the 1st day of paralysis and (b) on the right in mice following the onset of paralysis.

o———o Acute fiber necrosis

•———• Inflammatory phenomena

△———△ Regeneration of muscle fibers
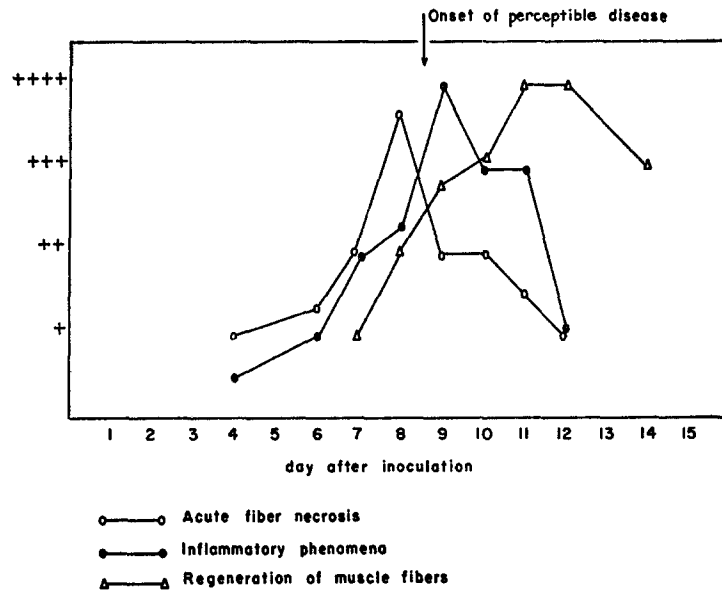
TEXT-FIG. 4. Extent of degenerative, inflammatory, and regenerative changes after inoculations, in relation to onset of disease.

the degenerating segments show frank hyaline necrosis on the 1st day of paralysis. The inflammatory exudate varies in direct proportion to the number of muscle fibers affected. After the 1st day of paralysis it declines slowly, and has not wholly disappeared by the 5th day. Removal of the products of necrosis with the formation of "sarcolemmic tubes" follows closely upon homogenization of the contents of the injured muscle segments and reaches its peak on the 2nd day (or possibly by the end of the 1st day). By the 3rd day of paralysis most of the phagocytic débridement has been accomplished, and most of the histiocytic cells have wandered out of the "tubes."

Regeneration.—Although the local nuclear increase preceding the first stages of segment injury (referred to as sarcoplasmic hyperplasia in the older literature) may be regarded as the abortive onset of regeneration, it may also be viewed as resulting from an initial proliferative stimulus which finally leads to necrosis. Effective regeneration is first recognized beginning with the alterations in the form of the muscle nuclei in the intact fiber ends. By the time that hyalinization has occurred, the vanguard of the ingrowing regenerating fiber as well as apparently isolated sarcoblasts have begun to grow into the necrotic area (1st to 2nd day of paralysis). Histocytic phagocytes penetrate into the hyalinized segment usually soon after the first slips of regenerating muscle. In the cell-filled sarcolemmic tube, the regenerating elements can usually be distinguished from the histiocytes by their distinctive nuclear form, very basophilic cytoplasm, and strand-like or linear growth pattern. Evacuation of the hyaline material and phagocytes from the sarcolemmic tube, a process which is under way on the 2nd day of paralysis and nearly complete by the 3rd day, leaves the field to the regenerating fibers. These, guided by the persisting sarcolemmic structures, proceed to fill up the defective area. If all the new fibers were to attain maturity, the resulting muscle would have many times more bulk than the original. Plainly, some of the newly formed strands must atrophy and disappear after the 3rd day of paralysis. By the 2nd day, some regenerating fibers begin to form the specialized contractile elements (myofibrils) and then slowly lose their basophilia as myohemoglobin accumulates. By the 5th day after the onset of paralysis, the new fibers are equipped with myofibrils, delicate A discs, and in some cases Z discs. After 11 days only the presence of occasional "mummified" remnants testifies to the former injury, and the wide spacing, delicate striations, central nuclei, and slenderness of the fibers bear witness to the youth of the restored muscle. An attempt has been made in Text-figs. 3 and 4 to chart in graphic form the degenerative, inflammatory, and regenerative changes with the passage of time.

DISCUSSION

Viral Aspects.—The course of events described is that obtaining when one Coxsackie virus, the Conn.-5, is inoculated intraabdominally. Results with the

Ohio-1, which also produces an encephalitis, are similar in that virus is either in low titer or even not detectable in the blood at the time of paralysis. However, with the Texas-1 or High Point strains, which rarely affect the brain, there is a marked viremia on the first day of illness. In fact the titer of virus in the blood, about 10^{-7}, is second only to that in muscle and exceeds that found in brain, liver, and intestines.

The absence in Conn.-5 infection of detectable virus in the brain on the 2nd day after inoculation, when all the other tissues examined already showed evidence of virus multiplication, suggests the necessity of a rich viremia being established in order to carry virus into the brain. It was surprising to find that the intestinal contents were as rich in virus as the intestinal wall, even on the 2nd day after inoculation when virus first made its appearance in the mice. In retrospect it would have been useful to have carried out tests on separate parts of the intestine and the contents of each part. In view of the widespread occurrence of virus in the tissues selected for study, some of which showed no histopathologic changes, the interpretation of the present study would have been aided if more tissues had been selected to determine if any are devoid of virus. With such a rapidly growing virus, it may be necessary to follow the appearance and quantitative levels of virus at intervals shorter than 24 hours, particularly in the first days after inoculation in order to gain further insight into the pathogenesis of the disease.

Histologic Aspects.—It has already been pointed out that manifest paralysis serves only as an approximate indicator to the actual onset or stage of development of the muscle and nervous system lesions in any single animal. Attention must also be called to the limitations of the technique, in which complete serial sections were not used and every muscle could not be examined, for the purpose of verifying the presence or absence of anatomic changes. The failure to find muscle lesions in two paralyzed mice without encephalitis is attributed to this.

The rapidity of the sequences occurring in infected muscle is remarkable. The initial attack produces numerous lesions of approximately the same stage of development. The relative infrequency of intermediate stages between the first tinctorial and proliferative changes and final complete hyalinization is evidence for the speed of degeneration of affected muscle segments. Why only certain segments of certain fibers are involved initially, why small numbers of segments are subsequently attacked, and what factors determine the resistance of the remaining fibers or of other muscles, are things which remain to be learned. Inflammation occurs as a response to necrosis. It follows closely upon the development of necrosis, always tarrying somewhat behind in onset and subsidence, and parallels in degree the extent of necrosis. By the end of the 2nd day most of the necrotic débris has been removed, inflammation is subsiding, and regeneration is in active progress. Regeneration is recognizable from the

end of the 1st day of paralysis, and is for the most part completed within 5 to 7 days; new myofibrils are occasionally found as early as the 2nd day. These degenerative and inflammatory changes are similar in kind to those seen in a wide variety of muscle injuries (10–19).

Rustigian and Pappenheimer (17), investigating the myositis in adult mice resulting from intramuscular injections of viruses of the mouse encephalomyelitis group, found that regeneration began on the 4th day after severe myositis. Following experimental muscle injuries in adult rabbits, necrotic fragments are present and being phagocytosed after 3 to 5 days (13). Regeneration is first seen 2 days following injury in the form of continuous sarcoplasmic strands, which ultimately (weeks to months) reconstitute the injured part (14–16). Durante (11), epitomizing the findings of earlier investigators, gives the following chronology: nuclear proliferation, 4 to 6 hours after trauma; sarcoblasts, 3 to 5 days; young fibers, after the 6th day; striation of young fibers, 3rd week to 3rd month. In Zenker's degeneration of rectus muscle in man consequent on infectious disease, Forbus (12) found that regeneration begins synchronously with degeneration and that complete restoration of the necrotic part occurred without scarring 15 to 20 days after onset of disease.

In the infected suckling mice these transformations are far more rapid than has hitherto been observed in comparable lesions of adult muscle. That differences in the type of regeneration in different species occur has been shown by Schminke (10); neither is it surprising that regeneration should be faster in the young animal. It is difficult to assess the relative roles of the nervous system and muscle disease in the production of signs, although paralysis does certainly occur in the absence of encephalomyelitis. This is particularly true with the other immunological types of C virus, viz., Texas-1.

From an examination of the tabulated data, particularly Table III, the impression is gained that with more days elapsing after inoculation, or with longer apparent incubation periods, there is a higher incidence of lesions in all susceptible tissues. This same rule appears to hold with respect to severity of lesions. However, this may prove to be true only with inoculation of a small dose of virus, for these animals were inoculated with only 10 ID_{50} doses.

Correlation of the Incidence of Perceptible Disease, Virus Level, and Lesions in Muscle.—From data listed above, Text-fig. 5 was constructed relating, on a temporal basis, the incidence of obvious disease, the amount of virus present in muscle, and the extent of lesions in this tissue. Virus appeared on the 2nd day after inoculation, and reached its maximum titer on the 4th day, maintaining this level through the 8th day. As with other agents (20, 21) pathologic changes appeared after virus had reached high levels. Lesions were first noted on the 4th day, when there was a peak viral concentration (the titer being 10^{-7}), and reached their maximum intensity and number on the 8th day. The virus titer during this period did not fall below 10^{-6}. Mice began showing signs of disease on the 5th day with the highest incidence of disease also occurring on the 8th day. After the 8th day, the number and intensity of the lesions quickly fell off,

but the continued appearance of scattered fresh necrosis on the 9th to 12th days, after the initial first crop, may be related to the continuing high virus titer, which remains above 10^{-4} during this interval. The regenerative phase, which began 1 day after the first lesions were noted, reached its maximum development on the 9th and 10th days. It is noteworthy that the regenerating sarcoblasts suffered no injury, despite viral concentrations ranging in titer from 10^{-7} to 10^{-4} on the 8th to 12th days. With the decreased myositis there was a concomitant decrease in the incidence of perceptible disease. Mice which
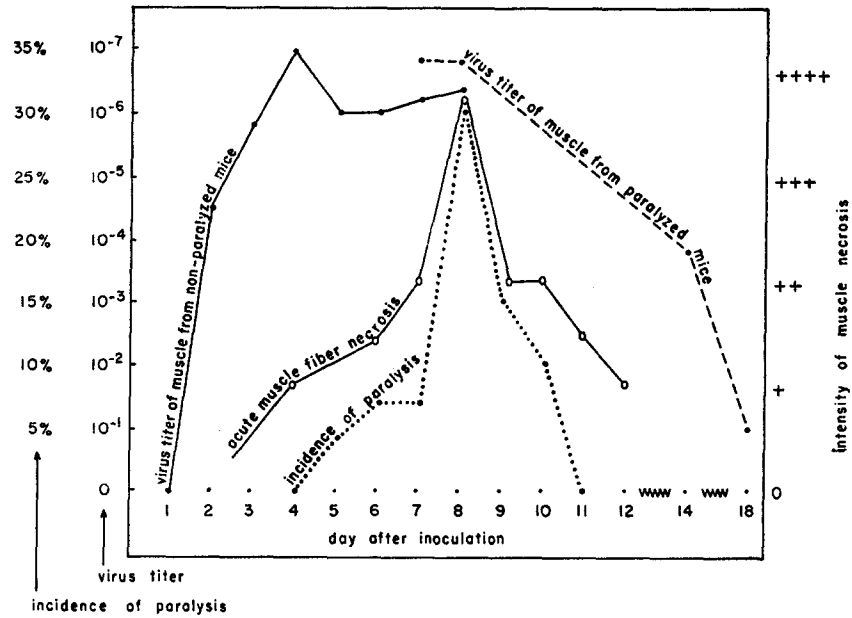
TEXT-FIG. 5. Correlation of virus concentration of muscle tissue in non-paralyzed and in paralyzed mice with intensity of muscle fiber necrosis and with incidence of overt disease.

survived the paralysis were found to have virus present in the muscle (and brain) 8 days after its onset, although at a greatly reduced level.

Although we have assumed that the virus titer obtained with "muscle" was due to the virus in the muscle itself, it is obvious that the tissue used contained variable amounts of fat, which was also found to be susceptible to injury in the course of the infection.

Mention should be made of the differences in the histopathology of the lesions in the 1 day old from that in the 4 to 5 day old animals. Most obvious were the acute hepatitis and pancreatitis in the younger mice, first noticed by Pappenheimer (8), and the sparing of these tissues in the older animals. In some animals, it appeared that the pancreatitis and hepatitis were so severe that

they resulted in overt disease before lesions in the muscle and central nervous system developed. When present, the myositis in these younger mice often was localized and mild. In view of the fact that the virus multiplied in the liver of the 4 to 5 day old mice without producing lesions, the question may be raised as to the significance of the virus level in relation to pathologic changes in this tissue. In newborn mice, does the virus concentration in the incubation period build up to higher levels in the pancreas and liver than in the 4 to 5 day old animals?

The information derived from virus titration correlated with histologic study, makes more pointed certain fundamental questions in the pathogenesis of viral disease. It has been demonstrated as in the case of the intestine, that an organ or tissue may be completely resistant to any anatomically demonstrable injury, while harboring rapidly multiplying virus which attains high titer. Moreover, susceptible tissues, such as muscle and central nervous system, develop lesions only in a fraction of cases although the virus apparently attains comparable concentrations during the incubation period in all cases. These observations stimulate curiosity concerning the factors which operate in different tissues to cause cellular injury as against those which only sustain virus growth.

RECAPITULATION

Following the intraabdominal inoculation of the Conn.-5 strain of Coxsackie virus into 4 to 5 day old mice, the appearance of virus in selected tissues was studied. A correlation was made of the incidence of perceptible disease, the amounts of virus present in muscle, and the extent of the lesion in this tissue.

On the 2nd day after inoculation multiplication was well under way in all tissues examined except the brain. The titers of blood, heart, liver, muscle, intestinal wall, and intestinal contents were all at the same level, $10^{-3.4}$ to $10^{-4.5}$. The level of virus in the blood fell off sharply after the 5th day, while high titers were maintained in the other tissues through the 8th day. Although the titer in muscle was not much higher than in other tissues, it was consistently the tissue with the greatest concentration of virus. This was found to be the case with obvious disease as well as in those sacrificed before disease became apparent.

In paralyzed mice, the highest level of virus (10^{-7}) was present in the muscle and brain. It was in these tissues alone that virus persisted in paralyzed mice through the 9th day of illness.

Muscle was the only tissue found in which the progressive development of a lesion reached its maximum intensity at the time of peak incidence of overt disease. Lesions were first noted in muscle on the 4th day after inoculation, 2 days after the initial multiplication of virus, and reached their maximum intensity on the 8th day. Signs of disease were first observed on the 5th day with

the maximum incidence also occurring on the 8th day. With the first appearance of paralysis, muscle necrosis when found was always well advanced in one or more muscles.

After the 8th day, the number and intensity of the acute-phase muscle lesions quickly declined, with the regenerative phase, which began 1 day after the first lesions were noted, reaching its maximum on the 11th to 12th days after inoculation. With the decrease in the myositis, there was an attendant decrease in the incidence of perceptible disease. Mice which survived the paralysis were found to have virus present in the muscle several days after onset although at a greatly reduced level (titer of 10^{-1}). At this time, limb muscle consisted chiefly of maturing regenerating fibers with interspersed intact adult fibers. The evolution of the muscle lesion has been described in some detail, from onset of muscle necrosis to early restitution.

Encephalomyelitis, found in 61 per cent of the paralyzed animals, presented only an acute picture. There was little consistent progression of central nervous system injury 2, 3, and 5 days after onset of disease, beyond the rarefaction of the necrotic zones.

Generalized fat necrosis occurred in 83 per cent of the paralyzed mice. As it appeared full blown when present, there was no opportunity to study its evolution. Calcification occurred early. Myocardial necrosis occurred in the mice but to a lesser degree.

Hepatitis and pancreatitis were found only when newborn (1 day old) mice were inoculated with virus.

SUMMARY

The quantitative distribution of the Conn.-5 strain of Coxsackie virus in different tissues was determined by serial titration at intervals after inoculation of 4 to 5 day old mice. High titers were reached by the 2nd day in blood, heart, liver, muscle, intestine, and its contents, and these were maintained through the 8th day, except for the blood, in which the virus level fell earlier. In paralyzed mice, muscle and brain attained the highest titers and it was in these tissues alone that virus persisted through the 9th day of illness.

The pathology of the infection has been briefly described. In particular, the evolution of morbid changes in striated muscle was correlated with the concentrations of virus in muscle. Acute muscle necrosis first occurred when there was a peak viral concentration (4th day), and reached maximal intensity on the 8th day. Scattered acute lesions continued to appear while the virus titer remained above 10^{-4}, from the 9th to 12th day. With the decrease in the myositis, there was a concomitant decrease in the incidence of perceptible disease. Inflammation was found to follow upon the development of necrosis, and subsided slowly. Regeneration began very early, became exuberant, and led finally to restitution of the muscle.

We are indebted to Dr. Henry Bunting for his advice in these experiments and for his assistance in the interpretation of the lesions. A more detailed description of the lesions produced by the Conn.-5 and other strains will be forthcoming by Godman, Bunting, and Melnick (9). We also wish to acknowledge the assistance of Dr. Lisbeth M. Kraft, Miss Eva Zabin, and Dr. S. Otani.

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EXPLANATION OF PLATE 12

FIG. 1. Selective involvement of iliac, sartorius, quadriceps, gastrocnemius-soleus, and hamstring muscles of hind limb. The other muscles, which appear darkly stained, are intact. Conn.-5 strain. × 20.

FIG. 2. Florid, acute, muscle fiber necrosis showing hyalinization of most segments and interstitial edema. Sarcolemmic nuclear proliferation is apparent. The longitudinal striae of the prehyaline stage are present in one segment (upper left). Conn.-5 strain, 1st day of paralysis. × 200.

FIG. 3. Two sarcolemmic tubes, with numerous mononuclear phagocytes and remnants of hyaline material within the sarcolemmic sheaths. Sarcoblastic slips, originating from the intact muscle periphery, are growing into the tube on the right. The demarcated segmental involvement is apparent. Conn.-5 strain, 2nd day of paralysis. × 340.

FIG. 4. Recent, acutely degenerated segment in a muscle showing a marked, predominantly regenerative picture. There are numerous slender sarcoblastic slips with typical nuclei aligned in rows, which are growing into emptied sarcolemmic hulls. In the interstitium there is a receding inflammatory exudate in which many histiocytic cells are visible. Conn.-5 strain, 3rd day of paralysis. × 320.
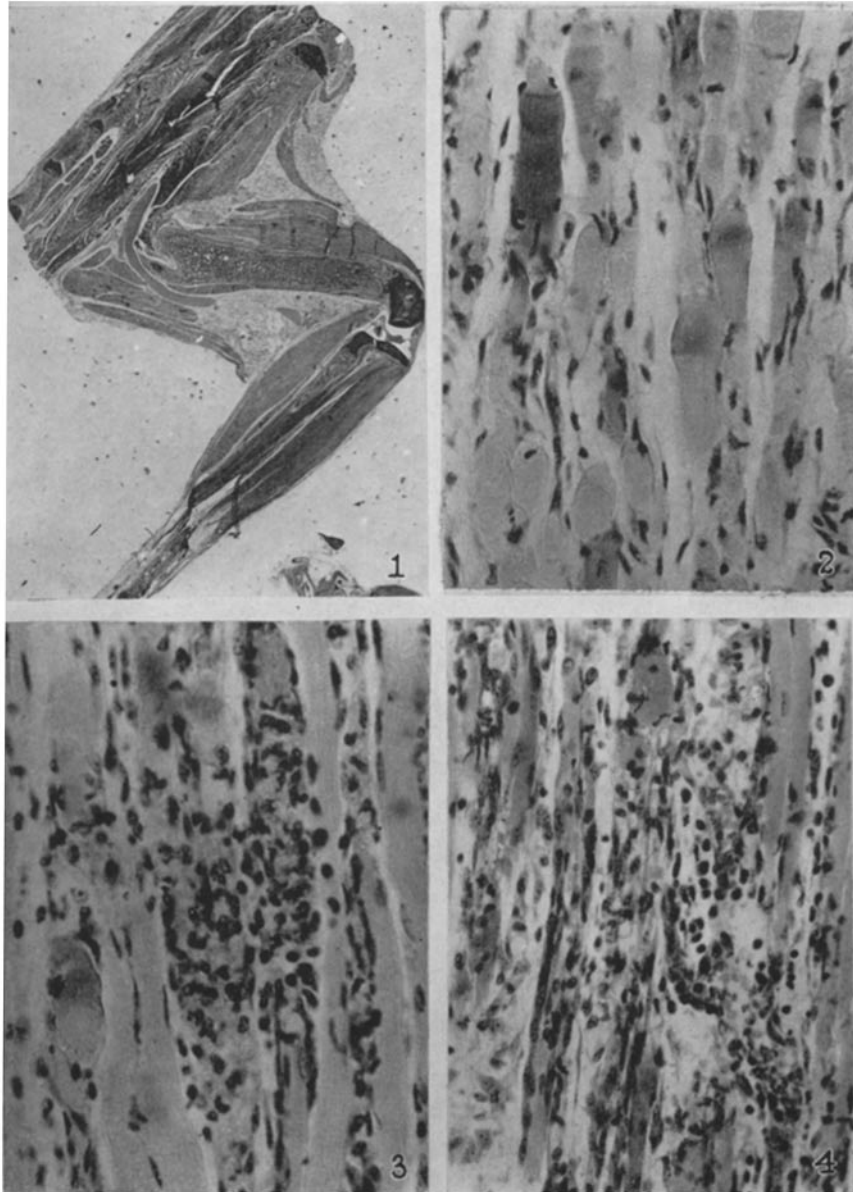
(Melnick and Godman: Pathogenesis of Coxsackie virus infection)