PASSAGE OF COXSACKIE VIRUS (CONNECTICUT-5 STRAIN) IN ADULT MICE WITH PRODUCTION OF PANCREATIC DISEASE*

By ALWIN M. PAPPENHEIMER, M.D., LAWRENCE J. KUNZ, AND SHEILA RICHARDSON

(From the Department of Bacteriology and Immunology, Harvard Medical School, Boston)

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A large series of young adult mice were given repeated intraperitoneal injections of Conn.-5 strain of Coxsackie virus in the form of carcass suspensions of infected sucklings, for the purpose of obtaining immune serum. Within a few days following the first injection, a number of the mice were found dead and partly eaten. Others were obviously ill, but still others presented a relatively normal appearance and behavior.

A mouse which had received three intraperitoneal injections seemed to be moribund 3 days after the last inoculation and was sacrificed for histologic examination. It was unexpected and interesting to find that the pancreas had undergone almost complete destruction, and that no significant lesions were found in other organs or tissue. We were stimulated therefore, to pursue the matter further. It soon became apparent that pancreatic disease of greater or lesser severity developed in virtually 100 per cent of adult mice inoculated intraperitoneally with suspensions containing this agent in adequate dosage.

These observations were in contradiction to the generally accepted view that viruses of the Coxsackie group are pathogenic only for suckling animals. Indeed, the susceptibility of *immature* mice and hamsters has been regarded as a distinguishing characteristic of the Coxsackie viruses (1–3). Although Dalldorf and Melnick have suggested that older animals may not be completely resistant, neither these authors nor any other worker in the field has reported the propagation of the virus in fully grown animals.

It will become plain from our studies that these statements do not apply unequivocally to the Conn.-5 strain of Coxsackie virus. It will be shown: (*a*) that this agent consistently produces pancreatic disease in adult mice; (*b*) that it can be propagated in series in adult mice with suspensions of pancreatic tissue; and (*c*) that the pancreas is selectively affected irrespective of the route of inoculation.

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Materials and Methods

Virus.—The Conn.-5 strain (4, 4*a*) of Coxsackie virus was received through the kindness of Dr. J. L. Melnick in December, 1949. The present studies were initiated with the 3rd passage of this material through infant mice in our laboratory.

Mice.—Mice used in all the experiments were bred in the Department of Bacteriology and Immunology, Harvard Medical School. They were started from the stock of Mr. Victor Schwentker of Tumblebrook Farm, Brant Lake, New York. No attempt has been made to compare the susceptibility of this strain with mice from other sources. The age of each mouse used in the experiments was known with certainty.

Suspensions of virus propagated in suckling mice were prepared as described by Melnick (5). Mice which had been inoculated 2 to 3 days previously (at the age of 0 to 1 day) with Conn.-5 virus were killed by decapitation and stored at −20°C., until suspensions were made. After thawing, the skin, limbs, tail, and viscera were removed. The carcasses, after being washed with ether, were then ground with alundum in a mortar. Sufficient 0.15 M phosphate NaCl buffer at pH 7.2 containing 500 units of penicillin and 500 μg. of streptomycin per cc. was added to the ground tissue to make a 10 per cent suspension of mouse carcass. The suspensions were refrigerated overnight and then centrifuged in the refrigerated International PR-1 centrifuge at 2500 R.P.M. for 10 minutes. The supernatant fluid was removed, distributed in pyrex glass ampoules which were then glass-sealed, and stored in the CO_2 cabinet.

These preparations are referred to as "muscle-bone suspensions" and consist essentially of extracts of muscle, bone, cartilage, and spinal cord. Suspensions of "normal muscle and bone" were prepared in the same manner when the tissues were harvested from uninoculated mice 2 to 3 days old.

Morbidity

In the experiment in which the pancreatic disease was first noted, 99 mice several weeks of age or over were inoculated intraperitoneally twice a week with 0.1 cc. of muscle-bone suspension of infected suckling mice. The first inoculation consisted of 5 per cent suspension; all subsequent inoculations were made with 10 per cent suspension of the same material. The purpose of this experiment was, as has been said, to obtain immune antiserum, and the procedure followed that described by Melnick (5). During the first few days, 13 mice were found dead and were discarded. One mouse, previously referred to, was found moribund on the 11th day after the first inoculation. By the 33rd day, 24 additional mice had died, making a total of 37 mice which succumbed as a result of the inoculations.

The incidence of pancreatic disease in this series is 100 per cent if we include amongst the positive cases the 37 mice which died spontaneously, and were unsuitable for examination. Of the remaining 62, all showed gross lesions of the pancreas and 31 of these, which were examined microscopically, had pancreatic lesions of greater or lesser severity.

As has been said, a certain proportion of the animals showed no obvious signs of illness. Amongst these were 20 mice which had survived for 93 days after the first inoculation. The entire pancreas was removed, fixed in Bouin's fluid, stained in bulk with sudan IV, and cleared with glycerine. With this method it was obvious in the gross that all had more or less replacement of the gland with

adipose tissue (Fig. 1). The amount of persisting glandular tissue, easily recognized by its solid grayish pink appearance, varied from roughly one-quarter of the bulk of the original pancreas to 5 per cent or less. It tended to be concentrated about the larger ducts, which were readily detected under the dissecting microscope. It is evident from these observations that a considerable reduction in the pancreatic exocrine tissue may occur without producing noticeable signs of disease.

Signs of Disease

The signs of illness which have been noted are first of all a marked loss of body weight, usually associated with arrest of growth. The sick animals, as one might surmise, seem weak and listless and their backs are hunched. The coat is rough. The palpebral fissures are often narrowed, probably because of periorbital edema. Signs pointing to involvement of the nervous and muscular systems are never noted. There is no paralysis, tremor, or spasm.

The weight loss has proved to be a sensitive index of the extent of pancreatic destruction. Although no weights were taken in this first group of animals, in subsequent experiments the mice were weighed weekly or oftener. Using as a basis the data from 48 mice it was found that the average weight loss 1 week after the first injection was 3.9 gm.; at the end of the 2nd week 5.1 gm.; at the end of the 3rd week the 21 survivors had lost about 2 more gm., making the total weight loss 6.9 gm. (Chart 1) This is roughly 32 per cent of the initial average weight of the animals. A few of the animals gained in weight several weeks after the initial fall. This was probably due to the occurrence of edema, since at autopsy free fluid was found in the peritoneal and pleural cavities and the subcutaneous tissues appeared to be edematous. This edema was probably associated with a low protein content of the serum. A pool of the sera from 9 mice suffering from pancreatic disease had a total protein content of 4.6 per cent in contrast to serum of normal mice in which the protein content was 6 per cent.[1] That this loss in weight is not due to the introduction of the tissue components or products of autolysis will be clear from the control experiment to be described below.

In view of the extensive pancreatic lesions, it is perhaps surprising that the feces did not differ in consistence or color from those of normal mice. Only exceptionally were they unformed and paler than normal feces. The normal appearance was probably due to the low fat content of the Purina fox checkers which comprised the diet of the animals. A determination of the total fecal fat content of the normal and infected mice showed no significant difference in the two groups. However on the diet used the fecal fat content was extremely low (less than 0.5 per cent). Further experiments on high fat diets are indicated.

[1] We are indebted to Dr. John R. Pappenheimer for these determinations which were made by the falling drop method of Barbour and Hamilton (6).

MEAN WEIGHTS OF NORMAL AND INFECTED ADULT MICE

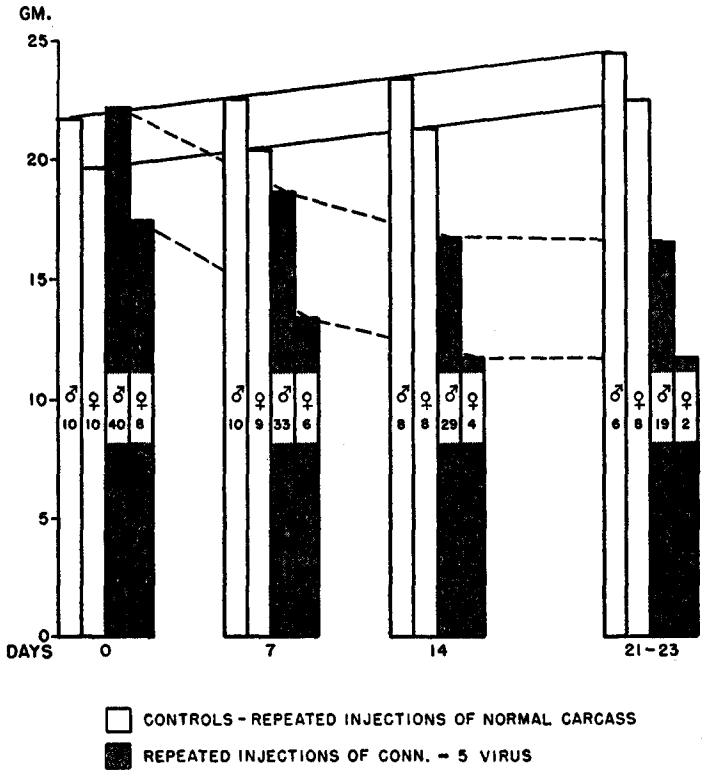


CHART 1

Pathology

Gross Lesions.—The most interesting and severe gross lesions were those found in the pancreas. These were readily visible to the naked eye and with experience proved to be well correlated with the histologic alterations.

In the earliest cases, examined within a few days of the first injection, the organ was swollen, edematous, and pale in contrast to the solid pink texture of the normal pancreas. At this stage too one frequently found circumscribed opaque whitish fat necroses in the perirenal adipose tissue or in the mesenteric and pelvic fat. The plaques were often several millimeters in size. They were confined to the intra-abdominal fat tissue, unlike the lesions in embryonic fat of suckling mice which affect the cervical, axillary, and interscapular fat pads by preference. They thus appeared to be associated with the acute pancreatic necrosis, rather than with direct viral activity.

As the disease progressed and the interval between the first injection and the time of death or sacrifice increased, the pancreas became atrophic, appearing as a transparent film of connective tissue, or when fat replacement had occurred, as a mass occupying the region of the pancreas, but indistinguishable from the remaining adipose tissue.

A few animals had bloody fluid in the upper intestine; the source of the bleeding was not discovered. A few also, as has been stated, were edematous and there was excess of clear fluid in the peritoneal and pleural cavities.

The *liver*, in mice dying early, was the seat of fat infiltration, usually peripheral, the center of the lobules being reddish. Later the liver resumed its normal appearance. The *spleen* in the early phases of the disease was enlarged and congested; later it became reduced in size. The *thymus* in the emaciated animals underwent extreme atrophy.

In contrast to the suckling mice, no gross lesions were noted in the heart, lungs, skeletal muscles, brain, or peripheral fat lobules.

Microscopic Lesions

Pancreas.—Within 3 or 4 days after a single injection, there occurs a massive necrosis affecting the greater portion of the acinar tissue (Fig. 2). The acinar structure is barely discernible. The individual cells lose their nuclei, or such nuclei as remain are pycnotic and fragmented. The cytoplasm is eosinophilic and coarsely granular; zymogen granules are missing or dispersed amongst the necrotic detritus. At this stage, there is little inflammatory reaction, but the interlobular septa are edematous. The islands of Langerhans and the large and small pancreatic ducts escape destruction.

Within the next few days, there occurs a profuse cellular reaction. Most of the cells, both within the necrotic lobules and in the interlobular septa, are monocytic, but a few polymorphonuclears are present, and may invade the dead cells. The resorption of the necrotic tissue proceeds rapidly, and the cellular reaction continues to increase (Fig. 3). In a number of preparations, taken 10 or 12 days after injection, bluish staining masses of necrotic material, or possibly inspissated secretion, are surrounded by histiocytes, some of which have coalesced to form multinucleate giant cells (Fig. 4); the connective tissue cells at this stage have taken on the character of fibroblasts, and are apparently proliferating. The edematous interlobular septa still contain large numbers of mononuclear cells, and in a few preparations, eosinophiles were quite abundant. There is no hemorrhage or vascular thrombosis.

At a somewhat later stage, the inflammatory cellular reaction has faded (Fig. 5). Now one finds some residual groups of hypertrophic acini, concentrated small ducts, and scattered islands of Langerhans surrounded by broad mantles of cells, histiocytic or fibroblastic in origin. Many of the cells are polygonal and vacuolated, taking on the character of lipoblasts.

As the process continues, these cells change into adult fat cells, and the entire

organ becomes replaced by adipose tissue in which the persistent ducts and islands afford the only clue to the original nature of the tissue (Fig. 6). About these, there now appear scattered histiocytes containing clumps of brownish pigment, which proves to be "ceroid" in nature (Fig. 7). It is brilliantly red when the sections are stained with Ziehl-Neelsen carbolfuchsin followed by decolorization in 5 per cent hydrochloric acid in 95 per cent ethanol. Under the fluorescence microscope, the pigment has a bright, slightly yellowish fluorescence (Fig. 8). The possible significance of this pigment, which is deposited also in small amount in the spleen and intestinal lymphoid tissue, will be discussed later.

Destruction of the acinar tissue is not always complete. Small compact nodules of normal pancreatic tissue may remain in one portion of the section. More commonly small groups of well preserved acini, or single acini, are scattered through the fat. The cells composing these are very definitely hypertrophied, both nuclei and cytoplasm sharing in the enlargement (Fig. 9). This is evident when one compares the photographs of normal and hypertrophied acini shown in Figs. 10 and 9, both taken at the same magnification.

In none of our preparations have we found any indication of regenerative activity on the part of the acinar epithelium. The small intralobular ducts, however, may show mitoses and limited proliferation.

It is evident that the amount of functional exocrine tissue which has escaped destruction, determines whether the mice can survive in a state of comparative well being, or whether they will succumb to chronic pancreatic insufficiency. We have made no effort to estimate the precise amount of acinar tissue necessary for survival, or for normal health; but it is certain that a very considerable portion of the pancreatic tissue can be lost without causing fatal disease. With very rare exceptions, we have found pancreatic disease in mice inoculated with an adequate dose of virus, even when the weight loss was minimal and the appearance and behavior indistinguishable from those of a normal mouse.

Fat Necroses.—In animals succumbing or killed soon after the first injection, it was usual to find opaque areas of fat necrosis scattered through the intra-abdominal fat. Histologically, these were composed of necrotic fat cells without nuclei, filled with a pink-staining coagulum. The necroses were sharply circumscribed, and often marginated by a wall of inflammatory cells (Fig. 11). Calcification did not occur, but the necrotic fat cells rapidly became surrounded by young connective tissue, and eventually were completely replaced by fibroblastic growth (Fig. 12).

Although necrosis and inflammation of adipose tissue frequently occur in suckling mice infected with the Conn.-5 and other strains of Coxsackie virus (7, 2, 8) there are striking differences in the location, histopathology, and presumably also the cause, of the fat lesions in suckling and adult mice. In the sucklings, the lesions are found in the lobules of embryonic fat in the cervical region, axillae, and interscapular fat pad. In the adults, mature fat tissue only

is affected, and the lesions are restricted to the fat within the abdominal cavity. Calcification of the necrotic fat invariably takes place after 4 or 5 days in the suckling lesions, but it has not been found in the lesions of adult mice.

It seems highly probable that the adipositis in infant mice is due to a preferential localization of the virus in the immature fat lobules; indeed, virus may be recovered from the affected fat tissue (9). Although pancreatic necrosis occurs in a high percentage of mice infected during the first few days of postnatal life, peripheral fat necrosis is frequently found in the absence of pancreatic lesions. The intra-abdominal fat necroses in the adult mice are unquestionably the familiar result of the massive necrosis of pancreatic tissue, with liberation of proteolytic and lipolytic enzymes.

The new born mouse has very little adipose tissue within the peritoneal cavity. This is presumably the reason why intra-abdominal fat necrosis has not been seen in newborn mice with pancreatic lesions.

Liver.—In mice dying early in the course of the disease, the liver is often the seat of marked fat infiltration, sometimes peripheral, but often involving the entire lobule. Individual liver cells in the central portion of the lobule are degenerated, and in some instances there is quite extensive central necrosis. These changes appear to be transitory, or they occur only in rapidly fatal cases. In the animals surviving, the fat infiltration is no longer found. In many livers, the central veins are surrounded by a zone of large liver cells which have lost their normal basophilia, and are less closely spaced than at the periphery of the lobule. The exact interpretation of this altered staining is not apparent.

Spleen.—In the acute phase of the disease, the lymphoid cells of the Malpighian follicles are fragmented, and the chromatin particles phagocyted by the reticular cells. The pulp is congested. As the disease progresses, the spleen becomes atrophic, and an abundance of hemosiderin is found scattered through the pulp. In many of the chronic cases, acid-fast ceroid pigment is also present.

Thymus.—In the emaciated animals, the thymus undergoes extreme involution. No lesions of consequence were discovered in other organs or tissues. The central nervous system, as might have been anticipated from the lack of neurologic signs, was not affected. It was interesting to find that the testes showed active spermatogenesis, despite the extreme emaciation and weakness of the mice. In only an occasional animal did the testes show reduction of spermatogenesis, with the appearance of spermatid-containing giant cells in some of the tubules. The ovary also, was not abnormal, and several of the infected animals became pregnant and gave birth to normal litters.

No lesions were found in skeletal muscle, even at the site of local intramuscular injection.

EXPERIMENTAL

Controls.—The question arose as to whether the pancreatic lesions were a specific effect of the virus or whether they might represent a non-specific result of the injection of tissue suspensions, autolytic products of which might conceivably include substances toxic for the pancreas. To answer this question ten male and ten female mice were given six biweekly inoculations of 0.1 cc. of a 10 per cent muscle and bone suspension of normal 2 day old mice.[2] The mice were

[2] The first inoculation consisted of a 5 per cent suspension of infected muscle and bone; all subsequent injections were made with 10 per cent suspension of the same material.

weighed before inoculation and at weekly intervals. As shown in Chart 1 the initial weight was maintained or a slight gain in weight was recorded. There were no external signs of illness. After each injection, one or more mice were sacrificed for pathologic examination. The pancreas was invariably normal and no lesions were found elsewhere. This showed conclusively that the tissue suspension *per se* was not responsible for the pancreatic lesions.

Effect of Single and Multiple Injection.—Since the pancreatic disease was first observed in a mouse which had received three spaced injections of virus suspension, it was necessary to ascertain whether multiple inoculations were requisite for the production of the lesions. This question is answered by the following experiment (Table I):

TABLE I

Effect of Single and Multiple Inoculations on Incidence of Pancreatic Disease

Group	No. of injections	Mean weight loss			No. sick or dead	Pancreatic lesions		
		Interval after 1st injection (days)						
		7	14	18		No. examined	Gross	Microscopic
		gm.	*gm.*	*gm.*				
A	1	5.0	4.2	4.9	8/8	8	8	8
B	2	5.0	5.8	4.0	8/8	7	7	7
C	3	3.6	7.2	6.6	8/8	6	6	6
D	4	4.0	5.6	4.4	7/8	7	7	7
E	5	3.5	6.9	7.2	8/8	4	4	4
F	6	2.1	3.8	2.7	8/8	7	6	6

Six groups of eight animals each were given respectively, 1, 2, 3, 4, 5, and 6 injections of the standard suspension of Conn.-5 virus and weights and signs of illness were recorded. Mice from each group were examined at various intervals. There was no obvious difference in the behavior of the different groups—those receiving a single injection showed weight loss and signs of chronic illness identical with those receiving multiple injections. Furthermore it was found that the character of the lesions depended upon the period of survival following the first inoculation and that a repetition of the injections in no way modified the evolution of the lesions. In no instance did animals surviving a week or more after the first injection show fresh necrosis of the still remaining acinar tissue. Indeed it would seem that the cells which have been spared after the first inoculation are resistant to subsequent contact with the virus. The possibility cannot be excluded that antibodies developing soon after the first injection may neutralize the virus within the peritoneal cavity and prevent its access to the surviving pancreatic cells.

Quantity of Virus Required for Production of Lesions.—It was of interest to

determine what quantity of virus, in terms of LD_{50} for suckling mice, was necessary to produce pancreatic disease in adult mice.

Serial tenfold dilutions of muscle and bone suspension of infected suckling mice were injected intraperitoneally in 0.1 cc. amounts into groups of eight 6 week old mice. Weight records were kept and one half of each group of mice were sacrificed on the 4th day after inoculation; the surviving mice were sacrificed for pathologic study on the 11th day after injection of the virus material.

In Table II are tabulated the occurrence of lesions visible in the gross, the results of histologic examination, average weight loss of the animals, and the occurrence of death in the various groups of inoculated mice. Since adult mice do not regularly experience fatal illness with the Conn.-5 virus, it has been necessary to resort to consideration of weight loss and of gross and microscopic lesions to determine the infectivity of a given virus preparation. It is obvious from Table II that there is excellent correlation between the occurrence of microscopic lesions and the gross signs of illness mentioned above.

The end-point of infectivity (ID_{50}) of the virus preparation for adult mice, using the criteria described, is calculated to be $10^{-3.8}$. The LD_{50} end-point of the same material for 0 day old mice is of the order of 10^{-7}, indicating that 1000 times less material is required to infect suckling mice than is needed to produce pancreatic disease in mice that have been weaned. Since the adult mice were roughly 20 times the weight of the newborn, a correction factor might justifiably be introduced to take into account this disparity. Melnick (3) has suggested that the capacity to produce disease in 15 to 20 day old mice might be a function of the quantity of virus present in the inoculum, referring to the ability to infect 15 to 20 day old mice with a high-titer virus strain (Texas, 1948). The present evidence, however, seems to exclude the necessity of high concentrations of virus for the production of pancreatic disease in adult mice, at least with the Conn.-5 strain, since only 400 to 1000 doses of virus infective for suckling mice are required. It is worth noting that, although fewer mice become infected with higher dilutions of virus, the pancreatic lesions in those mice in which the virus does become established are just as severe as in the mice inoculated with concentrated virus. This suggests that there are individual variations in resistance which become manifest only when the infective dose is reduced.

Production of Lesions by Various Routes of Inoculation.—The question arose as to whether direct contact of the virus with pancreatic tissue was necessary to evoke lesions of this organ, or whether the pancreas was selectively affected regardless of the route of administration of the virus. In suckling mice, the Conn.-5 strain, however introduced, produces lesions in the central nervous system, skeletal muscle, myocardium, lungs, and peripheral fat lobules, as well as in the liver and pancreas. Would pathologic changes be found in these tissues in adult mice also, if the virus were inoculated by routes other than peritoneal?

TABLE II

Titration of Virus in Adult Mice

Dilution of virus	Mouse No.	Weight		Gross lesions	Microscopic lesions
		Loss	Days		
10^{-1}	1	+	(4)*	+	++++
	2	+	(11−D)‡	+	Not examined
	3	+	(4)	+	++++
	4	+	(11)	+	++++
	5	+	(4)	+	++++
	6	+	(11)	+	++++
	7	+	(11)	+	++++
	8	+	(4)	+	++++
10^{-2}	11	+	(4)	+	++++
	12	−	(4)	+	++++
	13	+	(11)	+	++++
	14	+	(11)	+	++++
	15	+	(11)	+	++++
	16	+	(4)	+	++++
	17	−	(11)	−	−
	18	+	(4)	+	++++
10^{-3}	21	+	(4)	+	++++
	22	−	(11)	−	−
	23	−	(11)	−	−
	24	−	(11)	±	++++
	25	?	(4−D)	+	Not examined
	26	−	(8−D)	+	++++
	27	+	(4)	+	++++
	28	+	(4)	+	++++
10^{-4}	31	−	(4)	−	−
	32	+	(4)	+	++++
	33	−	(11)	−	−
	34	−	(11)	−	−
	35	−	(4)	−	−
	36	−	(11)	+	++++
	37	−	(11)	−	−
	38	−	(4)	−	−
10^{-5}	41	−	(11)	−	±
	42	−	(11)	+	++++
	43	+	(11)	−	−
	44	+	(4−D)	+	Not examined
	45	+	(11)	−	−
	46	−	(4)	−	−
	47	+	(4)	+	++++
	48	+	(4)	+	++++

* Number in parenthesis denotes days after inoculation when mouse died or was sacrificed.

‡ −D = died spontaneously.

Groups of six to eight mice were inoculated respectively by the intracerebral (I.C.), intramuscular (I.M.), intravenous (I.V.), and subcutaneous (S.C.) routes with a muscle and bone preparation of infected suckling mice. The concentration and quantity of inocula were as follows:—

Route of inoculation	Dilution of material	Quantity of inoculum
		cc.
I.V.	10^{-2}	0.25–0.5
I.C.	10^{-1}	0.03
I.M.	10^{-1}	0.1
S.C.	10^{-1}	0.5

All mice were weighed as usual and observed for signs of illness or death. At least one-half of the mice in each group were sacrificed at the end of 2 weeks and gross and microscopic observations of tissues were made.

The results of this experiment can be briefly stated. Infection, as indicated by weight loss and pancreatic lesions, occurred in each group. However, of the seven mice receiving intracerebral injections, only two showed weight loss, and of four examined only these two had pancreatic lesions.

The pathologic changes in all mice examined were restricted to the pancreas. This demonstrated convincingly that in adult mice this organ alone is severely affected by the Conn.-5 strain, whether infection take place by way of the blood stream or by direct contact of the virus with the pancreatic tissue.

It is of particular interest, in view of the emphasis which has been placed on the myositis in suckling mice, that in the adult animals injected intramuscularly, there were no lesions of the skeletal muscles at the site of inoculation or elsewhere.

Propagation of Conn.-5 Virus in Serial Passage in Adult Mice

That the virus can be successfully passed in adult mice by inoculation of suspensions of pancreatic tissue, is shown in the following experiment.

The pancreas was removed from adult mice 4 days after intraperitoneal injection of virus harvested from infected sucklings. A 10 per cent suspension was prepared in buffered saline to each cc. of which 500 units and 500 μg. of penicillin and streptomycin had been added. After centrifugation at 1800 R.P.M. for 10 minutes, the supernatant fluid was inoculated in 0.1 cc. amounts into six 7 week old mice. Within 4 days, four of the six mice had suffered loss of weight and showed the usual signs of illness. The mice were sacrificed at this time, the pancreas removed, and the suspensions prepared for passage in the same manner. The virus has been maintained through five passages thus far in sexually mature mice as shown in Table III. Since weight loss and gross pathology have proven to be reliable criteria of pancreatic disease, these were taken as evidence of virus activity. The histologic examination of selected animals confirmed the gross findings.

It is apparent that the virus has been maintained in adult mice through at least five passages by injection of pancreatic suspensions. The virus retains its

pathogenicity for sucklings after repeated passage in adults. Pancreatic suspension obtained at the fourth passage was injected into five litters of 0 to 1 day old mice, in various tenfold dilutions. It proved to be infectious in dilution of 10^{-6}. The pathology in these mice was typical of the disease as it appears in sucklings.

We have not attempted to assay the virus content of various organs and tissues as has been done for suckling mice by Melnick and his associates (8). The time of maximal multiplication, therefore, has not been determined, and the 4 day interval used in these experiments was chosen merely because at this time necrosis of pancreas was most extreme.

That the virus does not persist in the atrophied pancreas found in later stages of the disease is suggested by the following observation:—In the first experiment reported in this paper, one mouse was found dead 7 days after the sixth inoculation of infected muscle and bone preparation, which was 25 days after the initial injection of this material. A suspension of the viscera, brain, spinal cord,

TABLE III

Propagation of Conn.-5 Virus in Adult Mice

Passage No.....	1	2	3	4	5
	7/8*	6/6	4/6	18/19	8/9

* Numerator indicates number of mice with pancreatic disease as judged from gross pathology and weight loss; denominator indicates number of mice injected.

and skeletal muscle was prepared and inoculated into nine 0 to 1 day old mice and eight 3 week old mice by the intraperitoneal route. None of the mice showed any signs of illness during the period of observation which lasted longer than a month.

Identification of Conn.-5 Virus as the Agent of Pancreatic Disease in Adult Mice

Evidence that the agent which in these experiments causes pancreatic disease in adult mice is identical with the Conn.-5 virus has been obtained by serologic studies.

Equal volumes of 1/2000 dilution of muscle and bone suspension of infected suckling mice were mixed with 1/5 dilution of the following sera:—Conn.-5 immune, Ohio-R immune, and a pool of sera from animals which had been immunized with six inoculations of normal muscle-bone suspensions. After incubation at room temperature for 1 hour, 0.1 cc. of each mixture corresponding to about *thirty* infectious doses, was inoculated intraperitoneally into groups of 6½ week old mice.

Although the number of mice used in this experiment is limited, the results are clear cut and indicate complete protection in adults with specific immune serum (Table IV). Parallel testing of aliquots of these serum-virus mixtures in

suckling mice showed that the 1/10 dilution of the Conn.-5 antiserum protected against 10.000 ID_{50} of the virus. The same dilution of normal serum gave no protection.

TABLE IV

Neutralization of Pancreatic Disease by Conn.-5 Antiserum

Serum-virus mixture	Adult mice	Suckling mice
Virus + Conn.-5 serum	0/6*	1/8
Virus + Ohio-R serum	5/6	N.T.
Virus + normal serum	5/6	7/7

N.T. = not tested.

* Numerator indicates number of mice with pancreatic disease as judged from gross pathology and weight loss; denominator indicates number of mice injected.

In a second experiment, it was shown that Conn.-5 virus could be neutralized at several passage levels by serum collected from mice which had survived the pancreatic disease induced by Conn.-5 virus.

The serum, harvested 10 days after inoculation of adult mice with a suspension of pancreas in the fourth adult mouse passage series, neutralized in 0 to 1 day old mice the following virus preparations:—

(*a*) 17th passage in baby mice, received directly from Dr. J. L. Melnick

(*b*) Our own 3rd passage virus maintained exclusively in baby mice

(*c*) Virus obtained at the 4th passage in adult mice.

The virus-serum mixtures were incubated at room temperature for 30 to 60 minutes before inoculation and consisted of equal volumes of 1/5 dilution of serum and virus containing approximately 100 LD_{50} for suckling mice. Similar mixtures containing, in place of the immune serum, antiserum prepared against normal muscle-bone suspension, failed to neutralize the virus.

The protective capacity against the Conn.-5 virus of this serum obtained from mice surviving a single injection strongly supports the claim that pancreatic disease in these experiments has been produced by the Conn.-5 strain of Coxsackie virus rather than by an unknown agent or factor other than the virus in question.

Further evidence that pancreatic disease in adult mice is caused by the Conn.-5 virus and not by some extraneous agent accidentally introduced, is shown by the following experiment.

Six female mice, aged 6½ weeks, were inoculated with a suspension of Conn.-5 virus obtained from material received directly from Dr. J. L. Melnick in February, 1950, as seventeenth mouse passage. It had been stored in the CO_2 cabinet for a year, without further passage in this laboratory.

7 days after inoculation of this material, five of the mice had lost an average of 4.4 gm. in weight, and gross pathology of the pancreas was obvious when the mice were sacrificed.

This experiment excludes the possibility that the pancreatic disease was due to a second viral agent present as a contaminant in the material used for inoculations.

Susceptibility to Pancreatic Disease in Relation to Age

It has been our experience, in working with the Powers' strain of Coxsackie virus (9), that pancreatitis is produced in suckling mice only if inoculations are made during the first few days of life. This is true also of the Conn.-5 strain as shown in Table V. After the 4th day, pancreatic lesions were no longer regularly produced. Similarly, Melnick and Godman state that "Hepatitis and pancreatitis were found only when newborn (1 day old) mice were inoculated with [Conn.-5] virus." (8).

TABLE V

Incidence of Pancreatic Disease in Newborn Mice

Powers' virus						
Age when inoculated	0	1	2	3	4	5
No. of mice	29	6	6	29	12	7
No. with pancreatitis	28	5	6	7	2	0
Percentage	93	83	100	24	15	0
Conn.-5 virus						
No. of mice	29	5	7	20	13	12
No. with pancreatitis	27	4	7	4	2	0
Percentage	93	80	100	20	15	0

In a preliminary experiment, we have attempted to define the age susceptibility more precisely, by inoculating groups of eight or ten mice at 0, 7, 15, 22, 28, 35, and 42 days of age. The results of this experiment, based on the gross criteria—spontaneous death, weight loss, and macroscopic lesions of the pancreas—are interesting, and indicate the desirability of further study (Chart 2).

It is obvious that there is a period of relative resistance during the early nursing period, beginning a few days after birth and extending to at least the 15th day. At the end of lactation, the mice again become highly susceptible and remain so at least until they have reached the age of 8 or 9 weeks and are fully mature. The unusual number of deaths in the 22 day old group may have been due to the concomitant occurrence of severe diffuse hepatitis which was found in the three animals examined histologically.

Weight Gain after Administration of Predigested Food

It appeared obvious that the signs of illness observed in mice which had received infectious inocula were due to the pathologic condition of the pancreas and that when death occurred it was probably a result of pancreatic insufficiency. Thus, most of the mice lost considerable weight during the course of the

disease, autogenous fat appeared to diminish, and in some cases edema developed which was associated apparently with a low protein content of the blood plasma. Mice which survived the disease process exhibited hypertrophy of pancreatic acinar cells, probably compensatory in nature which produced sufficient enzymatic secretion to sustain life. It will be recalled, also, that in long term experiments, there was a grouping of deaths occurring about 3 weeks after infection was initiated—probably due to malnutrition secondary to pancreatic disease.

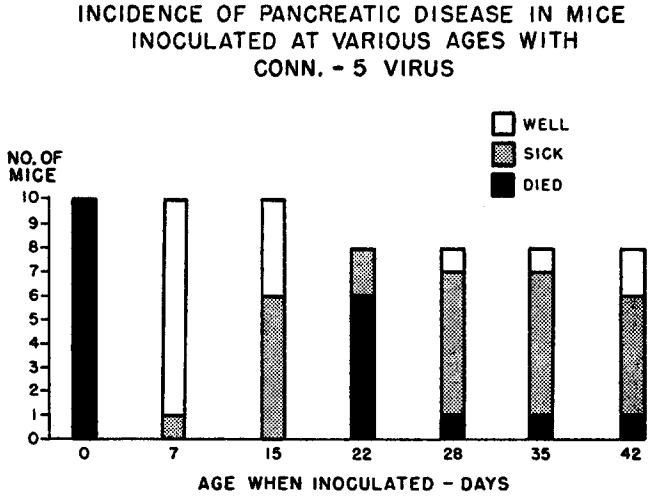


CHART 2

A preliminary experiment dealing with the nutrition of normal mice and mice suffering from pancreatic disease supports this concept.

The regular diet of the mice, Purina fox checkers, was digested over a period of 2 weeks with minced hog pancreas. After removal of most of the water the digested material was mixed with Difco yeast extract to give a final concentration of 1 per cent of the latter. The tough viscous mixture was then formed into balls. Small balls were prepared in the same way from powdered, undigested fox checkers with 1 per cent yeast extract supplement.[3]

Groups of four infected adult mice and of two normal mice of the same age were placed on these two diets, and another group on the regular whole fox checker regime. The infected mice had been inoculated 7 days previously and had already lost weight when the nutrition experiment was begun. The regular diet was withheld from all mice in the experiment for 18 hours before the controlled diets were fed. The mice were allowed to eat *ad lib.* and the food was replenished when necessary. The record of the weights of the mice measured at intervals during the experiment is presented in Table VI. It must be stated that the pellets of digested food did not attract the mice as well as the regular diet probably because of the consistence of the material, and it will be noted that the normal mice did not maintain the expected weight gain during the experiment.

[3] This experiment was suggested by Dr. H. E. Umbarger of this department and the diets were prepared by him.

A comparison of the weights of the various groups of animals, although very few mice were used, strongly suggests that the administration of previously digested food compensates for the loss of the digestive enzymes, indicating that the signs of illness observed in adult animals inoculated with Conn.-5 virus are a result of insufficiency of the exocrine pancreatic secretions. As

TABLE VI

The Effect of Diet on the Weights of Mice Inoculated with Conn.-5 Virus

	Diet	Original weight	Weight change			
			Before diet started	Interval after diet started		Total weight change
				5 days	8 days	
		gm.	gm.	gm.	gm.	gm.
Infected mice	Regular	21.1	−1.9	−2.9	−3.9	−5.8
		22.4	−3.2	−2.4	−4.3	−7.5
		19.8	−3.2	−2.6	−3.1	−6.3
		19.4	−1.9	−2.8	−4.5	−6.4
	Regular + yeast extract	20.7	−2.7	−1.5	−2.5	−5.2
		20.9	−1.5	+0.3	−1.5	−3.0
		20.0	−4.0	−1.3	−2.8	−6.8
		21.6	+0.4	+1.0	−0.2	+0.2
	Digested food + yeast extract	21.7	−3.1	+2.6	+3.5	+0.4
		20.0	−3.7	+1.0	+2.7	−1.0
		24.0	−3.7	+1.2	+2.1	−1.6
		19.4	−2.3	+2.1	+2.9	+0.6
Normal mice	Regular	26.5	+0.9	+0.7	+0.5	+1.4
		25.5	+0.3	+1.8	+0.8	+1.1
	Regular + yeast extract	25.8	0.0	+1.0	+1.1	+1.1
		24.2	+1.6	+13.0*		—
	Digested food + yeast extract	24.5	+0.6	−0.7	+0.4	+1.0
		24.5	+1.5	−2.0	−1.1	+0.4

* This mouse was pregnant and delivered a litter of mice 2 days after this weighing was made.

might have been expected, gross pancreatic lesions were found in all groups of inoculated mice.

DISCUSSION

It has been shown that the Conn.-5 strain of Coxsackie virus, by whatever route inoculated, regularly produces in sexually mature mice, severe changes in the pancreas. These begin as massive necrosis of acinar tissue, usually affecting the gland in its entirety, but sometimes sparing individual acini, or even small lobules. The necrosis reaches its apogee about the 4th day after inoculation, and

a fair proportion of the mice succumb at about this time; but the majority survive the initial damage, and in these, one can trace the further evolution of the disease.

The necrotic tissue is rapidly resorbed, and this process is attended by a violent cellular reaction, in which large mononuclear histiocytes constitute the dominant component. Fragments of necrotic tissue or inspissated secretion may become surrounded by giant cells, or be ingested by macrophages. The resorption of the necrotic material proceeds apace, and at 10 days, there remain only large and small ducts and islands of Langerhans, which because of the destruction of the acinar tissue, become concentrated. Histiocytic infiltration of the original lobules and septa is still present, but the cells are becoming massed about the ducts and islands, and some are taking on the character of lipoblasts. There is not much fibroblastic growth.

In the terminal or chronic phase, the histiocytic reaction fades out. The pancreatic tissue is represented only by ducts and islands, separated, often widely, by mature adipose tissue, or merely by collapsed stroma. Probably the lipoblasts develop into mature fat cells. In those cases in which destruction of the acini has not been universal, those which have been spared undergo striking hypertrophy. At no stage have we found evidence of active regeneration of acinar tissue.

This, in brief is the sequence of changes which end in more or less complete loss of acinar tissue, and which bring about in the affected mice a state of chronic pancreatic insufficiency. Since the islands of Langerhans resist destruction, one may surmise that diabetes does not ensue, but no blood sugar determinations have as yet been made. Death may occur at any time, but some animals may survive for a long period despite the great weight loss and obvious nutritional deficiency. Still others, and they are in the minority, maintain a state of relative well-being, presumably because sufficient pancreatic tissue has escaped destruction to cover their requirements.

There is an interesting feature in the histopathology, which deserves a few words of comment. This is the accumulation of acid-fast, fluorescent brown pigment within histiocytes about the degenerating pancreatic tissue, and less abundantly, in the spleen and intestinal lymphoid tissue. A considerable body of evidence is at hand linking the formation of "ceroid" pigment in the tissues with a state of vitamin E deficiency (10–13). That the lack of external pancreatic secretion should result in failure of absorption of fat-soluble vitamins is to be expected. The presence of active spermatogenesis in these mice does not negate the possibility that they are suffering from vitamin E deficiency, since the mouse, unlike the rat, does not undergo testicular atrophy as a result of tocopherol lack (14, 15). Nor does muscular dystrophy occur in adult mice, except after prolonged deficiency (16). We have found no anatomical evidence of vitamin A deficiency, in the form of metaplastic epithelial changes in bronchi, glandular ducts, or renal pelvis.

The physiologic and biochemical changes resulting from the state of chronic pancreatic insufficiency demand further study.

From these observations, several considerations of general interest merit discussion.

Adult, sexually mature mice are not, as has been generally assumed, completely resistant to infection with this strain of Coxsackie virus. On the contrary, they react in a highly specific way by the development of acute and chronic lesions of the pancreas. With the exception of the liver, in which the lesions are transient and probably contribute but in small part to the general disease picture, the pancreas alone is susceptible. The intra-abdominal fat necroses are secondary to the pancreatic necrosis. The emaciation, and the anasarca which occurs in some animals, are likewise not direct results of the viral infection, but referable to the pancreatic insufficiency.

It has been demonstrated that the virus multiplies in the pancreatic tissue, and that the infection can be passed from one adult mouse to another by injection of suspensions of the infected organ.

The selective localization of the lesions in the pancreas is in surprising contrast to the widespread distribution of lesions in suckling mice. In these, central nervous system, skeletal muscles, heart, lungs, and peripheral fat lobules are often affected. There is a period during lactation in which it is difficult or impossible to produce pancreatic lesions. The precise limits of this resistant phase have not been determined but mice inoculated at 7 days have rarely developed the disease. At 15 days, however, pancreatitis again appeared and at the end of the nursing period—22 days—the mice were found to be highly susceptible.

The reason for this interesting transient immunity is not apparent, and will have to be elucidated by further experiments.

Of more general interest, perhaps, is the fact that virus infection may eventuate in complete atrophy of an organ, or more accurately stated, in complete disappearance of its glandular component. Surely no one, seeing only the end results of the process—more or less complete absence of the acini, with fat replacement, and lack of all inflammatory reaction—would be inclined to ascribe the condition to previous viral infection.

It is pertinent here to recall that exactly similar changes—complete acinar atrophy with lipomatous replacement—have been described in children. We are referring here, not to the familiar cases of pancreatic fibrosis, usually regarded as being due to obstruction of ducts by inspissated secretion, but to the rare cases of complete fat replacement described by Høyer (17) and previously by Rössle (18), Gross (19), and Hantelman (20). Speculation as to the possible cause of such a condition has not included virus infection. But in the light of our observations, such an etiologic factor may well be suspected. In 1913, Apolant (21), working with various chemotherapeutic agents in Ehrlich's laboratory, found in three adult mice which had been injected with different sub-

stances, pancreatic atrophy with fat replacement. The drawing which illustrates his paper might have been made from our preparations.

In infant mice pancreatitis has been produced by a number of serologically unrelated strains of Coxsackie virus (7). Whether strains other than the Conn.-5 are capable of eliciting pancreatic disease in adult mice, remains to be investigated. It would also be interesting to know whether freshly isolated strains are pathogenic for adult mice or whether this property has been acquired by repeated mouse passage.

CONCLUSIONS

1. With Conn.-5 strain of Coxsackie virus, pancreatic disease can be regularly produced in adult mice.

2. The lesions consist of widespread necrosis, followed by repair; there occurs more or less complete loss of glandular acini, with fatty or fibrous replacement. The islands of Langerhans and pancreatic ducts persist.

3. Injection of virus suspensions by the intraperitoneal, subcutaneous, intramuscular, or intracerebral route is followed by selective necrosis of the pancreas.

4. The liver, in the earlier stages of the disease, is the seat of fat infiltration. There may be necrosis of individual hepatic cells, but the diffuse hepatitis described in suckling mice does not occur. In the later stages of the disease, the liver is not significantly altered.

5. Localized areas of fat necrosis, scattered through intra-abdominal adipose tissue, are usually present in the acute phase of the disease. These undergo fibrosis without calcification.

6. No lesions have been found in the skeletal muscle, even at the site of intramuscular injection. Central nervous system, heart, lungs, and peripheral fat lobules show no lesions comparable to those described in suckling mice.

7. Multiplication of virus takes place in the pancreas. Serial passage in adult mice, by injection of pancreas suspensions prepared from organs removed on the 4th day after infection, is readily accomplished. Five consecutive passages in adult mice have thus far been carried out. Pancreas suspension from 4th passage material produced typical disease in suckling mice when diluted 10^{-6}. No virus could be demonstrated in pancreas obtained 25 days after inoculation.

8. Complete protection against the pancreatic disease is obtained when the virus is neutralized, before injection, with Conn.-5 antiserum. Normal mouse serum and antiserum against the Ohio-R strain of Coxsackie virus have no protective effect.

9. Mice surviving the initial necrotizing effect of the virus, develop chronic pancreatic insufficiency. This is manifested by extreme weight loss—in some cases, 40 per cent or more of the body weight—and by hypoproteinemia, sometimes leading to anasarca.

10. The substitution of fox-chow which has been predigested with hog pancreas brings about a restoration of weight.

11. The possibility is considered that similar lesions of the pancreas in human beings may be due to virus infection.

BIBLIOGRAPHY

1. Dalldorf, G., *Science*, 1949, **110,** 594.
2. Dalldorf, G., *Bull. New York Acad. Med.*, 1950, **26,** 329.
3. Melnick, J. L., *Bull. New York Acad. Med.*, 1950, **26,** 342.
4. Melnick, J. L., Shaw, E. W., and Curnen, E. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71,** 344.

4*a*. Curnen, E. C., *Bull. New York Acad. Med.*, 1950, **26,** 335.

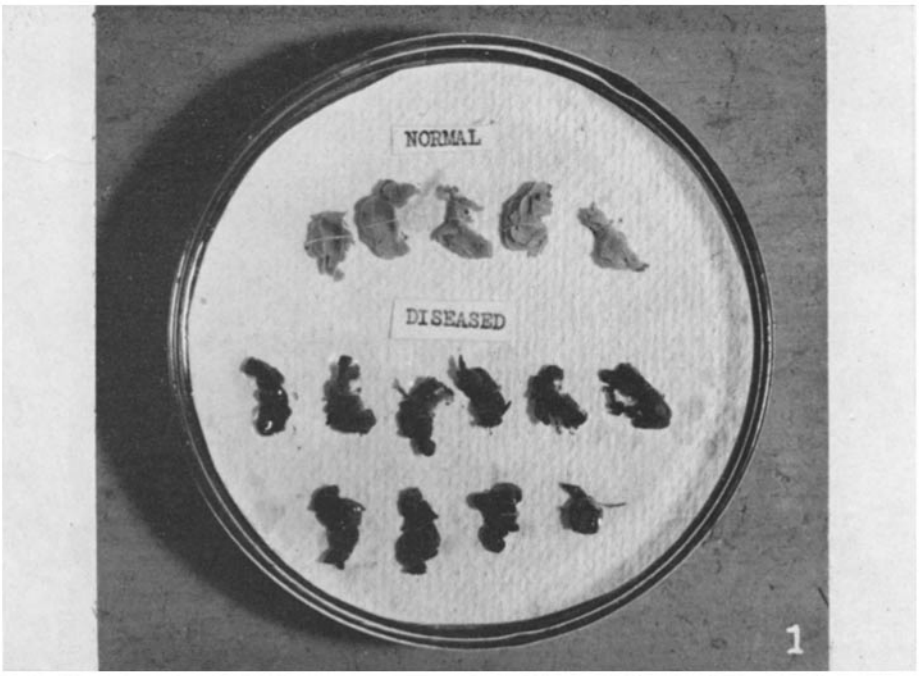
5. Melnick, J. L., and Ledinko, N., *J. Exp. Med.*, 1950, **92,** 463.
6. Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.* 1926, **69,** 625.
7. Pappenheimer, A. M., Daniels, J., Cheever, F. S., and Weller, T. H., *J. Exp. Med.*, 1950, **92,** 169.
8. Melnick, J. L., and Godman, G. C., *J. Exp. Med.*, 1951, **93,** 247.
9. Cheever, F. S., Daniels, J. B., and Hersey, E. F., *J. Exp. Med.*, 1950, **92,** 153.
10. Pappenheimer, A. M., and Victor, J., *Am. J. Path.*, 1946, **22,** 395.
11. Victor, J., and Pappenheimer, A. M., *J. Exp. Med.*, 1945, **82,** 375.
12. Mason, K. E., and Emmel, A. F., *Anat. Rec.*, 1945, **92,** 33.
13. Vitamin E Symposium, *Ann. New York Acad. Sc.*, 1949, **52,** 63.
14. Bryan, N. K., and Mason, K. E., *Am. J. Physiol.*, 1940, **131,** 263.
15. Pappenheimer, A. M., *Am. J. Path.*, 1940, **18,** 169.
16. Menschick, *et al.*, *Ann. New York Acad. Sc.*, 1949, **52,** 94.
17. Høyer, A., *J. Path. and Bact.*, 1949, **61,** 93.
18. Rössle, R., *Beitr. path. Anat. u. allg. Path.*, 1921, **69,** 163.
19. Gross, F., *Jahrb. Kinderheilk.*, 1926, **112,** 3F-62.
20. Hantelman, W., *Virchows Arch. path. Anat.*, 1931, **282,** 630.
21. Apolant, H., *Virchows Arch. path. Anat.*, 1913, **212,** 189.

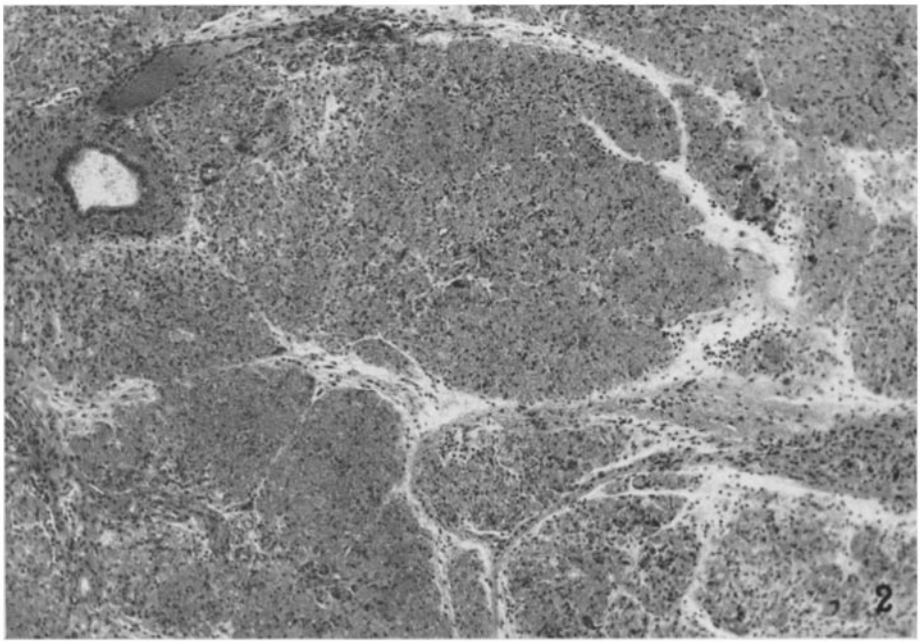
EXPLANATION OF PLATES

PLATE 13

FIG. 1. In the upper row are the pancreases from five normal adult mice. In the lower row are those of ten adult mice which had received six injections of Conn.-5 virus intraperitoneally and were sacrificed 93 days after the first injection. At this time they showed no obvious signs of illness. The organs were dissected out and, after fixation in Bouin's fluid, stained in bulk with sudan IV. Because of the extensive replacement of the parenchyma with adipose tissue, the pancreas is stained deeply red (appearing black in the photograph) in contrast to the gray color of the normal glands. One can discern remains of the original parenchyma in the form of small, circumscribed greyish nodules.

FIG. 2. Mouse 4423: one intraperitoneal injection of 0.1 cc. 5 per cent suspension of infected carcass; dead 4 days after inoculation. Massive necrosis of all pancreatic acinar tissue; early inflammatory reaction. Hematoxylin and eosin. × 89.



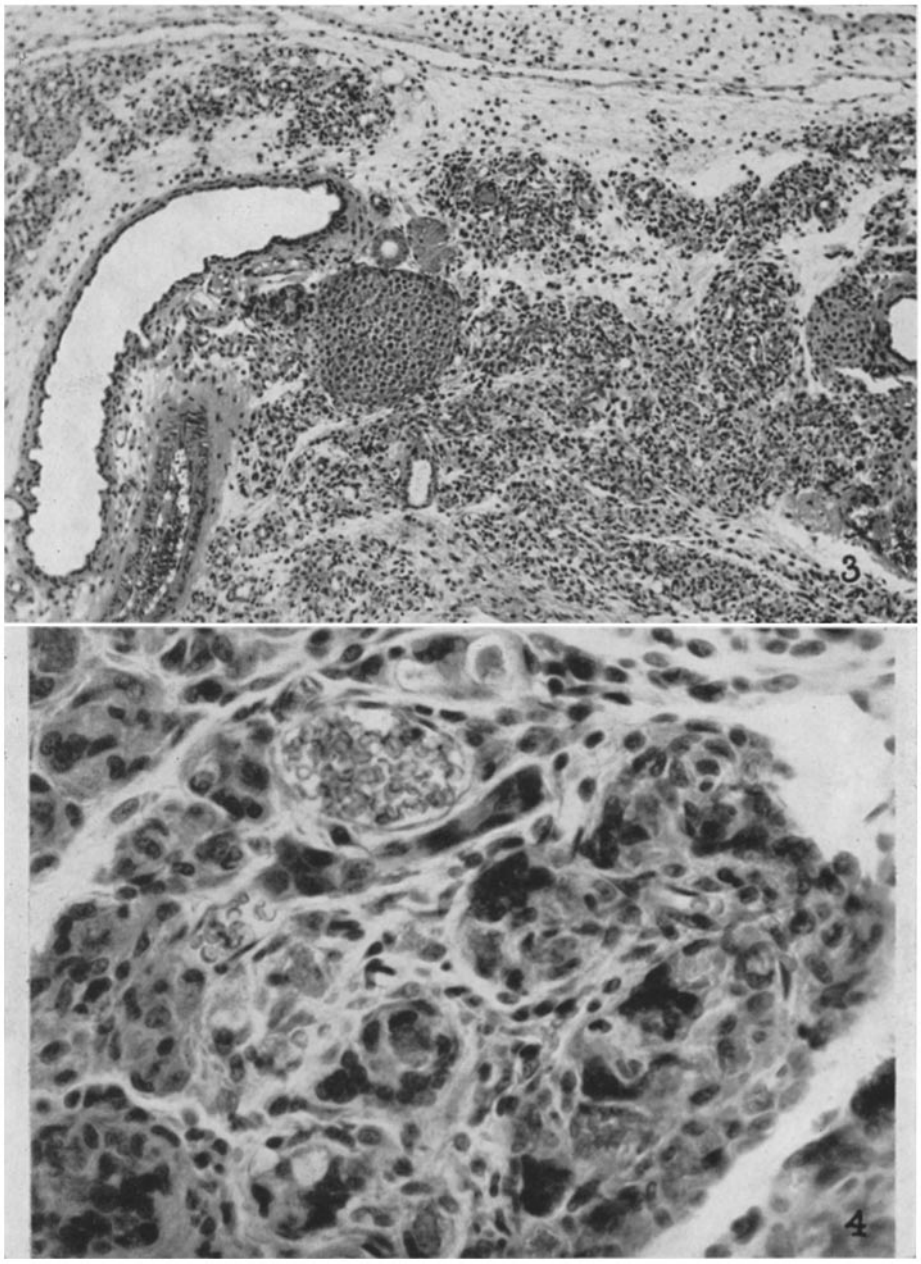


(Pappenheimer *et al.*: Passage of Coxsackie virus and pancreatic disease)

PLATE 14

FIG. 3. Mouse 4367: sacrificed 11 days after intraperitoneal injection of 10 per cent suspension of infective material. Pancreas shows almost complete resorption of necrotic material, dense infiltration of original lobules with mononuclear cells, persistence of ducts and islands of Langerhans. Hematoxylin and eosin. × 112.

FIG. 4. Mouse 4430: sacrificed 8 days after first inoculation of infective material. Necrotic cells and small masses of inspissated secretion (?) are surrounded by fibroblasts and giant cells. Hematoxylin and eosin. × 498.

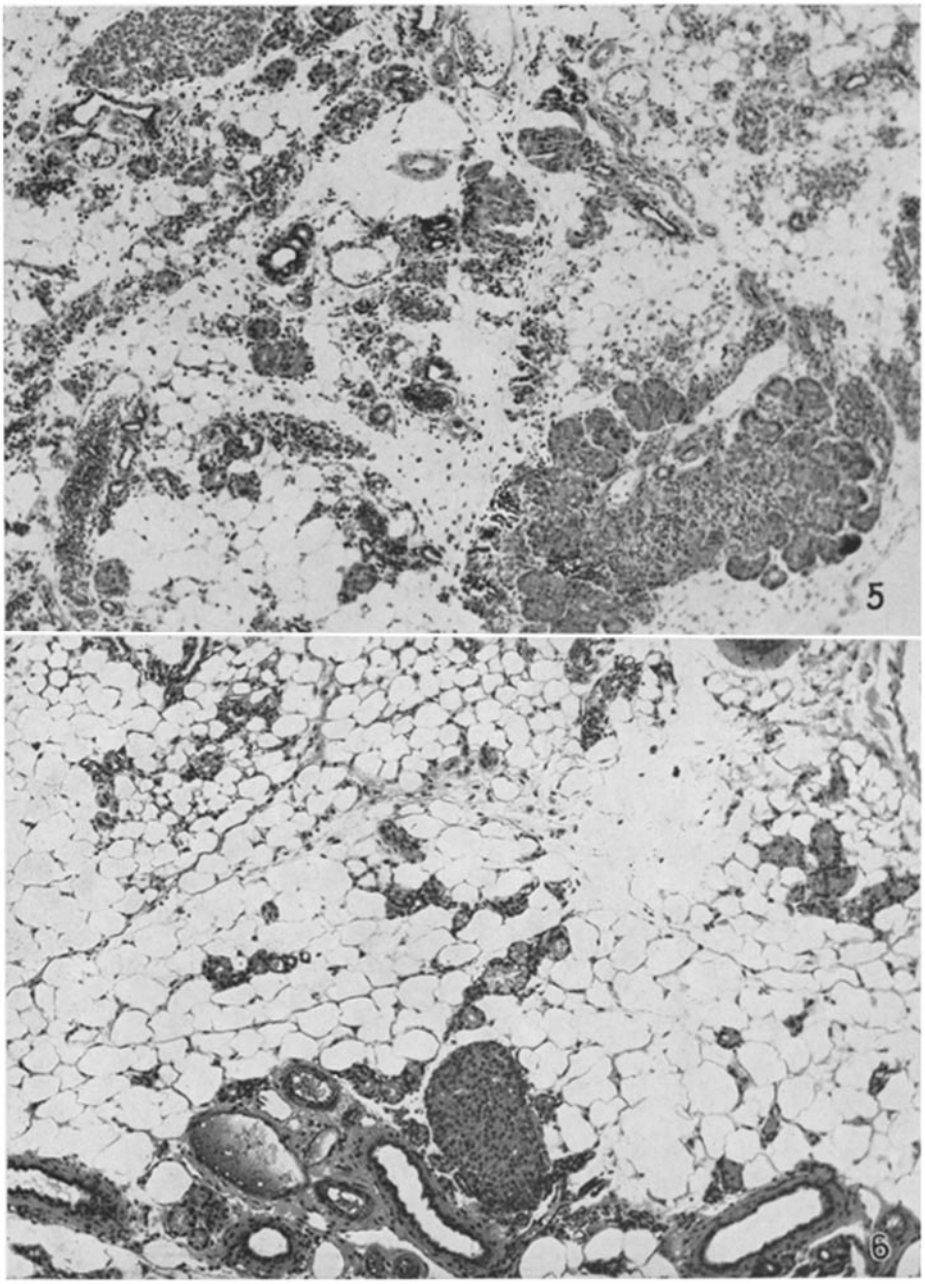


(Pappenheimer *et al.*: Passage of Coxsackie virus and pancreatic disease)

PLATE 15

FIG. 5. Mouse 4391: sacrificed 27 days after intraperitoneal injection of infective material. The greater portion of the pancreatic acinar tissue has been resorbed, but groups of hypertrophic acini still remain, as well as ducts and islands. There is fat replacement of the destroyed glandular tissue. Hematoxylin and eosin. × 114.

FIG. 6. Mouse 4431: sacrificed 40 days after intraperitoneal injection of infective material. There is virtually complete loss of acinar tissue with fat replacement; islands and ducts remain. The animal was hunched and emaciated. Hematoxylin and eosin. × 114.

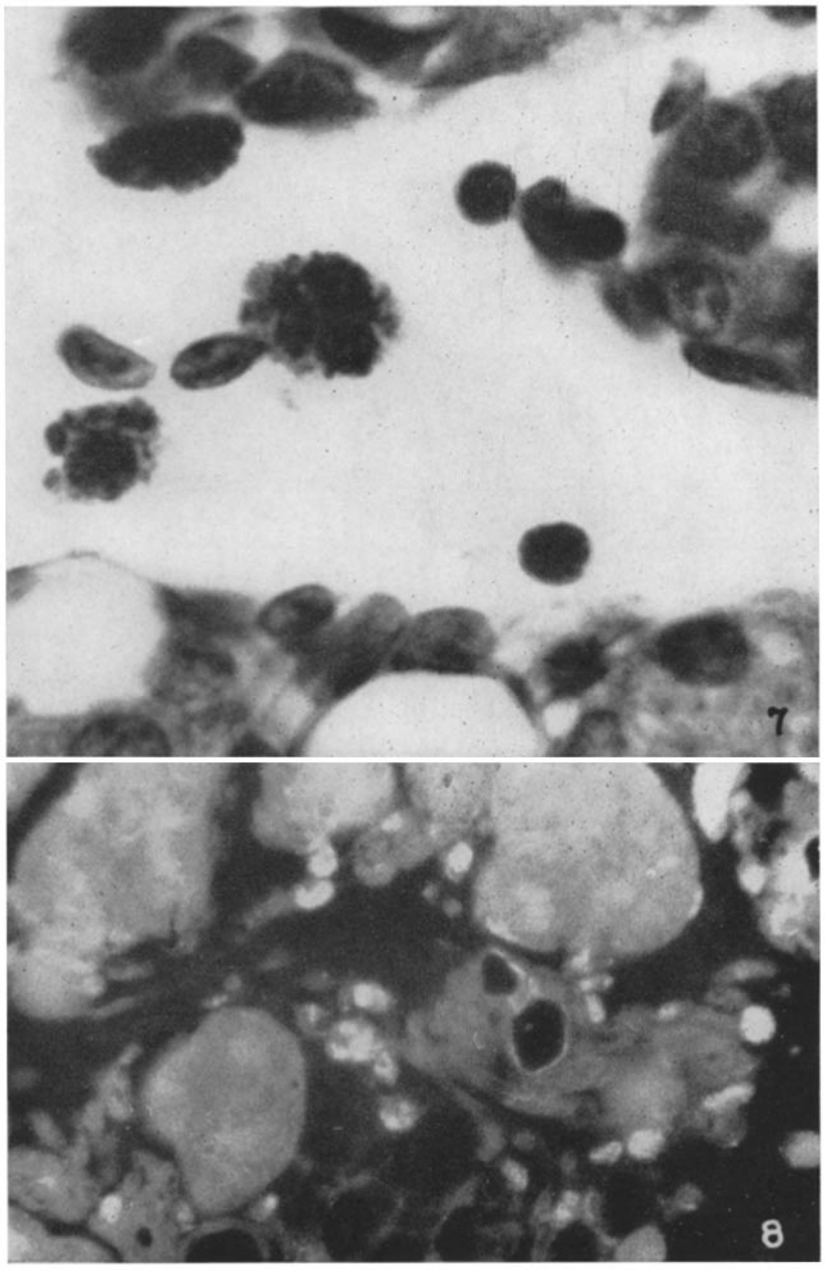


(Pappenheimer *et al.*: Passage of Coxsackie virus and pancreatic disease)

PLATE 16

FIG. 7. Mouse 4395: sacrificed 28 days after intraperitoneal injection of infective material. Histiocytes in atrophic pancreas contain clumps of acid-fast pigment (ceroid). Ziehl-Neelsen carbolfuchsin stain. × 2200.

FIG. 8. Mouse 4400: the histiocytes containing ceroid pigment are photographed with the fluorescence microscope described by Coons and Kaplan (*J. Exp. Med.*, 1950, **91,** 1). The photograph was made from a hydrated formalin-fixed paraffin section, mounted in glycerin. The pigment-containing histiocytes exhibit bright fluorescence. × 560.

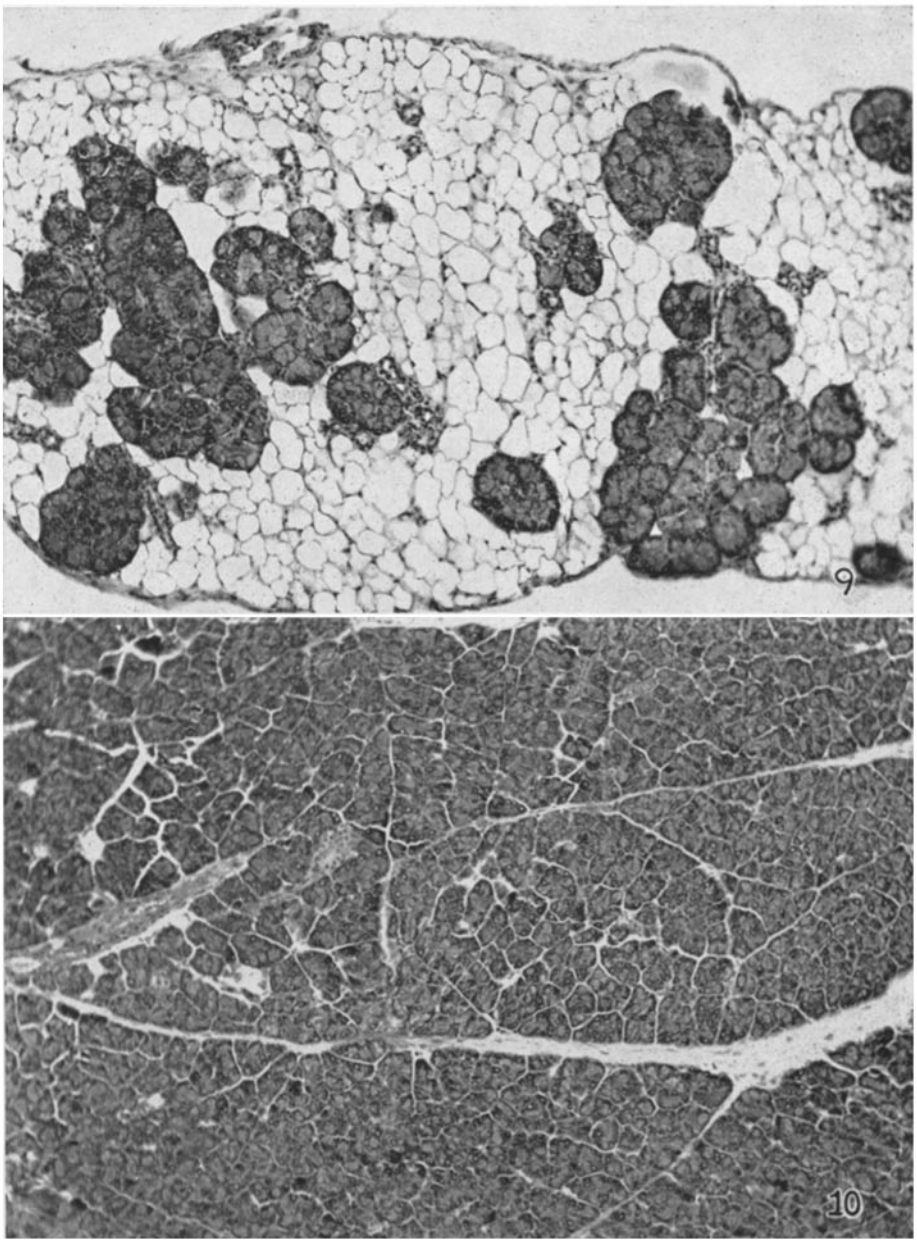


(Pappenheimer *et al.*: Passage of Coxsackie virus and pancreatic disease)

PLATE 17

FIG. 9. Mouse 4417: sacrificed 33 days after intraperitoneal injection of infective material; the animal appeared in good condition when killed. There are groups and single pancreatic acini separated by adipose tissue. The cells show marked hypertrophy as compared with those of normal gland (Fig. 10). Hematoxylin and eosin. × 97.

FIG. 10. Mouse 4410: normal adult pancreas for comparison with Fig. 9. Hematoxylin and eosin. × 97.

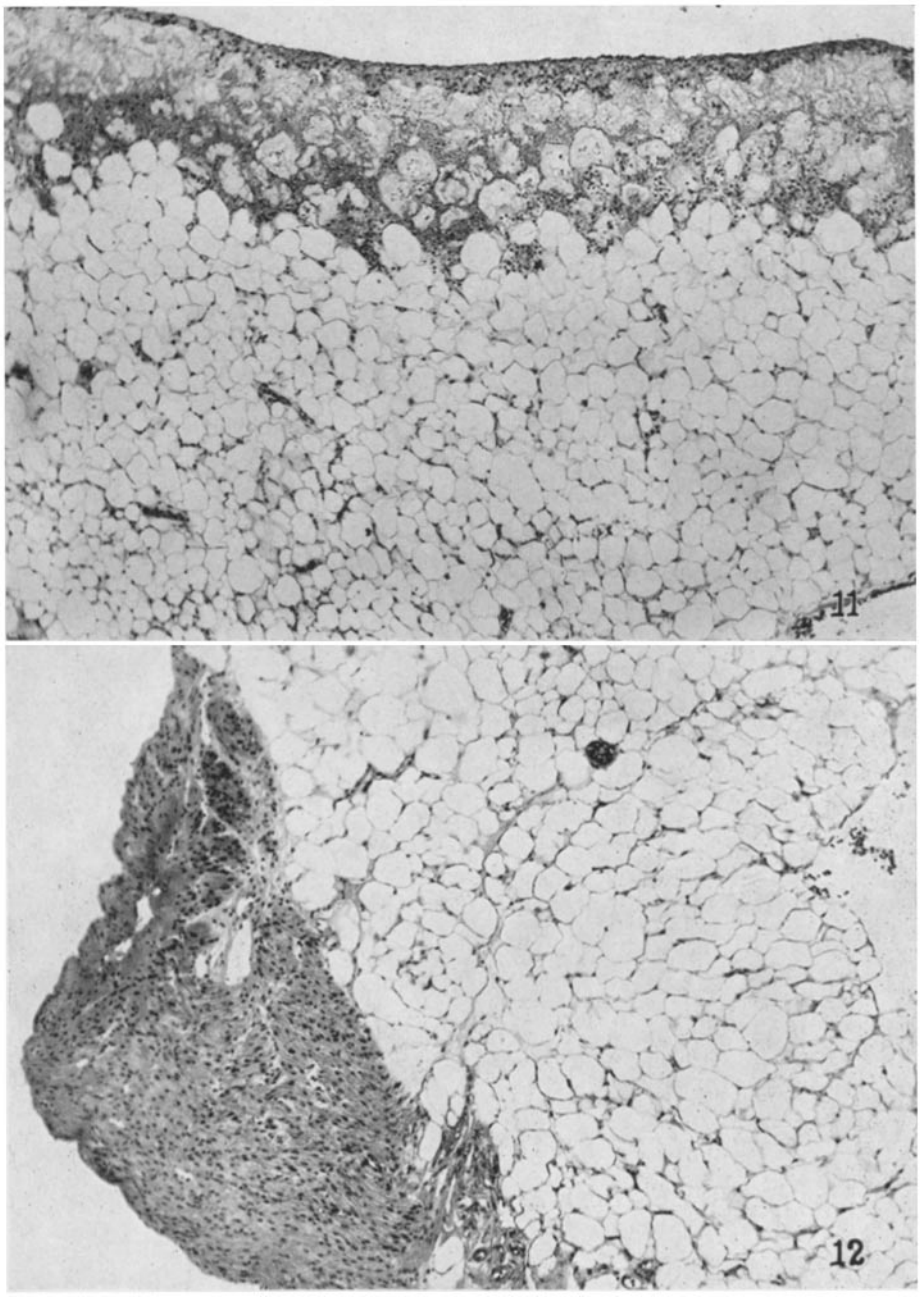


(Pappenheimer *et al.*: Passage of Coxsackie virus and pancreatic disease)

PLATE 18

FIG. 11. Mouse 4430: killed 8 days after first inoculation of infective material. Necrosis of intra-abdominal fat with inflammatory reaction. Hematoxylin and eosin. × 84.

FIG. 12. Mouse 4418: sacrificed 33 days after intraperitoneal inoculation of infective material; appeared healthy when killed; pancreas showed preservation of about 30 per cent of acinar tissue. Healed fat necrosis of intra-abdominal fat. Hematoxylin and eosin. × 82.



(Pappenheimer *et al.:* Passage of Coxsackie virus and pancreatic disease)