17OHCS(16), a finding not confirmed by the present experiments. The discrepancy may be explained by the fact that the experiments of Biglieri and Ganong were performed on acutely prepared animals and involved only a small number of animals. Increase in secretion of 17OHCS was observed after carotid constriction in only one of 16 other intact dogs prepared 24 hours before the experiment(4).

Summary. Changes in secretion of 17OHCS were measured in dogs with chokers about both carotid arteries and isolated vagus nerves, one day after operative preparation. Vagotomy stimulated secretion of 17OHCS; sham vagotomy did not. Carotid constriction stimulated secretion of 17OHCS in vagotomized, but not in intact, animals. These findings suggest that the adrenal cortical response to hypotension may be mediated in part by vagal pathways and by carotid baroceptors.

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Differences in Interferon Content in Tissues of Mice of Various Ages Infected with Coxsackie B1 Virus.* (29086)

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The work to be reported here was undertaken in an attempt to explain why the infant mouse almost universally succumbs to infection with Coxsackie viruses, whereas the older animal responds with the production of antibody but displays no symptoms of illness. Through the study of this convenient model, information might be obtained that would further our understanding of the factors that influence the pathogenesis of viral infections and their relative importance in the immature and the mature host. The Coxsackie virus model acquires additional interest since newborn infants infected with group B strains may develop a fatal infection characterized by myocarditis and encephalitis that is reminiscent of the disease in mice.

Previous investigators have tended to ascribe the severity of Coxsackie virus infection in the infant mouse to the failure of the immature animal to form antibody. The observations upon which these tentative conclusions were based include the demonstration that younger animals developed antibody at a later time after infection than did older mice(1), and that the virulence of the

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infection in adult animals could be increased by treatment with steroids(2,3), radiation(4, 5) or by stressful events, such as exposure to cold(6) or pregnancy(7). Kunin(8) has presented data indicating that infection occurred in the central nervous system of infant mice inoculated with a neurotropic strain of Coxsackie B virus whereas this did not occur in adult animals. He was able to correlate infection with the ability *in vitro* of suspensions of immature brain to adsorb the virus. The brain cells of the older animal appeared to lack the receptor sites necessary for initiation of infection.

The Coxsackie B1 virus employed in our experiments regularly produced death in suckling mice within 3 days. No antibody could be detected in the blood of older animals before 4 days after inoculation. Thus it seemed unlikely that, even if the infant animal had a normal ability to form antibody, control of viral multiplication could be achieved by this mechanism quickly enough to alter the course of events. In addition, assay for virus of the various organs of mature mice, including brain and muscle, showed that multiplication did occur in them, indicating that receptor sites for this strain of virus were present. Thus we were led to look for some explanation, other than defective antibody formation or presence of receptor sites, for the difference in response to infection of the mice of different ages.

Since the discovery of interferon by Isaacs (9) there has been much speculation concerning its role in the pathogenesis of viral infections. It has been considered possible that the production of this substance may be of primary importance in controlling viral proliferation before antibody can be elaborated. Most pertinent to the problem with which we were concerned was the observation by Isaacs and Baron(10) that *in vitro* the cells from young chick embryos were not capable of elaborating as much interferon as cells from older embryos. Furthermore, Sawicki(11) has demonstrated that interferon was produced in greater amounts in the lungs of mature mice infected with Sendai virus than in the lungs of suckling mice infected with this agent.

These findings led us to assay the tissues of mice of various ages infected with Coxsackie B1 virus for their content of interferon. The results summarized here revealed that tissues of infected suckling mice contained no more than minimal amounts of interferon in contrast to the significant quantities found in those of older animals.

Materials and methods. Mice: Albino mice from an inbred colony originally derived from the Harvard strain were used throughout this study. Mice ranging in ages from 24 hours to 8 weeks were employed.

Virus: The Coxsackie B1 virus, designated as Connecticut 5 strain was obtained from the American Type Culture Collection in 1958 and passed in our laboratory in suckling mice. A pooled 10% torso-brain suspension, which had a titer of 10^6 50% lethal doses (LD_{50}) per 0.03 ml for suckling mice was stored at —70°C and used for all experiments. The titer of the virus suspension was repeatedly checked and found to be stable.

Virus dilutions were made in sterile Hanks' balanced salt solution (BSS) with antibiotics added. Unless stated otherwise an inoculum of 100 LD_{50} per 0.03 ml for mice 24-48 hours old was used in all experiments, and injected intraperitoneally. The quantity inoculated was 0.03 ml per suckling mouse and 0.1 ml for mice 4 weeks and older.

Preparation of tissues. Mice were sacrificed with ether at appropriate time intervals. Bloods were obtained for antibody determination by the method described by Hamre *et al*(12). Liver, spleen, brain, muscle and kidneys were removed aseptically. All instruments were wiped, dipped in 70% alcohol and flamed between the removal of each organ to avoid cross contamination. The specific organs of all animals within a group were pooled. The number of animals per group varied between 6 and 12 for adult mice and from 20 to 30 for suckling mice. Twenty per cent suspensions of all organs, with the exception of spleen which was made up to 10%, were prepared by grinding the

tissue with an appropriate volume of Hanks' balanced salt solution (BSS) in a Virtis homogenizer. They were then centrifuged at 2000 rpm for 20 minutes and the supernatant assayed for content of virus and interferon. Suspensions to be tested for interferon were stored at —4°C whereas those for virus titration were stored at —20°C.

Tissue culture titrations of virus were performed in monkey kidney cells grown in Melnick's medium A containing 0.5% lactalbumin hydrolysate in Hanks' BSS and 2% calf serum. Serial decimal dilutions of tissue suspensions were made in Hanks' BSS and 0.2 ml was inoculated into each tube. Cultures were observed for one week and the highest dilution showing cytopathic effect (CPE) in one or both tubes was taken as the endpoint. Virus titers are expressed as log to the base 10 of the dilution per gram of wet tissue.

Serum antibody was determined by standard neutralization technique in tissue culture with 2-fold dilutions of serum and a virus inoculum of 100 50% tissue culture infectious doses ($TCID_{50}$) per tube.

Interferon assay. (a) Cultures: Suspensions of primary mouse cells were prepared by trypsinizing decapitated mouse embryos 16 to 19 days old and grown in 16 oz prescription bottles for one week in Melnick's medium A containing 10% calf serum. Cultures were then trypsinized and cell suspensions transferred into 4 oz prescription bottles in a concentration of 800,000 cells/1 ml. These secondary cultures were grown at 35°C for 48 hours and were ready to be used.

(b) Virus: Vesicular stomatitis virus (VSV) was obtained from Dr. W. Henle and passed in this laboratory through several cultures of secondary mouse embryo cells. Stock virus employed in these experiments was prepared from infected mouse embryo cultures. At a time when the cultures showed 2-3+ CPE, the cell sheets were scraped, pooled and spun at 1500 rpm for 10 minutes, the supernatant filled into ampules and stored at —70°C.

(c) Preparation of mouse tissue suspensions for interferon assay: Suspensions were stored at —4°C until processed. Since infectious Coxsackie B1 virus was found to interfere with vesicular stomatitis virus in mouse embryo cell cultures without producing cytopathic effect, these suspensions had to be rendered free of infectious Coxsackie B1 virus. They were dialyzed against 0.2 M pH 2 HCl-KCl buffer for 24 hours and thereafter against pH 7.2 phosphate buffer for another 24 hours. Suspensions were centrifuged at 2000 rpm for 20 minutes and a clear supernatant was thus obtained. This treatment preserves interferon and destroys the interfering ability due to infectivity of most viruses. However, since Coxsackie B1 virus retained considerable infectivity after 24 hours at pH 2, the suspensions were exposed to ultraviolet (UV) light for 20 minutes in petri dishes containing 5 ml each. When inoculated into monkey kidney cell cultures, suspensions treated in this way failed to produce cytopathic effect after one week.

UV inactivation was done on the same day as interferon titrations. Dilutions were made in Melnick's medium A, and 5 ml of the material to be assayed was introduced into each bottle. Since concentrated suspensions proved to be toxic to the cultures, the lowest dilution that could be tested was 1:2 of the original 20% or 10% tissue suspension.

Secondary mouse embryo cells, 48 hours old, were washed once with 6 ml Hanks' BSS and inoculated with the tissue suspensions, using 2 bottles per dilution. They were incubated for 24 hours at 35°C, then washed again with 6 ml of Hanks' BSS and challenged with VSV, using 100 pfu (plaque forming units) in 0.1 or 0.2 ml of inoculum delivered onto the dry cell sheet. The VSV was allowed to adsorb for 1 hour at 37°C while the bottles were rotated frequently. Cultures then were overlaid with 4 ml of plaquing medium containing equal amounts of 1.5% Noble special agar and medium 199 with 4% calf serum. Forty-eight hours later the cultures were stained with neutral red, using a 0.01% concentration; 3 hours later plaques were counted. In this assay system the presence of an interfering substance

TABLE I. Virus and Interferon Content* of Tissues from Mice 6 Weeks Old Inoculated with 300 LD_{50} Coxsackie B1 Virus.

Day after inoculation	Liver		Spleen		Brain		Muscle		Kidney	
	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon
1	neg	< .3	neg	< .6	neg	< .3	1.4	< .3	neg	< .3
2	5.4	.3	7.7	1.3	2.4	.3	3.4	< .3	5.4	.3
3	4.4	.3	3.7	2.3	1.4	< .3	2.4	2.0	2.4	< .3
4	4.4	3.0	4.7	ND†	2.4	2.0	3.4	3.0	4.4	3.0
5	3.4	3.0	3.7	3.3	neg	< .3	neg	2.0	2.4	3.0

* Virus and interferon content is expressed as $\log_{10}$ titer per gram of tissue in this and subsequent tables.

† Not done.

manifested itself in two ways: by reduction in plaque number and by reduction in plaque size. The dilution of suspension producing reduction of plaques in size or number was taken as the endpoint and interferon titers are expressed as the log of the dilution per gram of wet tissue.

Results. The data presented below are from single experiments, but are representative of several duplicate experiments.

6-week-old mice: In Table I are summarized the results of titrations for virus and interferon in the tissues of 6-week-old mice at various times after inoculation with 300 (LD_{50}) of Coxsackie B1 virus. No signs of illness were observed in any of the animals. Virus could be detected in the tissues over an interval of 5 to 7 days but the titers were not high, particularly in brain and muscle. However, sufficient virus was recovered to indicate that multiplication did occur in these animals. It will be noted that significant levels of interfering activity were found in each of the organs tested. Interferon was present by the second day after inoculation and its presence or absence roughly paralleled that of virus.

The findings in mice of the same age, but inoculated with a larger dose (30,000 LD_{50}) of virus, are shown in Table II. Virus was present in the tissues of these animals on the day after inoculation; approximately 24 hours earlier than was the case for those that received the smaller inoculum. By the second day titers of 10^6 were recorded in liver, spleen and muscle. Again, the viral content of the brain was comparatively low with a maximum titer of 10^2. It is interesting that virus did not persist in the tissues of these animals any longer than in those given an inoculum 100 times smaller. The interferon content of the tissues was somewhat higher than that observed in the previous group of animals but the difference was not striking.

TABLE II. Virus and Interferon Content of Tissues and Proportion of Animals with Serum Antibody of Mice 6 Weeks Old Inoculated with 30,000 LD_{50} Coxsackie B1 Virus.

Day after inoculation	Liver		Spleen		Brain		Muscle		Kidney		Proportion of animals with antibody*	Titer of antibody†
	V	I	V	I	V	I	V	I	V	I		
1	1.4	.7	3.7	<.6	neg	<.3	2.4	<.3	1.4	<.3	0/12*	
2	7.4	1.0	7.7	3.3	3.4	<.3	7.4	2.0	5.4	ND	0/12	
3	5.4	4.0	6.7	3.3	2.4	2.0	4.4	2.0	4.4	1.0	0/12	
4	6.4	3.0	4.7	1.3	3.4	ND	4.4	ND	4.4	2.0	9/12	$(4)^2$, $(8)^3$, $(16)^4$†
5	4.4	1.0	3.7	ND	3.4	2.0	neg	1.0	neg	.7	ND	
7	neg	<.3	1.7	<.6	1.4	.7	"	<.3	"	ND	8/12	$(4)^1$, $(8)^4$, $(16)^3$
8	"	ND	1.7	ND	neg	ND	"	ND	"	"	12/12	$(4)^4$, $(8)^1$, $(16)^2$, $(32)^3$, $(64)^1$, $(128)^1$
21											8/10	$(64)^1$, $(128)^2$, $(256)^5$

* Numerator: No. of animals with antibody titer of 1:4 or greater. Denominator: No. of animals tested.

† Exponent: No. of animals. No. in parentheses: inverse of titer.

TABLE III. Virus and Interferon Content of Tissues of Mice 24-28 Hours Inoculated with 100 LD_{50} Coxsackie B1 Virus.

Day after inoculation	Liver		Spleen		Brain		Muscle		Kidney	
	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon
2	7.4	<.3	7.7	<.6	3.4	<.3	6.4	<.3	5.4	<.3
3	7.4	.7	9.7	<.6	5.4	<.3	5.4	<.3	5.4	<.3

The correlation between presence of virus and of interferon is also apparent. Tests for neutralizing antibody to Coxsackie B1 virus in the serum revealed that antibody first appeared on the 4th day after inoculation. At this time 9 of 12 animals possessed antibody in titers of 4 to 16. By the 8th day all animals displayed antibody and the titers were somewhat higher, ranging from 4 to 128. However, it is to be noted that on the 21st day 2 of 10 animals tested had no antibody in their sera. No ready explanation can be given for this finding.

Thus, the mature, 6-week-old mice inoculated with 300 or 30,000 LD_{50} of Coxsackie B1 virus sustained an inapparent infection with multiplication of virus in various organs and concomitant appearance of interferon in the tissues. Antibody was first detectable in the blood 4 days after inoculation and by the 6th to 8th day virus was no longer demonstrable.

One- to 2-day-old mice: In contrast to the mature animal, mice inoculated with Coxsackie B1 virus during the first 2 days of life developed paralysis and died within 3 to 4 days after inoculation. The virus and interferon content of the tissues of newborn mice inoculated with 100 LD_{50} of virus is presented in Table III. By the third day after inoculation titers of 10^7 were found in the liver and spleen. Virus concentrations in the brain, muscle and kidney were 10 to one thousand-fold less. Although the virus content of the tissue suspensions from the newborn mice was higher than in the adult animals, the most striking difference was in the amount of interferon. Except for the liver, no interferon was detected in the organs of the newborns. Suspensions of liver removed on the third day after inoculation did have some interfering capacity at a dilution of 1:5. Thus, mice inoculated with Coxsackie B1 virus in the first 48 hours of life experienced a uniformly fatal disease with viral proliferation to high titer in the tissues, particularly of liver and spleen. However, little or no interferon was elaborated.

6-day-old mice: When mice were inoculated with 100 LD_{50} of Coxsackie B1 virus at 6 days of age the majority developed symptoms of encephalitis 7 to 8 days later with a mortality of 15% to 20%. As shown in Table IV viral proliferation occurred to approximately the same extent as in the younger animals. In contrast, however, low levels of interferon could be demonstrated in liver, spleen and muscle by the third day after inoculation and in the brain on the eighth day. No interferon was found in the kidney.

2-week-old mice: Approximately 10% of 2-week-old mice inoculated with the same dose of virus as 6-week-old animals (300

TABLE IV. Virus and Interferon Content in Tissues of Mice 6 Days Old Inoculated with 100 LD_{50} Coxsackie B1 Virus.

Day after inoculation	Liver		Spleen		Brain		Muscle		Kidney	
	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon
2	6.4	< .3	6.7	< .6	3.4	<.3	5.4	<.3	5.4	<.3
3	8.4	.7	7.7	1.0	3.4	ND	6.4	.7	5.4	<.3
4	6.4	2.0	5.7	1.0	5.4	<.3	6.4	.7	4.4	<.3
8	neg	< .3	3.7	< .6	6.4	.7	6.4	<.3	3.4	<.3

TABLE V. Virus and Interferon Content in Tissues from Mice 2 Weeks Old Inoculated with 300 LD_{50} Coxsackie B1 Virus.

Day after inoculation	Liver		Spleen		Brain		Muscle		Kidney	
	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon	Virus	Interferon
1	2.4	<.3	2.7	< .6	neg	< .3	1.4	< .3	2.4	< .3
2	5.4	<.3	4.7	< .6	"	< .3	6.4	< .3	2.4	< .3
3	5.4	.7	5.7	2.3	2.4	1.0	6.4	.7	4.4	.3
6	6.4	.3	6.7	1.0	3.4	1.0	5.4	1.0	3.4	1.0
7	neg	.7	4.7	ND	neg	.3	3.4	.7	neg	.3
9	neg	<.3	neg	< .6	3.4	.7	4.4	1.0	"	.3

LD_{50}) displayed some decrease in activity and roughening of the fur on either the 7th or 8th day following inoculation but none died. The progress of viral proliferation in the tissues of these animals was not significantly different from that observed in 6-week-old animals inoculated with the same dose of virus (Table V). However, in the 2-week-old mice interferon was elaborated more slowly and in lower concentration.

Discussion. The data presented indicate that the mouse 2 days of age or less was not capable of producing more than minimal amounts of interferon in response to infection with Coxsackie B1 virus whereas the adult animal was able to do so. By 6 days of age mice no longer experienced a uniformly fatal infection and produced interferon in low concentration. The 2-week-old animal resembled the mature one of 6 weeks of age except that interferon titers in the tissues were slightly lower. Thus, the production of interferon seems to be a function of age and to parallel roughly the clinical sequelae of infection. The correlation between interferon production, titer of virus in the tissues and disease is graphically presented in Fig. 1. To simplify the Figure, the data concerning interferon and virus concentration are given only for the spleen on the third day after infection. It will be recalled that small amounts of interferon were detected in the liver suspension of newborn mice, but none in any of the other organs.

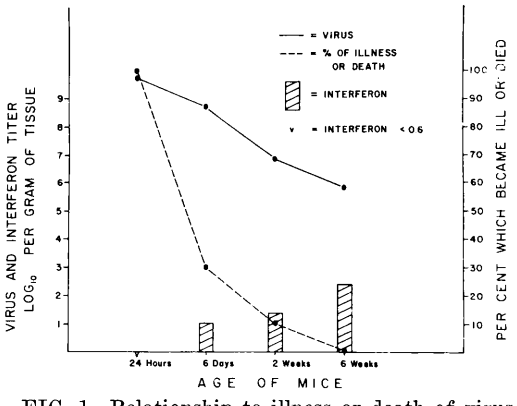

FIG. 1. Relationship to illness or death of virus and interferon titers in the spleen of mice of various ages on 3rd day after infection.

In attempting to evaluate the significance of these observations several considerations must be kept in mind. In this paper, for convenience, we have referred to the presence or absence of interferon, realizing that the actual substance responsible for the inhibition of viral growth has not been characterized fully. However, it was resistant to low pH, was not specific for the infecting virus, and was present only in infected animals. The possibility must be considered that interference was due to non-infectious virus in the tissue suspensions rather than to interferon elaborated by the infected cells of the animal. This seems unlikely since the suspensions from the newborn animals contained the highest concentration of virus, but lacked the capacity to interfere.

All determinations of virus and interferon content were done on suspensions prepared from pools of organs from a number of animals. Thus, no information is provided concerning values in individual animals and degree of variation that may occur. It would be of interest to know whether there was any correlation between interferon production and outcome of the infection in the 6-day-old mouse since some of them died, although the

majority survived. Also, virus titers must be regarded as rough approximations. The organs of 12 to 30 animals were included in a pool. Therefore, the titer of the pool would be no more than approximately 1 log less than that of the single specimen with the highest value.

That the mouse infected with Coxsackie virus during the first 2 days of life produces little or no interferon by the third day after infection does not necessarily mean that its cells are incapable of elaborating this substance, given sufficient time. It was not possible to test beyond the third day since most of the animals were dead by the fourth day. (When inocula greater than 100 LD_{50} were used suckling mice died before the third day.) Nonetheless, from our data as well as from those of Isaacs and Baron(10) for the chick embryo and those of Sawicki(11) for Sendai virus infected mice, it would appear that the newborn animal does differ markedly from the older one in the way in which interferon is released in response to infection. Whether or not this is significant in explaining the severity of certain infections in the young is yet to be determined. However, available evidence would support the hypothesis that the failure to produce interferon is one of the important handicaps under which the young individual suffers in coping with virus infections.

Summary. Mice 24 hours to 6 weeks old were infected with Coxsackie B1 virus. Their tissues were assayed for virus and interferon content. It was demonstrated that adult mice produced interferon in all the tissues which became infected, whereas suckling mice produced small amounts of interferon in their livers only. There is a direct relationship between interferon titers and rapidity with which virus disappears from the tissues. A hypothesis is offered that the difference in outcome of Coxsackie B1 infection in the suckling and older mouse is in large part to be explained by inability of the cells of the immature animal to elaborate interferon.

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Folate Activity in Tissues of Rats on Folate-Free Diets.* (29087)

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Recently we presented data on folate activity in blood and various tissues in rats kept on a standard diet(1). No detailed information is available on the effect of folate deficiency on the folate tissue stores, nor is it known to what extent blood folate activity reflects the state of the body folate stores. Increased urinary excretion of formiminoglutamic acid was found in folate-deficient rats after 20-40 days on a folate-free diet(2).

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