jections in rats treated with phenylhydrazine (10), and ESF production was inhibited in cobalt-treated rats receiving mercury-containing drugs(11). Considerable tubular necrosis was observable in these mercury-poisoned rats.

Although these investigators were concerned with determining the site of ESF formation, in the light of our studies(1-6) it is likely they were actually measuring the effects of the various erythropoietic stimuli and depressants on REF production by the kidney. The present studies indicate convincingly that the REF is distributed throughout the kidney, a conclusion based on our finding that approximately equal activity was present in the renal cortex and medulla in the 6 species of mammals examined.

The relative hypoxia that exists normally in the renal tubules(18) may serve as a mild continuous stimulus for the daily production of the REF in the normal animal. It would seem that more severe hypoxia induces augmented production of the REF as a result of greater stimulation of both the cortical and medullary sites of formation. Conversely, in situations resulting in renal hyperoxygenation, it would be anticipated that REF production in both the cortical and medullary regions of the kidney should be depressed. This point is presently under investigation.

Summary. The REF has been demonstrated in the kidneys of rats, rabbits, dogs, sheep, pigs and humans. In these 6 species, approximately equal quantities of the REF were found in the renal cortical and medullary tissues. Exposure of rats to hypoxia induced an increase in both cortical and medullary REF activity.

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Interferon Production in Neonatally Thymectomized Mice.* (32500)

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The host response to certain viruses may be altered by neonatal thymectomy. Evidence has been provided that neonatally thymectomized mice are more susceptible to coxsackievirus B5 infection(1), and in mice inoculated with the LDH agent, increased levels of serum lactate dehydrogenase as well

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as virus have been demonstrated(2). Mice subjected to thymectomy in the neonatal period also have a higher incidence of tumors when inoculated with polyoma virus(3-5). Because interferon has been shown to inhibit the replication of non-oncogenic viruses(6-8) and the development of neoplasia secondary to oncogenic viruses *in vivo*(9-11), it was of interest to determine if neonatally thymectomized mice are deficient in their ability to produce interferon.

Materials and methods. Viruses. Newcastle disease virus (NDV) [Hickman strain] grown in the allantoic cavity of 10 day old embryonated eggs was used to induce the formation of interferon. The titer of the viral preparation used in the present experiments was 2×10^9 EID_{50}/0.2 ml or 10^9 PFU/0.2 ml when titrated on a human conjunctival cell variant (clone 1-5C-4)(12). Vesicular stomatitis virus (VSV) [Indiana strain] served as the indicator virus in interferon titrations. The stock VSV was grown in a primary chick embryo cell monolayer and had a titer of 2×10^7 PFU/0.2 ml on the NCTC 2071 strain of L cells. Uniform plaque size was obtained.

Antiserum. NDV antiserum [Hickman] was prepared by 3 spaced injections of a rabbit with a total of 14.1 ml of an undiluted allantoic fluid virus preparation that had a titer of $2 \times 10^{8.8}$ EID_{50}/0.2 or 10^9 PFU/0.2 ml on clone 1-5C-4. The antiserum had a hemagglutination inhibition titer of 1:512 *vs* 4 hemagglutinating units of virus and was inactivated at 56°C for 30 minutes before use.

Animals. Pregnant female albino mice of the MF-1 specific pathogen free outbred strain(13) were the source of mice used in the present experiments (Manor North, Staatsburg, N. Y.). The pregnant mice were received in the laboratory during the last week of gestation and were kept in separate cages and given an *ad lib* diet of specific pathogen free baked biscuits (Price-Wilhoite Co., Frederick, Md.). In most cases chlordiazepoxide HCL (Librium) [60 mg/l] (Roche Laboratories, Nutley, N. J.) was added to the drinking water(2). The litters were housed separately from time of delivery to termination of the experiment.

Thymectomy. Newborn mice chosen at random from each litter were thymectomized within 24 hours after birth by a modification of the method of Sjodin(14). The mice were anesthesized by hypothermia and following the operation they were placed under a warm lamp for a minimum of 3 hours. When returned to the appropriate mother they were not handled and a "salt lick" was placed in the cage. Approximately one half of each litter was operated upon and the animals not operated upon remained with the mother during the thymectomy procedure. The animals were maintained in a quiet room and the use of Librium was discontinued within 7 days following delivery.

Animals subjected to sham operations were treated the same as the thymectomized mice except that no suction was applied to the thymus gland after the mediastinal contents were exposed. The completeness of thymectomy in all cases was assessed by serial sections of the contents of the anterior and superior mediastinum. Some partially thymectomized animals were easily identified at autopsy by gross examination.

Interferon production. All the mice in each litter were inoculated with 2×10^8 PFU NDV when 5-6 weeks of age. After inoculation, blood was obtained by open cardiac puncture under nembutal anesthesia. The blood from the control animals was pooled and the blood of the thymectomized animals collected individually. Blood was allowed to clot at 4°C for several hours and then centrifuged twice. The volume of serum obtained from the individually treated mice varied from 0.1 to 0.2 ml. Serum specimens thus obtained were stored in the frozen state (—20°) to prevent evaporation.

Several control mice were inoculated with 0.01 M phosphate buffered saline (PBS) and one completely thymectomized mouse and its litter mate controls were given an NDV preparation that had been ultracentrifuged (100,000 *g* for 2 hours) and then passed through a 100 mμ millipore filter. As a result this NDV preparation was not infective for chick embryos.

Tissue culture. The NCTC 2071 cell strain (chemically defined medium derivative of NCTC clone 929 (L), obtained through the courtesy of Dr. A. Greene at the Institute for Medical Research, Camden, N. J.) was used for the interferon titrations. The cells were grown in medium 199 with 5% heat inactivated fetal calf serum and antibiotics in 250 cc plastic flasks and 60 cc Petri dishes. (Falcon Plastic Co., Los Angeles). The dishes used for the interferon titration were seeded with 1.2×10^6 cells and incubated at 37°C (5% CO_2 atmosphere) for 12-20 hours until the monolayers were complete.

Interferon titration. Interferon titrations were performed by a modified plaque reduction technique(13). The L cell monolayers were incubated for 45-60 minutes following a VSV challenge of 80 PFU before the 1% agar in medium 199 with 1% inactivated fetal calf serum was added. After a 48 hour incubation period, monolayers were fixed with Bouin's solution (through the agar), washed, and then stained with 0.1% crystal violet. Titers were determined by interpolation by ratio when the end point fell between two dilutions.

Hematology. Some leukocyte and differential counts were performed on blood obtained by cardiac puncture after thoracotomy in nembutal anesthesized mice. Peripheral blood white and differential counts were done on the tail vein blood from unanesthesized animals. Because of the diurnal variation of peripheral leukocyte counts(15), all counts were performed between 11 A.M. and 3 P.M.

Histology. Spleen, thymus and lymph nodes were fixed in formalin solution. The slides for histologic examination were stained with hematoxylin and eosin.

Statistical analysis. The interferon titers were analyzed by a variance technique. In Table I the titers of the thymectomized and unoperated control animals in each litter are shown under the appropriate time period. Each time period formed the basis for a variance analysis. The analysis permitted calculation of the overall titer variation, the titer variation of the litters, and the variation of the titers among the thymectomized animals. The residual error quantity was taken as the variance of the difference between the titers of the treated and untreated animals from litter to litter. Analysis of variance was also done on the lymphocyte counts and the body weights of the mice. Chi-square testing was used to evaluate the incidence of cannibalism in Librium and water fed litters.

Results. Pathologic and physiologic effects of neonatal thymectomy.

Effects of librium. Cannibalism, when

TABLE I. Serum Interferon Titers in Neonatally Thymectomized and Control Mice by Litter.

Litter No.	Mouse No.	6 hr titers	Litter No.	Mouse No.	12 hr titers	Litter No.	Mouse No.	24 hr titers
1.	KOT 7	8192†	3.	TOT 1	4096†	7.	OOT 3	256†
	KOT 8	16384		TOT 2	4096			
	KOT 9	8192		TOT 3	6400		Control*	256
	mean	(10923)		mean	(4864)			
	Control*	16384		Control*	8192			
2.	KOT 17	16384	4.	ROT 6	3400	8.	MOT 16	160
	KOT 18	12300		ROT 10	2048		MOT 17	128
	mean	(14342)		mean	(2724)		mean	(144)
	Control*	8192		Control*	6000		Control*	217
			5.	ROT 5	2048	9.	C22-1	332
				Control*	2500		Control*	742
			6.	JOT 7	1700			
				JOT 9	1024			
				mean	(1362)			
				Control*	1600			

* Pooled sera from 2-4 litter mates.
† Units (reciprocal of dilution at the endpoint).

present, nearly always occurred during the first 24 hours following thymectomy and infant death was an extremely uncommon event after one week. Therefore, it was of interest to see if Librium did effectively prevent cannibalism during the first week following the thymectomy operation. Forty litters comprising 205 animals were observed. The drinking water of one half of the litters contained 60 mg/l of Librium. The percentage of survivors among the animals operated upon and returned alive to their mothers in the Librium and water fed groups was 53.84 and 42.04, respectively. The difference between these two percentages was nonsignificant. ($.2>p>.1$)

Because a significant degree of lymphocytopenia has been described in some strains of neonatally thymectomized mice(16,17) and because such lymphocytopenia has been used in evaluating thymectomy, it was necessary to see what effect Librium had on the lymphocyte counts in animals not subjected to operation. The cardiac blood lymphocyte counts were studied in Librium and water fed mice using 4 litters in each group (67 mice). The peripheral lymphocyte counts were determined in 4 litters fed Librium and 3 litters fed water (35 mice). Lymphocytopenia was not consistently demonstrated in either the peripheral or cardiac blood of Librium treated animals when the data were examined by analysis of variance.

Effects of thymectomy on lymphoid tissue. Cardiac and peripheral blood lymphocyte counts of the totally thymectomized mice were depressed in some of the litters; however a depression was not observed in all litters. The spleen weights in the thymectomized mice also were not consistently depressed, but the spleens of many of these animals contained areas of lymphocyte depletion similar to those described previously(18). The mediastinal lymph nodes in 89% of the totally thymectomized mice were either undemonstrable by serial section of the mediastinum or showed a marked depletion of lymphocytes in both the inner portions of the cortex and in most of the medulla. Similar findings were also noted on examination of lymph nodes taken from the axillae of totally thymectomized mice and, to a lesser extent, in those partially thymectomized (Fig. 1).

Debilitated mice or animals that were chronically infected as evidenced by abscess formation, (proved by gross or histologic examination), were not included in these experiments. Of the remaining mice (41 litters) the animals subjected to operation weighed, at the time of virus inoculation, on the average 1.71 g less than the litter mate controls. ($p>.01$). Six to 24 hours after the inoculation of NDV the difference in weight between the totally thymectomized and unoperated controls was 2.92 g ($.02>p>.01$). Fatal wasting disease was not observed by 5-6 weeks of age.

Variation of interferon titers in nonthymectomized litter mate mice. Eleven 6½ week old offspring from a litter that was not operated upon (9 males, 2 females) were inoculated with 2×10^7 PFU of NDV intravenously. After 7 hours, cardiac blood from 6 mice selected at random was collected in 2 pools each derived from 3 mice. The cardiac blood from the other 5 mice was processed individually. The 11 mice weighed from 27.0-33.0 g. The weight of the animals in the two pools averaged 30.3 and 32.8 g, and the average weight of the 5 mice treated individually was 30.7 g. The interferon titers of the individuals had a range of 2160-7200 units/2 ml; the 2 pools had values of 7200 and 8000 units/2ml. The average titers of the individual and pooled groups were 4032 units and 7600 units, respectively. The standard deviation of the pooled titers was 566 units; that of the individual titers was 2384 units.

These findings demonstrate little difference between the mean interferon titers of the individual and pooled groups and a small variation in the interferon titers of the two pools. Thus, the use of pooled serum specimens in the litter mate conrtols for the thymectomized mice is supported.

Interferon titers in neonatally thymectomized mice. When 5-6 weeks of age all the offspring in each litter were inoculated intravenously with 2×10^8 PFU of NDV. At the appropriate time following inoculation of NDV (6, 12, or 24 hours) cardiac puncture was

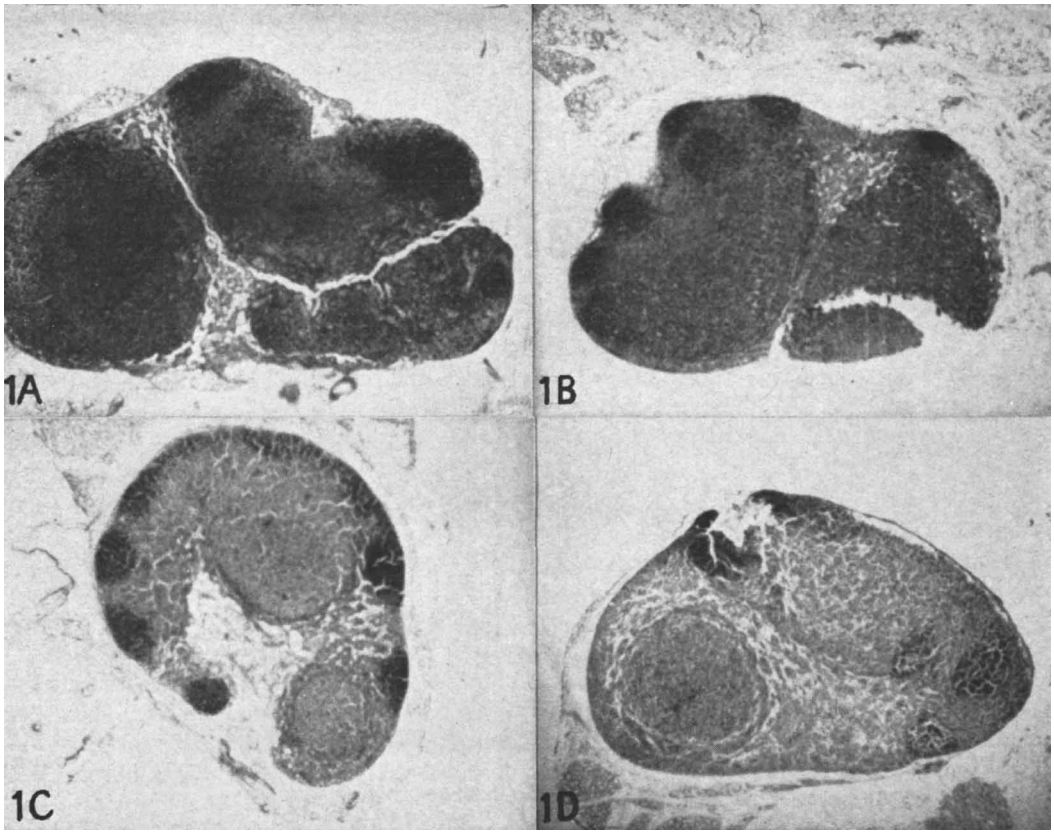

FIG. 1. Axillary lymph nodes from 5 week-old mice. (47 ×). A, C, and D are from litter mate mice. A. Lymph node from an unoperated control animal. B. Lymph node from a mouse subjected to a sham operation. C and D. Lymph nodes from partially and totally thymectomized mice, respectively, showing the marked depletion of lymphocytes in all of the medulla and in most of the cortex. On higher power, collections of large eosinophilic cells can be seen throughout the medullae of both sections.

performed. The blood of the control (unoperated) animals was pooled (2-4 mice) and that of the thymectomized mice collected individually. Following histologic confirmation of total thymectomy, the sera of the thymectomized and control animals from the appropriate litters were diluted and the interferon titers determined. In each case all the serum interferon titers for any one litter were determined using the same passage of L cells and at the same time. The interferon titers in each litter at each time interval were summarized (Table I) and variance analysis performed. No significant difference between the titers in the thymectomized and control mice at any one time period was demonstrated by analysis of variance even though the differences in mean titers at 12 and 24 hours were 1472 units and 186 units (Fig. 2), respectively. ($.1 > p > .05$ and $p > .2$).

In the variance analysis on these data a large residual or uncontrolled error term was found. This error term can be attributed to the gradual decrease in sensitivity of the L cells to interferon action (Table I, 12 hour samples) over a period covering more than 10 cell passages. An anerobic mycoplasma was identified in the L cells during this period and it is suggested that the drop in sensitivity of the cells to interferon could have been a result of this contaminant. However, the titers of interferon in each litter had been determined with the same cell passage and at the same time, so that comparison of the titers of

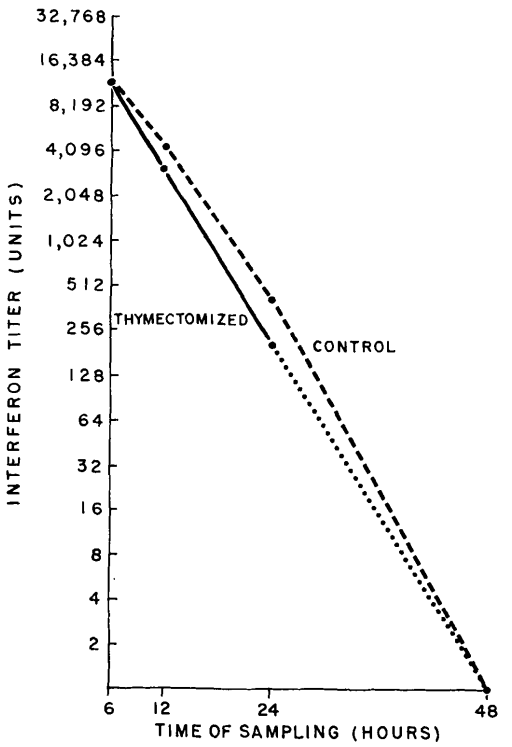

FIG. 2. Decrease of serum interferon titers with time.

the control and thymectomized mice has not been compromised.

Identification of interferon. No virus interference was produced by a 1/32 dilution of a 6 hour serum sample from a pool of 5 normal mice given PBS intravenously. Similarly, no interference could be demonstrated with a 1/64 dilution of the 6 hour serum samples from one totally thymectomized and 2 normal mice inoculated with the stock NDV after treatment of the preparation by ultracentrifugation (100,000 *g* × 2 hours) and passage through a 100 mμ millipore filter. The interfering material found in the sera of NDV-inoculated thymectomized or control animals was resistant to ultracentrifugation at 100,000 *g* × 2 hours, was inactivated by treatment with 500 μg/cc of trypsin (8-fold or greater loss of activity), was not effective in inhibiting VSV plaque formation in human foreskin cells and was not directly virucidal for VSV. The titer of the inhibitor was depressed slightly more than 2-fold by heating to 56°C for 55 minutes. Because of the necessity of analyzing the serum of individual animals, diluted specimens with a volume of one ml or less were dialyzed at pH 2.0 for 24 hours and then at pH 7.0 for 48 hours. When this small volume was used, significantly lower titers of the inhibitor in the sera from thymectomized (individual) animals were observed (as great as 5-fold). With larger volumes (4.0 cc) from the pooled control specimens, this did not occur. It was thought that NDV may have been present in the sera of totally thymectomized mice and may have induced the synthesis of interferon in the L cells during the titration procedure. Therefore, to determine if there was residual virus in the serum samples, 0.5 cc of a 1/25 dilution of rabbit NDV antiserum was added to each 2-fold dilution of the serum in a routine interferon titration. No depression of the titer of the inhibitor in the sera of either the totally thymectomized or control animals occurred. In addition, no infective or hemagglutinating NDV particles could be recovered from eggs inoculated with 1/10 dilutions of sera from totally thymectomized and control mice. The interferon titers in sham operated and control animals at 6 and 12 hours following the inoculation of NDV were not significantly different. No interferon was detected in the sera of normal animals 48 hours after virus inoculation.

The mean titers of interferon in the sera of the thymectomized and control mice are plotted against time in Fig. 2. The titers decreased exponentially with time after 6 hours. This suggests that interferon is cleared from the sera of mice according to a mass action principle.

Discussion. The ability of neonatally thymectomized MF-1 specific pathogen free mice to produce interferon in response to NDV, a virus not pathogenic for mice by the intravenous route, but known to produce high levels of serum interferon in this strain(13) was not impaired in the present experiments.

Although the blood lymphocyte counts in these neonatally thymectomized mice were not uniformly depressed, the lymphoid tissues were markedly altered. In addition to the expected histologic findings in many of the spleens, a high percentage of the mediastinal

and axillary lymph nodes from the thymectomized animals contained areas of lymphocyte depletion. Parrott *et al*(19) have emphasized that the lymphocyte depletion secondary to neonatal thymectomy occurs primarily in the cortex of lymph nodes, yet in the present experiments nearly all of the medulla as well as much of the cortex was involved. Law *et al*(18) found great variability in the changes in the lymphoid tissue in neonatally thymectomized C3Hf/Lw mice and the lesions they described in the medulla of the lymph nodes are similar to our findings.

The lack of a consistent depression of the lymphocyte count in all of the totally thymectomized mice studied in this outbred strain does not permit us to draw any conclusions as to the relationship of blood lymphocytes to NDV-induced interferon *in vivo*. However, other observations(20) have demonstrated that at most a two and one-half fold depression of the peak serum interferon titer may occur in mice given NDV intravenously 16 hours following 550 R total body irradiation. The peripheral lymphocyte counts of such mice were markedly depressed. Recently Jullien and De Maeyer(21) reported that no depression of serum interferon occurred in mice given NDV 24 hours after 1000 R total body irradiation. Nevertheless they found a marked depression of the serum interferon titers under the same conditions in mice given Sindbis virus and have suggested that NDV and Sindbis virus induce the production of interferon in two different cell populations. If a specific virus then, were found to induce selectively large amounts of interferon in the cells of the lymphoid tissues, it is conceivable that neonatal thymectomy in certain animal strains could lead to a significant depression of interferon production.

However, the evidence provided by the present limited study does suggest that a defect in interferon synthesis may not be the critical factor in the increased viral susceptibility that results from neonatal thymectomy.

Summary. No significant difference in serum interferon titers could be demonstrated in neonatally thymectomized and litter mate control mice inoculated with NDV intravenously. Although the blood lymphocyte counts of the thymectomized mice were not consistently depressed, there was other evidence that the lymphoid tissue of these animals was markedly altered.

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