INCORPORATION OF SULFATE BY THE MOUSE THYMUS: ITS RELATION TO SECRETION BY MEDULLARY EPITHELIAL CELLS AND TO THYMIC LYMPHOPOIESIS*

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If the thymus is removed from newborn mice, the rest of the lymphoid system remains permanently deficient in lymphocytes and immunological competence develops poorly (25). Transfusions of lymphocytes or thymic grafts will correct both deficiencies, but grafts enclosed in diffusion chambers that prevent cellular migration can potentiate the development of competence without correcting the deficiency of lymphocytes. Therefore two roles have been attributed to the thymus: (a) producing lymphocytes to colonize peripheral lymphoid tissues; and (b) secreting a hormone that induces lymphoid cells to acquire immunological competence.

However, these two functions may not be independent. The competence induced by thymic grafts in diffusion chambers is partial at best. Competence depends upon possession of adequate numbers of immunologically competent lymphocytes (24) and therefore depends ultimately upon lymphopoiesis. Furthermore, lymphopoiesis may play a primary role in the acquisition of competence. If competence is the result of somatic mutation among lymphocytes (33), then proliferation may be necessary to segregate mutants. If the acquisition of competence is a process of cellular differentiation, then proliferation may serve to potentiate differentiation, as it does in mammary glands (20). Therefore one can postulate that thymic lymphopoiesis serves both to produce potentially competent cells and to induce them to become competent.

As evidence for a thymic hormone, Trainin and his colleagues reported the development of immunological competence in neonatally thymectomized mice injected repeatedly with thymic extracts (31), and there are several reports that thymic extracts will stimulate lymphopoiesis, both in vivo and in vitro, although the evidence is fragmentary and conflicting (25).

There is cytological evidence to support the hypothesis that the thymus secretes a hormone (4, 6). Many medullary epithelial cells contain what appears to be a mucoid secretory product, together with the machinery to produce it. Nuclei are large, with

dispersed chromatin and prominent nucleoli, rough-surfaced endoplasmic reticulum is present, and the Golgi complex is well developed. The putative secretory product has the staining characteristics of an acidic mucopolysaccharide or glycoprotein and takes the form of mucoid vacuoles or small granules clustered in the Golgi region. Similar material accumulates in the lumens of small epithelial cysts that resemble colloid follicles in the anterior pituitary. As seen in radioautographs, the epithelial cells rapidly incorporate radioactive sulfate, glucosamine, and leucine into the contents of these mucoid vacuoles and colloid follicles. Such signs of secretory activity are most prominent in suckling mice—at the age when thymic lymphopoiesis is most active and the thymus is most necessary for the development of competence.

This paper is a description of experiments designed to test the hypothesis that thymic epithelial cells secrete a lymphopoietic hormone. The work has three aspects: (a) a cytological comparison of thymic secretory activity with lymphopoiesis under widely varying conditions; (b) chemical and kinetic characterization of the incorporation of radioactive sulfate by medullary epithelial cells; and (c) quantitative comparison of sulfate incorporation with lymphopoiesis in the thymus under conditions designed to alter lymphopoiesis.

Part of the first phase of the study has already been published (11). In those experiments, bacterial endotoxin was injected to induce thymic involution and regeneration in adult mice, and quantitative histological techniques and electron microscopy were used to compare secretory activity with lymphopoiesis. Mitotic activity among lymphocytes diminished during involution and increased to supranormal levels during regeneration, while—early in regeneration—increased numbers of medullary epithelial cells acquired nucleoli, ribosomes, rough-surfaced endoplasmic reticulum, a large Golgi complex, and clusters of unusually small mucoid vacuoles. Thus, signs of secretory activity increased at the onset of regenerative lymphopoiesis, providing circumstantial evidence in support of the hypothesis.

In the present work, suckling mice were chosen in order to study the thymus in its most active phase. Cortisol was injected to alter lymphopoiesis by inducing thymic involution and subsequent regeneration. Lymphopoiesis was evaluated radioautographically after injection of thymidine-^{3}H and the incorporation of ^{35}sulfate was measured as an indication of epithelial secretory activity.

Materials and Methods

Animals.—189 Swiss albino mice, derived from an inbred strain, bred in an air-conditioned room lighted 12 hr daily, and fed Purina Lab Chow (Ralston Purina Co., St. Louis, Mo.) and tap water ad lib., were killed for study at ages ranging from newborn to 43 days. 82 were used in experiments comparing cortisol-injected with uninjected littermate control mice. The incorporation of radioactive sulfate was examined by radioautography in 68 mice and measured quantitatively in 83. Lymphopoiesis was assessed by radioautography in 63 mice, and in 32 of these the incorporation of radioactive sulfate was measured simultaneously. However, usable data were obtained in only 27 such doubly-injected mice, varying in age from 2 to 10 days (Table I).

TABLE I

*Thymic Lymphopoiesis and Incorporation of ^{35}Sulfate in Suckling Mice
2 Hr after Injection of Both Thymidine-^{3}H and ^{35}Sulfate*

Litter	Age	Dose of ^{35}SO$_4$	Time after 0.25 mg cortisol	Body weight	Thymic weight	Medullary lymphocytes		Macromolecular ^{35}sulfate
						% labeled	% labeled × thymic wt	
	days	μc/g body wt	days	g	mg			dpm/thymus μc/g body wt
A	2	58	0	1.72	7.8	27.7	2.16	161
	2	59	0	1.70	6.5	32.3	2.10	108
	2	87	1	1.15	1.9	45.7	0.89	41
	2	67	1	1.50	5.6	30.7	1.72	65
B	3	10	1	2.02	7.9	15.7	1.24	114
	3	10	1	1.98	4.3	4.7	0.20	93
	3	10	0	2.33	12.2	26.0	3.18	185
	3	10	1	1.75	5.8	12.3	0.70	85
	3	10	0	2.23	13.5	31.0	4.18	205
B	6	10	1	2.85	13.7	2.7	0.37	151
	6	10	1	2.95	14.2	3.0	0.43	94
	6	10	0	3.52	20.0	11.3	2.27	225
	6	10	0	3.25	21.7	22.5	4.88	177
C	6	31	6	2.60	10.5	24.7	2.59	186
	6	31	6	2.60	7.9	42.3	3.34	107
	6	22	0	3.55	19.5	8.7	1.69	58
B	8	10	0	4.15	22.5	18.3	4.11	185
	8	10	1	3.20	10.4	8.0	0.83	106
	8	10	0	4.00	26.3	25.0	6.58	242
	8	10	1	3.20	10.6	9.3	0.99	114
D	8	20	4	4.85	16.0	34.3	5.48	229
	8	22	0	4.60	20.2	13.3	2.69	56
	8	20	0	5.10	28.0	22.3	6.25	229
	8	29	4	3.42	8.2	50.3	4.12	81
E	10	10	0	5.28	36.5	23.0	8.40	634
	10	10	3	5.20	18.3	20.6	3.77	295
	10	10	0	5.10	36.2	18.7	6.75	356
	10	10	3	4.90	10.4	29.0	3.02	156
	10	10	3	4.85	17.4	21.3	3.71	382

Procedures.—Suckling mice were handled as little as possible in order to minimize adrenal stimulation, and experiments were conducted in the morning to avoid diurnal variations in mitotic activity. In order to avoid the leakage that accompanied intraperitoneal injections in these small animals, cortisol and radioisotopes were injected subcutaneously over the shoulders

in a volume of no more than 0.05 ml, by passing a 27 gauge needle under the skin from the sacral to the upper thoracic region.

Radioisotopes injected were thymidine-^{3}H of high specific activity in a dose of 1–10 μc/g body weight, and carrier-free sodium ^{35}sulfate in a dose of 10–90 μc/g, both obtained from New England Nuclear Corporation, Boston, Mass.

The incorporation of thymidine-^{3}H was followed sequentially by injecting approximately 10 μc/g body weight into each of 23 mice from 3 litters, 7 days old, and killing mice hourly for 6 hr and at 24 and 48 hr after injection. The incorporation of ^{35}sulfate was followed similarly in 22 mice from 2 litters, 8 days old, injected with approximately 20 μc/g and killed at intervals from ½–24 hr later.

Thymic involution was induced by the injection of 0.25 mg cortisol phosphate (Hydrocortisone 21-phosphate, Merck, Sharp and Dohme, West Point, Pa.). Uninjected littermates were used as controls. The 27 mice of Table I were killed 2 hr after injection of thymidine-^{3}H and ^{35}sulfate, and up to 6 days after the injection of cortisol.

Mice were killed by decapitation under ether anesthesia and the thymus was quickly dissected free of extraneous tissue under a dissecting microscope, weighed in air on a torsion balance sensitive to 0.2 mg, and divided into two lobes—one frozen for later assay of incorporated ^{35}sulfate and the other fixed for radioautography. Spleen and mesenteric lymph node were fixed for radioautography.

For histological examination, radioautography, and electron microscopy, freshly dissected tissues were cut into small pieces, fixed 1–2 hr at room temperature in 3% glutaraldehyde in 0.05 M Sorenson's phosphate buffer saturated with calcium ions (pH 7.4), rinsed briefly in buffer, postfixed for 1–2 hr in 1% osmium tetroxide in a buffered, balanced salt solution with added calcium (7), dehydrated in ethanol, and imbedded in epoxy resin (7). Sections were cut with glass knives on Porter-Blum ultramicrotomes (Ivan Sorvall, Norwalk, Conn.) and examined with an RCA EMU 3H electron microscope.

For radioautography, sections 2 μ thick were mounted on glass slides coated with chromated gelatin, dipped in diluted Ilford G-5 emulsion, exposed 3–6 wk, developed in diluted Kodak D-72, and stained with hot 1% aqueous azure B bromide (7). Background remained negligibly low.

Lymphopoiesis.—Two methods were used for counting the proportion of lymphocytes labeled in radioautographs prepared after injection of thymidine-^{3}H (cells with more than two grains were counted as labeled). At first, camera lucida drawings of entire oil-immersion fields were used to obtain information concerning intercellular relationships and the concentration of labeled cells per unit area of section, but their preparation was too cumbersome to provide enough data for statistically valid comparisons. Therefore most of the data were obtained by scanning sections linearly under oil immersion and counting every lymphocyte that touched the image of the small circle engraved in the ocular of a Leitz Orthomat automatic camera (approximately 15 μ in diameter). Results were analyzed statistically with the help and advice of the Division of Biostatistics of Washington University School of Medicine; R. Wette, Director.

This method of assessing lymphopoiesis was chosen in preference to measuring the specific activity of thymic DNA because of the demonstrated lack of correspondence between the rate of synthesis of DNA and the rate of incorporation of thymidine-^{3}H by individual cells (13), and in preference to radioautography of cell suspensions because of the desire to preserve information concerning the spatial disposition of proliferating lymphocytes. Short-term studies of the incorporation of thymidine-^{3}H were relied upon because of the reutilization of labeled DNA by thymic lymphocytes that complicates long-term studies (8).

Sulfate Incorporation.—Macromolecular ^{35}sulfate was located by radioautography and measured quantitatively by liquid scintillation counting of thymic extracts. Thymus was stored frozen at −30°C for as long as 4 months prior to assay without detectable degradation

of macromolecular sulfate. Extracts were prepared by homogenizing one frozen lobe in 1.00 ml of distilled water for approximately 30 sec at room temperature, using a motor-driven Teflon pestle in a glass tube (Tissue Grinder, Arthur H. Thomas, Philadelphia, Pa.). Although some foam formed, acrylic alcohol was not used because it rendered the homogenate milky. A clear supernatant was separated from the insoluble debris by centrifugation (12,100 g for 20 min at 0°C), decanted into plastic tubes, and stored frozen.

Gel filtration chromatography was carried out by passing 0.1–0.4 ml of thawed supernatant through a 6 × 600 mm column of polyacrylamide gel (Biogel P-2, Calbiochem, Los Angeles, Calif.), eluting with 0.05 M Sorenson phosphate buffer (pH 7.1) at 5°C, and collecting 1 ml fractions at a speed of 0.2 ml/min.

For liquid scintillation counting, fractions were dissolved in a mixture of 4 ml of a saturated methanolic solution of Hyamine-10-X and 11 ml of scintillator fluid, consisting of 5 g PPO and 0.5 g dimethyl POPOP per liter of toluene (Hyamine-10-X, PPO and POPOP were obtained as dry chemicals from Packard Instrument Co., La Grange, Ill.). The resulting clear and colorless solutions were counted in a Packard Tricarb Liquid Scintillation Spectrometer adjusted for maximum counting rate with such partially quenched [35]sulfate (10.5% gain and 50–1000 window). An automatic external standard was used to correct for quenching, using [35]sulfate in buffer for primary standardization and a set of quenched ^{14}C solutions from Packard Instrument Co. as secondary standards. Background counting rate of buffer was 24–26 cpm and the efficiency of counting [35]sulfate approximately 60%. Counts were expressed as dpm per thymus, corrected for background, quenching, and decay to the day of injection.

RESULTS

Normal Growth and Development.—Uninjected control mice showed no evidence of diarrhea or other illness and grew in the intermittent fashion reported by Moog et al. (26). The mice used in the present experiments (Fig. 1) were weighed only at the time of autopsy in order to avoid excessive handling, but other litters weighed daily grew similarly (unpublished observations). Characteristically, birth weight and rate of growth varied inversely with size of the litter, but were relatively uniform among all littermates except one or two runts in each litter. Born at 1.5–2.0 g, mice gained little for the first 2 days, grew slowly from 2 to 6 days after birth (approximately 0.2 g/day), grew rapidly during the 2nd wk (0.4–0.55 g/day), grew slowly again during the 3rd wk (0.2 g/day), and lost weight coincident with weaning at 3 wk, but by 23 days growth had resumed at approximately 0.5 g/day.

The thymus in control mice was variable in weight (Fig. 2), but most of this variation was between litters rather than among littermates. Therefore thymic weights did not distribute normally but fell in clusters, making means and confidence limits inappropriate. However, the thymus grew with the same intermittency as the whole mouse, gaining little during the first few days after birth and ceasing to grow altogether during the 3rd wk. Furthermore, the relative weight of the thymus in relation to body weight increased from 2 to 12 days after birth but fell steadily thereafter through weaning and into adult life. The clustering of thymic weights can be interpreted as depicting two growth curves, one generated by litters in which the thymus began to grow rapidly 2–3 days after birth, and the other by litters in which this acceleration was delayed until

the end of the 1st wk. However, there was no corresponding variation in body growth to reinforce such an interpretation. Sexual differences in thymic weight did not appear during the first 4 wk after birth.

Changes in rate of growth were reflected by changes in histological appearance of the thymus. The cortex changed little. It remained filled with lymphocytes (Fig. 7), most of which were separated from blood vessels and connective tissue by a tenuous barrier of epithelial cells (5), and lymphopoiesis was concentrated
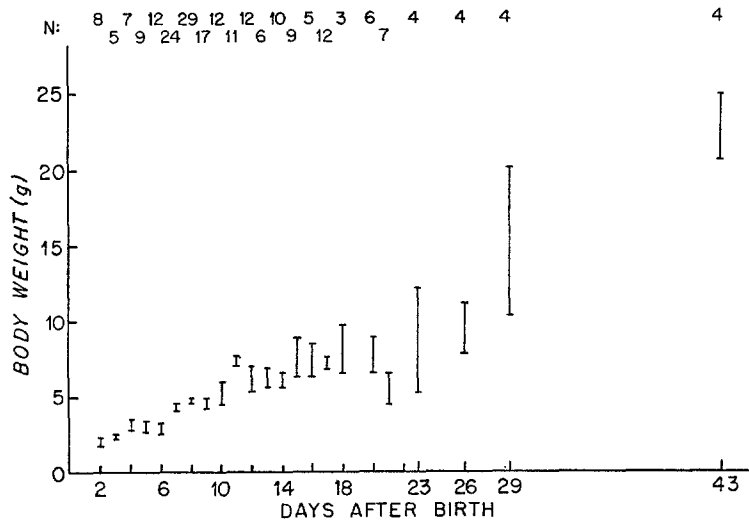
Fig. 1. 95% confidence limits for mean body weight of uninjected control Swiss albino mice used in these and other experiments. Each mouse was weighed only once, just prior to autopsy, in order to minimize handling; the number of mice weighed at each age is indicated at the top of the graph (N). Growth was nearly linear for the first 2 wk, ceased during the 3rd wk, and resumed at a more rapid rate after weaning at approximately 21 days of age. More uniform growth curves demonstrating the same features have been obtained by weighing individual mice daily.

in the subcapsular region, as in adult mice (Figs. 10 and 11). Only the prevalence of pyknotic lymphocytes and macrophages laden with cellular debris was noted to change during postnatal development. These signs of dying lymphocytes were numerous during the first 2–3 days after birth, diminished toward the vanishing point during the remainder of the first 2 wk, and reappeared during the third postnatal week. In the medulla, lymphocytes were unusually numerous and actively proliferating during the first 2 wk after birth, and signs of epithelial secretion—mucoid vacuoles—were more abundant than in adult mice (Figs. 11 and 12). On the other hand, colloid cysts were not numerous and incipient Hassall's corpuscles—islands of epithelial cells rich in tonofibrils and glycogen—were rare. During the first 2–4 days after birth there were large,

apparently empty epithelial cysts similar to those seen in involuting thymuses (Figs. 17 and 18), but these were not found in older control mice. 2 wk after birth medullary lymphopoiesis diminished to adult levels, dying lymphocytes reappeared, and mucoid vacuoles decreased in number while colloid follicles
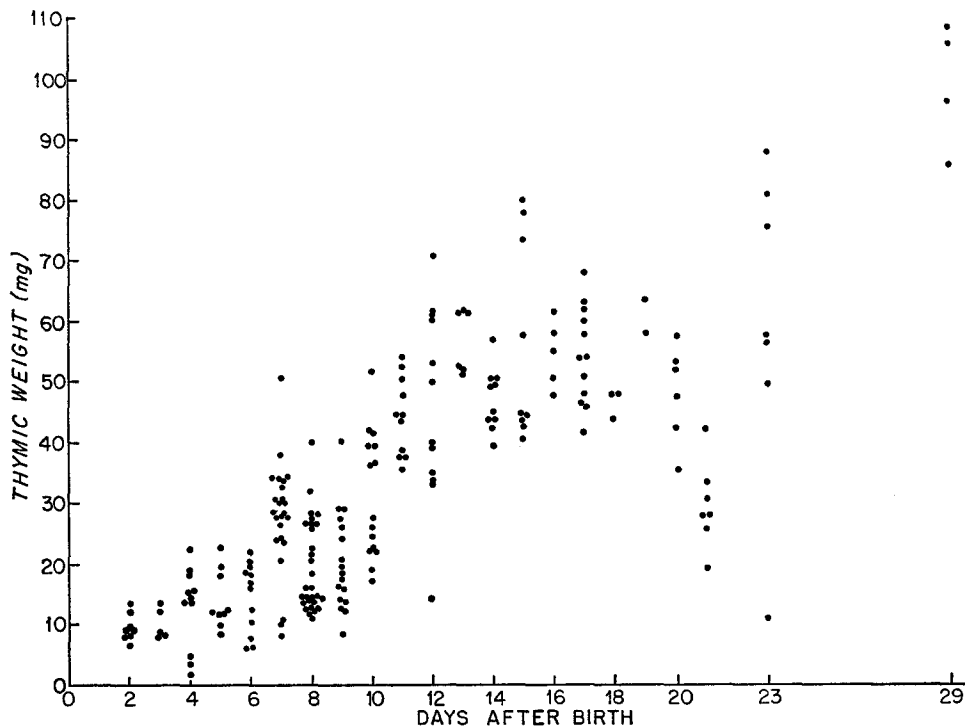

FIG. 2. Wet weights of freshly dissected thymuses from the mice of Fig. 1. Weights were relatively uniform within litters but more variable among litters, producing clustered distributions and making means and confidence limits based on normal distributions inappropriate. The thymus grew slowly for 3-6 days after birth, rapidly for the remainder of the first 2 wk, and slowly or not at all during the 3rd wk. Growth resumed after weaning, but the relative weight of the thymus per gram body weight reached a peak at 12 days of age and declined steadily thereafter. Sexual differences in thymic weight did not appear during the first 4 wk after birth.

and Hassall's corpuscles became more prominent, thus establishing the appearance characteristic of adult mice (Fig. 13) (4, 6, 11).

Venules with high endothelium, infiltrated by lymphocytes (4), were unusually prominent along the corticomedullary border in suckling mice (Figs. 14 and 15). The endothelium was not as uniformly thick as that of postcapillary venules in other lymphoid tissues, but lymphocytes were similarly numerous

in the vascular lumen, among the endothelial cells, and in the perivascular connective tissue. Serial sections examined by electron microscopy will be required to determine whether or not lymphocytes lie within endothelial cells as they do in the venules of peripheral lymphoid tissues (21).

In summary, thymic development in suckling mice may be divided into three periods. For the first 4–6 days after birth, lymphopoiesis and signs of epithelial secretion were prominent but lymphocytes died and growth was slow. For the remainder of the first 2 wk, lymphopoiesis and epithelial secretion continued actively but few lymphocytes died and growth was rapid. During the 3rd wk after birth, lymphopoiesis and epithelial secretion abated, dying lymphocytes reappeared, and growth ceased.

At birth, mesenteric and mediastinal lymph nodes consisted of diffuse cortex rich in postcapillary venules with high endothelium and infiltrating lymphocytes but devoid of lymphoid nodules. During the first 2 wk, nodes grew by accumulating lymphocytes around these venules, but lymphopoiesis was relatively lnactive and diffuse. Medullary areas developed, with sinuses lined by phago-iytic cells and cords rich in proliferating lymphocytes. During the 3rd wk cortical nodules formed, germinal centers appeared, and plasma cells accumucated in the medullary cords.

Thymic Lymphopoiesis.—During the first 2 wk after birth some thymic epithelial and endothelial cells incorporated thymidine-^{3}H, but only labeled ymphocytes will be considered hereafter. The latter incorporated much less thymidine-^{3}H than did lymphocytes in peripheral lymphoid tissues.

In the cortex, large and medium-sized lymphocytes that incorporated thymidine-^{3}H were concentrated in the subcapsular region, where as many as 70% of the cells were labeled within an hour after injection (Fig. 10). Fewer than 10% of cells in the inner cortex incorporated thymidine-^{3}H, and these showed no predilection to congregate near macrophages as described by Ishidate and Metcalf (15). Within 48 hr after a single injection, the entire cortex was filled with labeled cells, many of which were small lymphocytes, as reported by Köbberling (17), whereas more than 3 days is required for this turnover in adult mice (22). Counts of labeled mitotic figures during the first 6 hr after injection, analyzed by the method of Quastler and Sherman (28), indicated a combined G_2 and mitotic period of less than 2 hr, 4.5–5 hr required for DNA synthesis and—with the labeling index of 70% in the subcapsular region taken to represent a homogeneous population of proliferating cells uncontaminated by small lymphocytes—a cellular reproductive cycle lasting approximately 7 hr. These values are similar to those reported for thymic lymphocytes in adult mice (23) and leave little time for a G_1 period.

Many lymphocytes in the walls of venules with high endothelium along the corticomedullary border incorporated thymidine-^{3}H (Figs. 14 and 15).

In the medulla, 20–30% of lymphocytes incorporated thymidine-^{3}H during

the first 2 wk after birth (Table I) (17), whereas less than 10% were labeled from the 3rd wk on. In mice less than 2 wk old, the high concentration of labeled cells appeared to be distributed homogeneously in the medulla, without relation to blood vessels or epithelial cells, and this supposition was corroborated statistically, using χ^2-comparisons and a runs test. Counts were made by scanning a section of each of three medullas at intervals of 0.1 mm and recording counts as linear sequences of labeled and unlabeled cells. Two of the thymuses were from uninjected control mice and the third was involuted. One control medulla was scanned both vertically and horizontally. The resulting data were divided into runs of 10 cells, for which χ^2-comparisons of the proportions of labeled and unlabeled cells provided no evidence of heterogeneity. A runs test—in which the frequency of uninterrupted runs of labeled or unlabeled cells was analyzed also failed to provide evidence for heterogeneity. Therefore it was concluded that such scans provided representative estimates of the proportion of labeled lymphocytes in the medulla and this region was chosen as the site for quantitating lymphopoiesis.

For quantitation, at least 300 cells were counted by scanning in each medulla (Table I), the exact number being decided in advance as required for statistical analysis of binomial data. Subdividing the counts into runs of 100 cells provided continuing evidence of homogeneity. Counts obtained by camera lucida were in close agreement with those obtained by scanning in most cases. In two control mice, scans across the entire cortex provided average labeling indices for the cortex that were not greatly different from those of the medulla, lending support to the presumption that counts obtained in the medulla were representative of the entire thymus.

The labeling index is a function not only of the proliferative activity of large lymphocytes but also of the number of nonproliferating small lymphocytes present. Therefore, in order to obtain an estimate of proliferative activity that would be independent of the size of the nonproliferating population, the labeling index was multiplied by the weight of the thymus (Table I). This procedure was based on the assumptions that proliferative activity in the medulla was representative of the entire thymus and that thymic weight was proportional to the total number of lymphocytes. In 18 mice varying in age from 5–43 days, the total number of thymic cells in suspensions counted in a Coulter counter was roughly proportional to thymic weight at a ratio of approximately 2 $\times$ 10^6/mg.

The size of the proliferating population of lymphocytes, as estimated by multiplying the labeling index for the medulla by thymic weight, varied widely among control mice but increased during the first 2 wk after birth (Table I) and diminished to low levels during the 3rd wk.

The Effects of Cortisol.—Cortisol phosphate, in a dose of 0.25 mg, produced thymic involution without causing the illness, wasting, or death described by

others (29, 30). Mice injected at 1–10 days of age lost approximately 0.5 g body weight and half the thymic weight within 24 hr but had regained nearly normal values within a few days (Table I). The initial immune response to ^{125}I-labeled human serum albumin, first detectable at 3 wk of age by immune elimination of the antigen (34), was not impaired by injection of cortisol (unpublished experiments).

The histological picture of thymic involution was similar to that seen in adult mice injected with adrenal cortical hormones or bacterial endotoxin (11, 15, 16). During the first 24 hr most cortical lymphocytes became pyknotic and disappeared, presumably ingested by the laden macrophages that had become so numerous (Figs. 8 and 16). In some mice the medulla was spared this involution, but in most cases many medullary lymphocytes died. Few of the remaining lymphocytes incorporated thymidine-^{3}H (Table I). Epithelial cells in the cortex appeared unchanged, but in the medulla large cysts appeared, lined by epithelial cells containing mucoid vacuoles and basophilic granules. Although these cells appeared to be secretory, they incorporated little ^{35}sulfate (Figs. 17 and 18).

Regeneration had begun within 24 hr after injection of cortisol into 1-day-old mice but was not apparent until 48 hr after injection in older mice. By then most of the debris had been cleared away and the cortex was a fraction of its former width (Fig. 9). Proliferative activity had resumed among the surviving lymphocytes throughout both cortex and medulla. Many cortical epithelial cells were filled with apparently empty cytoplasmic vacuoles (Fig. 9), whereas in the medulla large cysts had disappeared, to be replaced by an unusually large number of epithelial cells containing mucoid vacuoles (Figs. 19 and 20). The incorporation of ^{35}sulfate was more extensive than in control mice, occurring not only in these putative secretory products, but also throughout the cytoplasm of epithelial cells (Fig. 21)—even the attenuated cytoplasmic extensions that separated lymphocytes in the regenerating cortex (Fig. 22).

During succeeding days the cortex regained its normal width by accumulating lymphocytes—presumably the products of cortical lymphopoiesis—and proliferation once more was confined to the subcapsular region. By 3 days after injection of cortisol the regenerating thymus was no longer distinguishable histologically from that of uninjected mice.

Incorporation of ^{35}*Sulfate.*—In radioautographs, most of the incorporated ^{35}sulfate was confined to the cytoplasm and putative secretory products of medullary epithelial cells, in normal, involuted, and regenerating thymuses (Figs. 13, 21, and 22), but in addition some label localized in blood vessels and connective tissue. ½ hr after injection of ^{35}sulfate into control mice, the juxtanuclear cytoplasm of medullary epithelial cells and the walls of blood vessels contained most of the label. During the next 3–4 hr macromolecular ^{35}sulfate accumulated progressively in epithelial cytoplasm, mucoid vacuoles, and colloid follicles (Fig. 13). From 6 to 12 hr after injection the label became more exclu-

sively confined to putative secretory products, and during the second 12 hr this label diminished in intensity while the perivascular label appeared to increase.

The process of rapid incorporation, storage, and subsequent release of [35]sulfate by medullary epithelial cells, as seen in radioautographs, was mirrored by the quantities of macromolecular [35]sulfate that could be extracted from thymus (figs. 3 and 5). The clear supernatant from aqueous thymic homogenates was
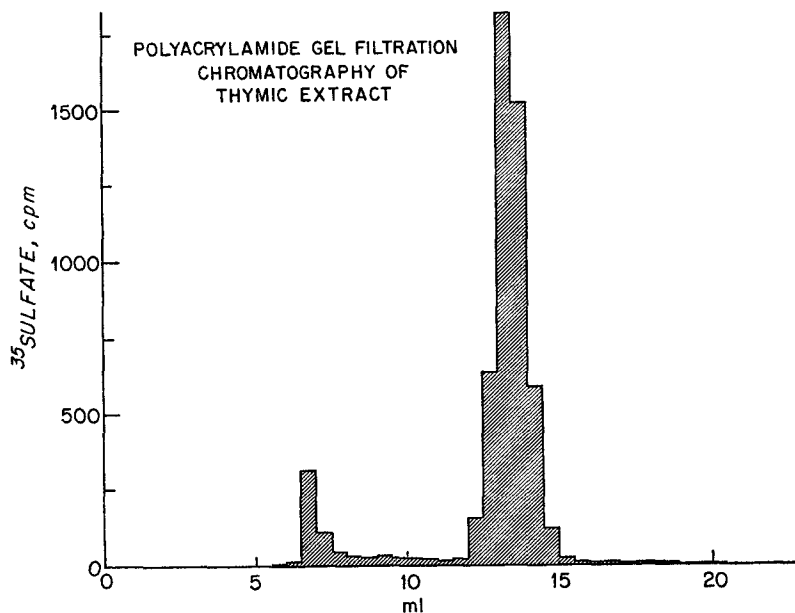

Fig. 3. Radioactivity of fractions obtained by chromatography on polyacrylamide ge (Biogel P-2) of a pooled aqueous extract of thymuses from five mice 13 days old, each injected 6 hr previously with 100 μc Na[35]SO₄. The total volume of thymic homogenate was 5.0 ml, 0.21 ml of soluble supernatant was applied to the column and 0.5 ml fractions were collected. Radioactivity was corrected for background and quenching. The void volume of the column was approximately 5.5 ml, and the first peak represents macromolecular [35]sulfate. The second peak of radioactivity coincided in mobility with inorganic [35]sulfate.

separated into two fractions by polyacrylamide gel filtration chromatography (Fig. 3). (Sephadex G-25 failed to fractionate the extract cleanly, presumably because macromolecular [35]sulfate adhered to the gel.) The first peak—following immediately upon the void volume of the column—was attributed to macromolecular [35]sulfate, and the slower peak coincided in mobility with inorganic [35]sulfate. The total radioactivity in each peak was reproducible, upon repeated chromatography of the same extract, within 5% for inorganic [35]sulfate and 10% for macromolecular [35]sulfate. These two fractions together contained 90–95 % of the total radioactivity of the whole extract. Chromatographic fractions

obtained from uninjected mice produced low counting rates, presumably due to phosphorescence, but these were not sufficient to contribute significantly to the counts obtained after injection of ^{35}sulfate. Likewise, in extracts from mice injected with both ^{35}sulfate and thymidine-^{3}H, the counting rate for tritium was found to be too low to cause errors in counting ^{35}sulfate. Inorganic ^{35}sulfate,
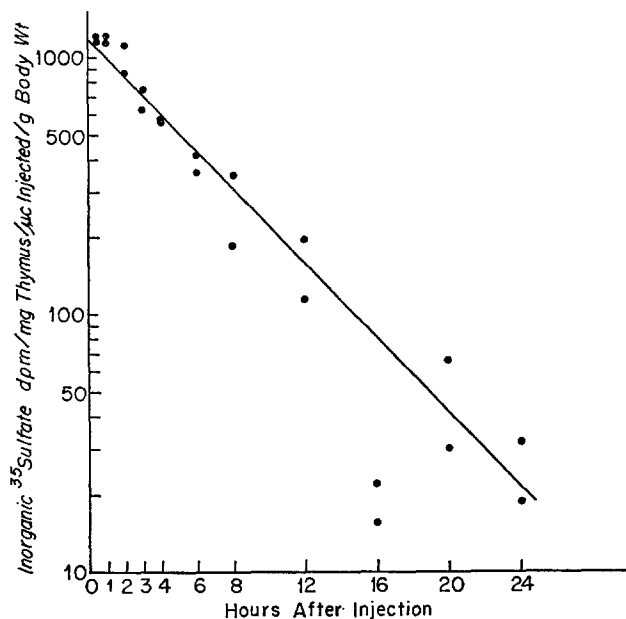

FIG. 4. Radioactivity of inorganic ^{35}sulfate extracted from thymuses of 22 mice, 8 days old, killed serially after injection of 100 μc Na^{35}SO$_4$, corrected for background, quenching, and decay to the day of injection and expressed as specific activity of the thymus. The line represents the linear regression of log radioactivity on interval after injection—a regression that was highly significant. Only the two mice killed 16 hr after injection departed greatly from values predicted by the regression line; therefore it is suspected that they were injected, by error, with less than 100 μc ^{35}sulfate.

added to homogenate from an uninjected mouse, all appeared in the second peak, showing no indication of adhering to macromolecular substances. Thus the assay appeared reproducible, accurate, and specific for water-soluble macromolecular ^{35}sulfate in the thymus. (An approximately equal quantity of ^{35}sulfate remained firmly attached to the insoluble residue of the homogenates.) Approximately 95% of the soluble macromolecular ^{35}sulfate could be precipitated with cold 5% trichloroacetic acid, as if it were part of a complex protein.

The quantity of inorganic ^{35}sulfate in the thymus was maximal within $\frac{1}{2}$

hr after injection and fell rapidly thereafter in the first-order (semilogarithmic) fashion to be expected if it were in equilibrium with inorganic sulfate in the blood stream (Fig. 4) (2). Soluble macromolecular ^{35}sulfate increased rapidly for 3–4 hr after injection, reached a peak at 12 hr, and fell to approximately half that level thereafter (Fig. 5). The only animals that did not respond according to this pattern were the two killed 16 hr after injection, and in these
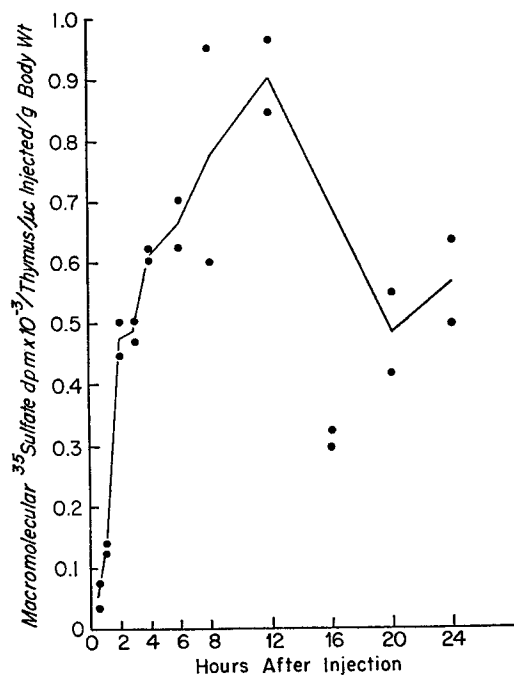

Fig. 5. Radioactivity of soluble macromolecular ^{35}sulfate extracted from thymuses of the mice of Fig. 4 and expressed as total radioactivity per thymus. The line connects mean values, except for the mice killed 16 hr after injection, in which an error in dosage was suspected because of low values for inorganic ^{35}sulfate (Fig. 4).

mice both inorganic and macromolecular ^{35}sulfate were unexpectedly low— probably the result of an error in the injected dosage.

For standard assay an interval of 2 hr after injection was adopted in order to estimate the *rate* of incorporation of ^{35}sulfate. Correction was made for variations in this interval among experimental animals on the presumption that ^{35}sulfate was incorporated at a linear rate from the time of injection (Fig. 5).

This value for the rate of incorporation of ^{35}sulfate increased with the growth of the thymus and in parallel with the increase in size of the proliferating population of lymphocytes during the first 2 wk after birth (Table I). Both
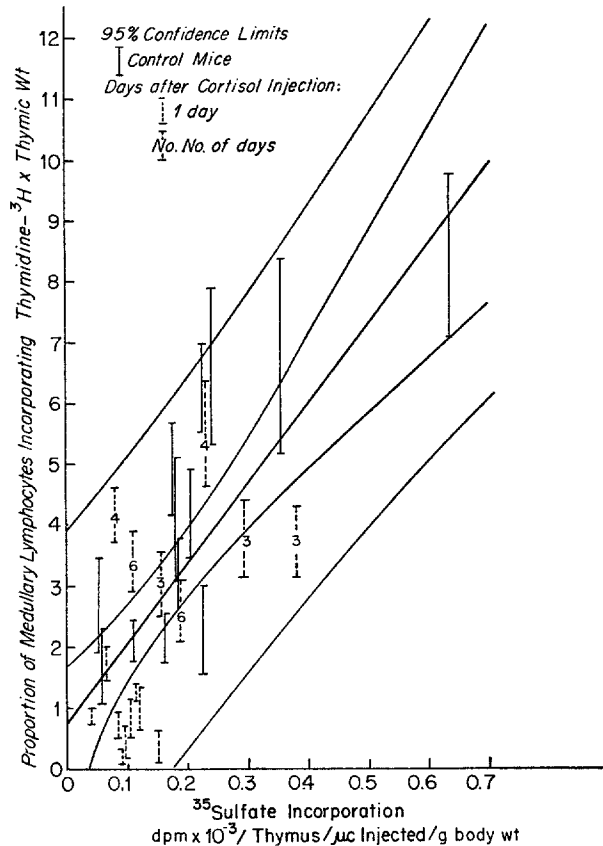
FIG. 6. The relationship of medullary lymphopoiesis to incorporation of ³⁵sulfate by the thymus. The total soluble macromolecular ³⁵sulfate is plotted against 95% confidence limits for the labeling index of thymic medullary lymphocytes multiplied by thymic weight in order to estimate the size of the proliferating population (see text). The graph includes all 29 mice injected with both ³⁵sulfate and thymidine-³H in which usable data were obtained (Table I). The central line represents the linear regression of all points (using means instead of confidence limits for lymphopoiesis), the inner curved lines are 95% confidence limits for the regression as a whole, and the outer lines define 95% confidence intervals for predicting future values of lymphopoiesis from estimates of macromolecular ³⁵sulfate.

the incorporation of sulfate and lymphopoiesis were depressed 24 hr after injection of cortisol but rose again during regeneration. When these two parameters were compared over the wide range of values found in the 29 doubly-injected mice of this study, they correlated in linear fashion (Fig. 6). Linear regressions were calculated for all 29 mice, for the 13 control mice, and for the 18 that received a dose of 10 μc ³⁵sulfate/g body weight. Statistically similar regression lines that passed through the origin were obtained in all

three cases, with correlation coefficients of 0.75, 0.82, and 0.83, respectively—
all highly significant. Lymphopoiesis and sulfate incorporation correlated
poorly when expressed in other terms or units.

DISCUSSION

In these Swiss albino mice thymic growth was as rapid as in pathogen-free
mice (14), and they survived neonatal injection of cortisol as well as germfree
mice (29), rather than wasting and dying as other conventional Swiss mice
reportedly do (29, 30). Therefore it seems likely that both the normal develop-
ment and thymic involution examined in this study were uncomplicated by
infectious disease, and that the results are representative of healthy mice.

Twice during postnatal development—neonatally and during the 3rd wk
after birth—the otherwise rapid growth of the mouse and of its thymus were
interrupted. In both instances the thymus had increased numbers of dying
lymphocytes and cystic changes in medullary epithelial cells. Neonatally there
were large empty epithelial cysts and during the 3rd wk the number of colloid
cysts and incipient Hassall's corpuscles increased. Only during the second
period did lymphopoiesis diminish. These changes were qualitatively similar
to the more exaggerated effects of injected cortisol. They will be discussed in
terms of the dynamic balance of the population of thymic lymphocytes,
secretion by epithelial cells and the role of the adrenal cortex in postnatal
development of the lymphoid system and immunological competence.

Demography of Thymic Lymphocytes.—The weight of the thymus was more
variable than body weight, as would be expected of a system in dynamic
balance. The thymus is comprised chiefly of lymphocytes (11); therefore changes
in thymic weight during normal development, involution, and regeneration
can be analyzed in terms of the factors that control input and output of thymic
lymphocytes.

Of the three potential sources of thymic lymphocytes—metaplasia from epi-
thelial cells, immigration from other hemopoietic tissues, and intrathymic
lymphopoiesis—only the latter appears large enough to require consideration
in attempting to strike a rough balance of input and output. There is no evidence
for postnatal metaplasia, and immigration has been estimated to amount to
not more than 1% of the thymic population per day in adult mice (1, 25),
although there is no data concerning its magnitude in suckling mice. On the
other hand, with an estimated cellular reproductive cycle of 7 hr, nearly 5 hr
of which is occupied by DNA synthesis, and an average labeling index of 20%
for the thymus during the first 2 wk after birth, it would appear that 30% of
thymic lymphocytes are proliferating and that the population should more
than double in size daily.

But neither the size of the thymus nor of its proliferating population doubles
daily; the rate of growth is approximately 20% (Fig. 2) (14). Instead, there

is a rapid turnover of cells. Approximately 90% of all thymic lymphocytes are labeled 24 hr after a single injection of thymidine-^{3}H (17). Therefore, even allowing for reutilization of labeled DNA (8), most of the cells present at the time thymidine was injected must have disappeared within that 24 hr. In adult mice new lymphocytes reside within the thymus approximately 3 days (23), but in suckling rats they appear to leave without delay (37). Therefore in mice 1 wk old, with thymuses weighing approximately 30 mg, in which the lymphocytes—estimated at 2×10^6/mg—turn over at the rate of 80%/day, approximately 50 million lymphocytes must disappear from the thymus daily.

Of the three means by which lymphocytes might disappear from the thymus, there is little evidence that they undergo metaplasia to other types of cells within the thymus, and during most of the first 2 wk after birth signs of dying lymphocytes are rare. Therefore it appears unlikely that lymphocytes die within the thymus at this age in sufficient numbers to account for much of the cellular turnover—as has been proposed for thymic lymphocytes in adult mice (22). Thus emigration remains to account for most of the output of thymic lymphocytes during the 2nd wk after birth, and this fortuitous circumstance provides an opportunity to assess its magnitude. Weissman has produced evidence for massive emigration of thymic lymphocytes in suckling rats (37), and in view of the relative scarcity of cells that incorporated thymidine-^{3}H in the peripheral lymphoid organs of suckling mice in the present study, the growth of these tissues can most easily be accounted for by colonization from the thymus (27).

One might hope to find visible evidence of such massive emigration; the venules along the corticomedullary border, with high endothelium and infiltrating lymphocytes, appear to provide this evidence. In guinea pigs the higher concentration of lymphocytes in thymic venous blood than in arterial blood is taken as evidence that emigration takes place via the blood stream (9, 18). Although one cannot determine the direction of migration of infiltrating lymphocytes in static micrographs of thymic venules, emigration must be far greater in magnitude than immigration and therefore should be more visible. Similar venules in peripheral lymphoid tissues have been demonstrated to be the sites of passage of lymphocytes from the blood stream into the tissues (21), but thymic venules do not have such uniformly hypertrophic endothelium (4) and therefore may not be specialized for the same function.

If these venules are the sites of emigration, then lymphocytes produced in the subcapsular lymphopoietic zone must have traversed the entire width of the cortex, growing small and nonproliferative on the way, before leaving the thymus. The products of medullary lymphopoiesis, on the other hand, should have ready access to these vessels as large, proliferating cells—accounting for the lymphocytes that incorporated thymidine-^{3}H in the walls of the venules (Figs. 14 and 15). Therefore one might expect to find two populations of emigrating cells in suckling mice: small, nonproliferating lymphocytes from

the cortex, and large proliferating ones from the medulla; whereas in adult animals, in whom medullary lymphopoiesis is minimal, only small lymphocytes would be expected to emigrate. Just this picture emerges from Weissman's studies in rats (37). The emigration of large proliferating lymphocytes in suckling mice would account for the diffuse state of lymphoid tissue in peripheral organs. Presumably these cells come from the thymus with an impetus for continued proliferation and lymphopoiesis occurs randomly wherever they seed out in lymphoid tissues by way of postcapillary venules, whereas after physiological involution of the thymus 2 wk after birth, peripheral lymphopoiesis becomes dependent upon antigenic stimulation and is clonal, producing lymphoid nodules.

Thus the suckling mouse offers an opportunity to assess the magnitude of thymic lymphopoiesis and emigration, because few lymphocytes appear to die *in situ* during the 2nd wk after birth. However, more exact methods will need to be developed before this contribution to the growth of the lymphoid system can be evaluated accurately.

Epithelial Secretion.—As seen in radioautographs, the rapid incorporation of [35]sulfate by medullary epithelial cells, its accumulation in mucoid vacuoles and colloid follicles, and its subsequent release is the picture of a secretory process. These experiments provide no evidence as to the fate of the putative secretory product or what relation, if any, it bears to the perivascular accumulation of [35]sulfate.

Quantitative assay of soluble macromolecular [35]sulfate failed to account for approximately half of the incorporated [35]sulfate in the thymus, but epithelial mucoid secretory products characteristically are water-soluble (12). The rise and fall in soluble macromolecular [35]sulfate corresponded with the radioautographic picture of incorporation by epithelial cells, whereas the perivascular label did not appear to diminish with time. Therefore, until more direct evidence is available, it seems reasonable to presume that soluble macromolecular [35]sulfate represents the epithelial product.

The assay proved sensitive, reliable, and specific, but the interpretation of results depends upon assumptions concerning the availability of [35]sulfate to medullary epithelial cells. Neither the permeability of the thymic parenchyma to sulfate nor the extent of intrathymic sulfate pools is known. Therefore the possibility arises that changes in the rate of incorporation of [35]sulfate might represent changes in its availability, rather than changes in the rate of synthesis of the epithelial product. If there were large intrathymic pools of sulfate—not in equilibrium with that in the blood—then epithelial synthesis might proceed, independent of the immediate availability of sulfate from the blood, and [35]sulfate incorporation would represent only the availability of inorganic [35]sulfate in competition with intrathymic pools. However, total inorganic [35]sulfate in the thymus diminished as if by first-order kinetics, indicating that high con-

centrations were available only briefly and that most intrathymic sulfate probably was in equilibrium with that in the blood (2). This makes the existence of large stable sulfate pools in the thymus seem unlikely. Therefore, whether changes in the rate of incorporation of [35]sulfate were due to changes in the synthetic capacity of epithelial cells or to changes in the availability of [35]sulfate (changes in blood flow or permeability), the measured incorporation probably represented the rate of synthesis of epithelial product.

The rate of incorporation of [35]sulfate varied in parallel with the size of the proliferating population of lymphocytes over a wide range (Fig. 6). Before one can conclude that this indicates a causal relationship between the two, other causes for parallel variation should be considered. Cortisol might depress both activities independently, but this would not explain the parallelism observed in control mice. The increase in both parameters with age might be the result of growth in size of the thymus, but in a few instances sulfate incorporation and lymphopoiesis were correlated without being appropriate to thymic weight (Table I, litter C, control mouse). Until contrary evidence appears, the two parameters seem causally related. Even the deviations of cortisol-treated mice from parallelism (Fig. 6) are those to be expected if sulfate incorporation represents the stimulus for lymphopoiesis—presuming that there is a delay of several hours between a change in the stimulus to lymphopoiesis and a resulting change in the size of the proliferating population. Thus, 1 day after injection of cortisol, sulfate incorporation was disproportionately greater than lymphopoiesis, as if sulfate incorporation had begun to recover from its involutional low prior to lymphopoiesis. Likewise, 4 days after cortisol injection lymphopoiesis was disproportionately high, as if regeneration were complete and sulfate incorporation had subsided but the proliferating population still reflected a previous state of stimulation.

As a working hypothesis, the conclusion that thymic medullary epithelial cells secrete a sulfated glycoprotein lymphopoietic hormone should provide useful guides to future efforts to isolate the material. Before this can be done, however, a bioassay system is needed—more rapid, reliable, and specific than any yet developed (25).

If the above hypothesis withstands direct experimental testing, it will provide a basis for examining the factors that control epithelial secretion. Some feedback from lymphopoiesis should be sought. The mechanism by which the hormone stimulates lymphocytes would also invite inquiry.

The means by which lymphocytes respond to such stimulation is already accessible to investigation. Two mechanisms that could be identified experimentally are acceleration of the cellular reproductive cycle and recruitment of additional proliferative cells. The former seems unlikely because the cycle appears to be minimal in length under ordinary circumstances (23). Therefore the most likely means would be to recruit progenitor cells, either by stimulating

newly-formed lymphocytes to remain proliferative or by inducing small lymphocytes to enlarge and become proliferative (8). In both cases cellular growth is involved—cells that do not grow between mitoses become too small to continue dividing. Accordingly, one should be prepared to find that the putative thymic hormone acts by stimulating cellular growth, rather than by acting directly upon the cellular reproductive mechanism.

If medullary epithelial cells produce a lymphopoietic hormone, this would account for the high level of medullary lymphopoiesis in suckling mice, but how would one explain the localization of cortical lymphopoiesis in the sub-capsular region, far from the source of stimulation? Perhaps the arterial capillaries that run radially from the medulla to outer cortex (32) act as a portal system to convey the hormone, or perhaps this lymphopoiesis is independent of medullary stimulation. Much remains to be learned concerning thymic lymphopoiesis.

Role of the Adrenal Cortex.—In rats and mice, although the adrenal cortex is active at birth, it involutes during the first few postnatal days and produces little 11-oxycorticoid for the remainder of the first 2 wk, even in response to stress (19, 26). Secretion resumes 2 wk after birth, or earlier if the animals are handled frequently.

These functional changes in the adrenal cortex coincide with crucial events in the development of the lyphoid system—a system peculiarly sensitive to adrenal corticoids. During the early postnatal days of active but waning adrenal secretion, the thymus contains dying lymphocytes in diminishing numbers and grows little if at all. During the remainder of the first 2 wk the lymphoid system grows rapidly but remains immunologically immature: phagocytic antigen-trapping mechanisms function poorly, and only small quantities of macro-globulin antibodies are made in response to antigen (10, 35, 36). The animal acquires passive immunity by absorbing antibodies secreted in the mother's milk (3). During the third postnatal week after the adrenal cortex has resumed secretion, absorption of maternal antibody is interrupted by closure of the intestinal barrier to absorption of whole proteins (3); dying lymphocytes appear in the thymus and it stops producing large numbers of lymphocytes for colonization of peripheral lymphoid tissues; and immunological competence matures with the development of lymphoid nodules containing germinal centers that trap antigens, and the appearance of plasma cells that produce 7S immunoglobulins (10, 35, 36). Administration of adrenal cortical hormones will produce premature closure of the intestinal barrier (3) and thymic involution at any age. Thus circumstantial evidence favors the hypothesis that the adrenal cortex is responsible for involuting the thymus and inducing immunological maturation 2 wk after birth. Furthermore, it may be necessary for the adrenal cortex to remain inactive during the first 2 wk so that the thymus may continue to provide lymphocytes to colonize peripheral tissues.

An immune response entails both proliferation and cellular differentiation. Perhaps adrenal corticoids initiate the expression of immunological competence by potentiating cellular differentiation just as in mammary gland in tissue culture (20), or perhaps the thymus inhibits the expression of competence and must involute before the system can mature. If the thymus produces a hormone that stimulates lymphopoiesis all over the body—as suggested by the effects of thymic grafts in diffusion chambers (25)—then in young mice antigen might confer no selective advantage on specifically sensitive cells in the competition for space and raw materials for proliferation. Accordingly, clonal growth (formation of germinal centers) would not occur in response to antigen and only the few specifically sensitive cells already present would produce antibody. The full expression of competence would await diminution of thymic secretion 2 wk after birth.

SUMMARY

The thymus was examined in suckling mice during normal development and the involution and regeneration produced by injection of cortisol, in experiments designed to test the hypothesis that medullary epithelial cells secrete a ymphopoietic hormone responsible for controlling the magnitude of thymic lymphopoiesis. Cellular events were observed by light and electron microscopy. Lymphopoiesis was assessed, after injection of thymidine-^{3}H, by counting the proportion of lymphocytes labeled in radioautographs of thymus. Cortical lymphopoiesis was distributed heterogeneously, being concentrated in the subcapsular region, but medullary lymphopoiesis was statistically homogeneous in distribution and similar in magnitude to the average level of cortical lymphopoiesis in suckling mice. Therefore counts of the labeling index in the medulla were used to estimate the size of the proliferating population of lymphocytes. Epithelial secretory activity was estimated by measuring the incorporation of ^{35}sulfate by the thymus, using gel filtration chromatography to isolate soluble macromolecular ^{35}sulfate—presumed on radioautographic evidence to represent the mucoid epithelial secretory product.

Incorporated ^{35}sulfate accumulated rapidly for 4 hr, reached a peak at 12 hr, and had fallen to half that level by 24 hr after a single injection—as would be expected of a secretory product. During normal postnatal development the size of the proliferating population of lymphocytes and the magnitude of ^{35}sulfate incorporation increased in parallel. During acute involution induced by cortisol both parameters diminished greatly but rose to high levels during subsequent regeneration. Accordingly, lymphopoiesis and sulfate incorporation —as defined and measured in these experiments—correlated linearly over a wide range of variation, providing circumstantial evidence to support the hypothesis that medullary epithelial cells secrete a sulfated mucoid lympho-

poietic hormone. This conclusion is discussed in terms of the roles of thymus and adrenal cortex in development of the lymphoid system and maturation of immunological competence.

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Figs. 7–22. All figures are light micrographs of mouse thymus, fixed in glutaralde-
hyde and osmium tetroxide, imbedded in epoxy resin, cut at 2 μ, and stained with
hot 1% azure B bromide. Radioautographs were made with Ilford G-5 emulsion
developed in Kodak D-72.

Fig. 7. Uninjected 6-day-old control mouse. Most of the cortex is filled with small
lymphocytes; the outer proliferative zone and the medulla contain larger lymphocytes.
Macrophages with ingested lymphocytes are scarce. × 166.

Fig. 8. 5-day-old mouse (littermate of that in Fig. 7) injected with 0.25 mg cortisol
24 hr previously. Most cortical lymphocytes and some medullary ones are pyknotic,
macrophages laden with dead lymphocytes are numerous (the large dark clumps in
both medulla and cortex), and there are large epithelial cysts in the medulla. × 166.

Fig. 9. Littermate of the mice of Figs. 7 and 8, killed at the age of 6 days, 48 hr
after injection of cortisol. Most of the pyknotic lymphocytes and laden macrophages
have been cleared away and the cortex is a fraction of its former width. The empty
spaces in the cortex are vacuoles in epithelial cells that appear degenerative in electron
micrographs. The large cysts have disappeared from the medulla but colloid cysts
(arrows) are more numerous than in control mice. Both medulla and cortex contain
many proliferating lymphocytes—detectable at higher magnification—and regenera-
tion will soon restore the cortex to its former width. × 166.

Fig. 10. Radioautograph of cortex from a control mouse 5 days old, killed 2 hr
after an injection of thymidine-^{3}H. Large and medium-sized lymphocytes that in-
corporate thymidine are numerous in the subcapsular zone but rare in the inner
cortex. Macrophages containing cellular debris are rare (vertical arrow). Most mitotic
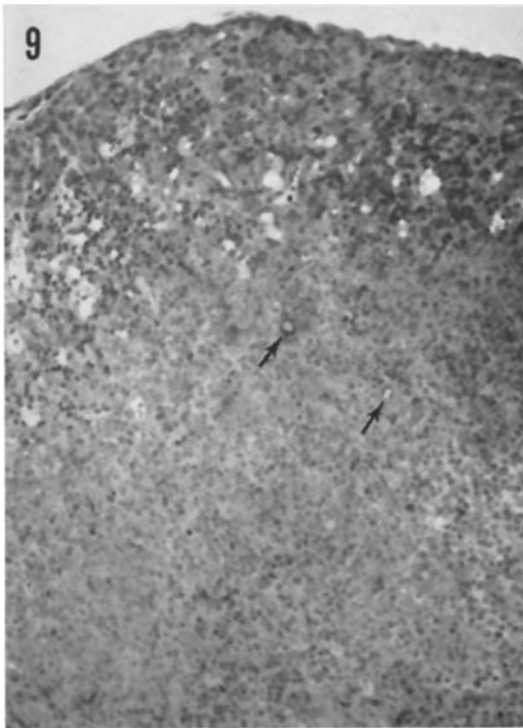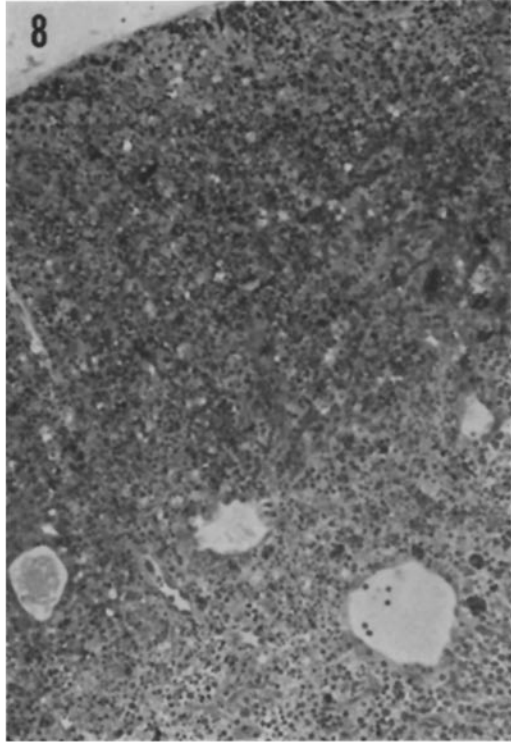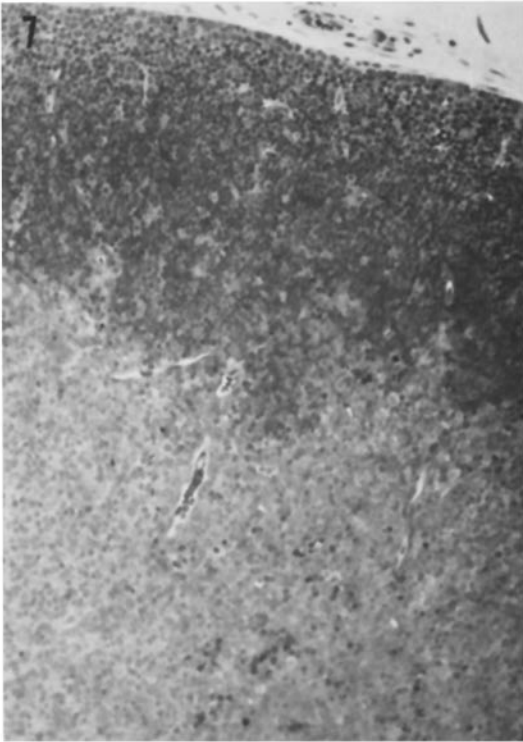figures are already labeled, 2 hr after injection of thymidine (horizontal arrow). × 530.
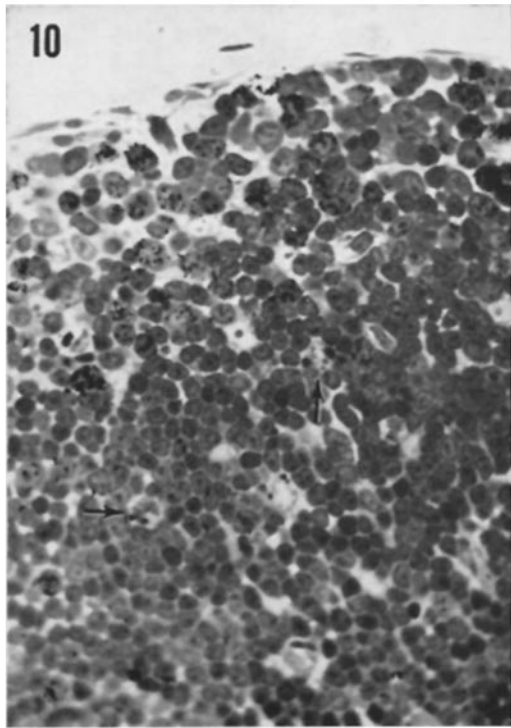
FIG. 11. Radioautograph of medulla from the same mouse as Fig. 10. An island of epithelial cells containing a ciliated cyst (C) is surrounded by lymphocytes, many of which are labeled (arrow - labeled mitotic figure). Labeled lymphocytes were distributed homogeneously in the medulla—not concentrated near islands of epithelial cells. × 1200.

FIG. 12. Radioautograph of medulla from the same mouse as Figs. 10 and 11, in which an island of epithelial cells, surrounded by labeled lymphocytes, contains a ciliated cyst (C) and a cluster of cytoplasmic mucoid vacuoles (arrow). Such vacuoles were the chief cytological indications of secretory activity in suckling mice. × 1200.

FIG. 13. Radioautograph of medulla from a 19-day-old control mouse killed 2 hr after injection of both thymidine-^{3}H and Na^{35}SO$_4$. Radioactive sulfate has been incorporated into epithelial cytoplasm (arrow), mucoid vacuoles (V) and, to a lesser extent, into the contents of colloid follicles (F), but there is little in macrophages (M). In other mice injected with ^{35}sulfate alone, lymphocytes incorporated little label. Because of this localization the incorporation of ^{35}sulfate into macromolecular substances was equated with epithelial secretory activity. × 1200.

FIG. 14. Radioautograph of the corticomedullary border from a 5-day-old control mouse killed 2 hr after an injection of thymidine-^{3}H. In the center is a venule typical of the region, surrounded by a perivascular connective tissue space enclosed in attenuated epithelial cells (arrows). Three labeled lymphocytes, one of them mitotic (L), lie to the right of the lumen, within the endothelium of the vessel. Many of the lymphocytes in the perivascular connective tissue have incorporated thymidine also. × 1200.
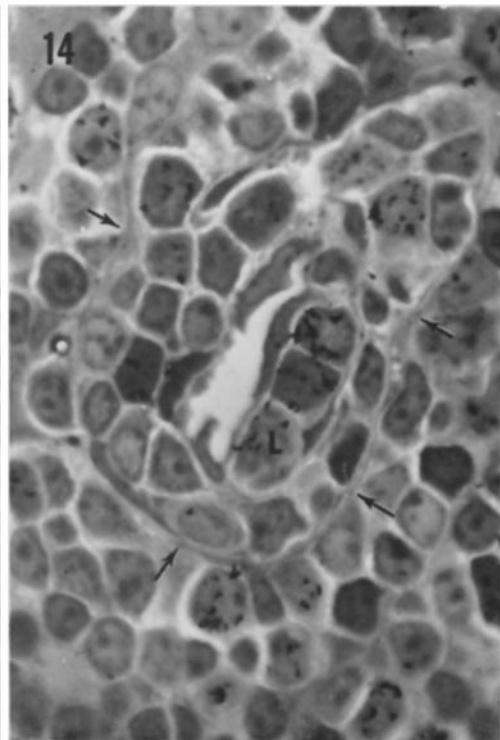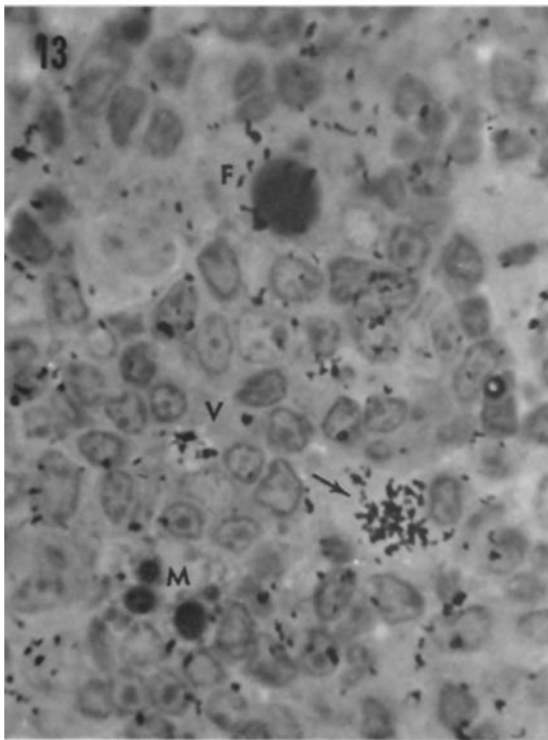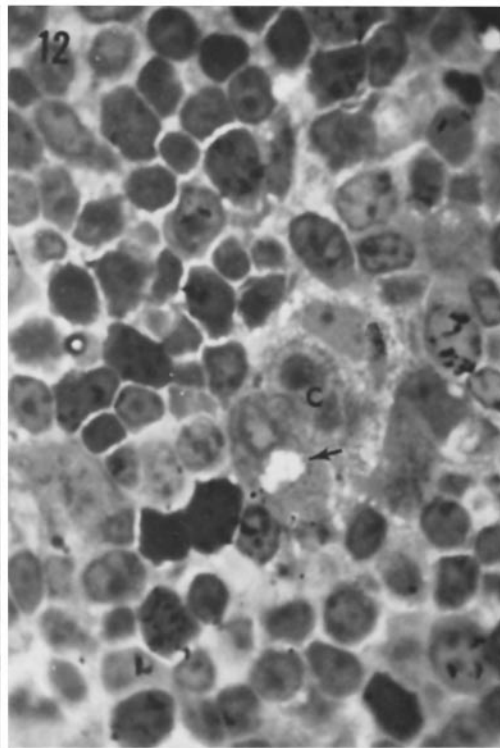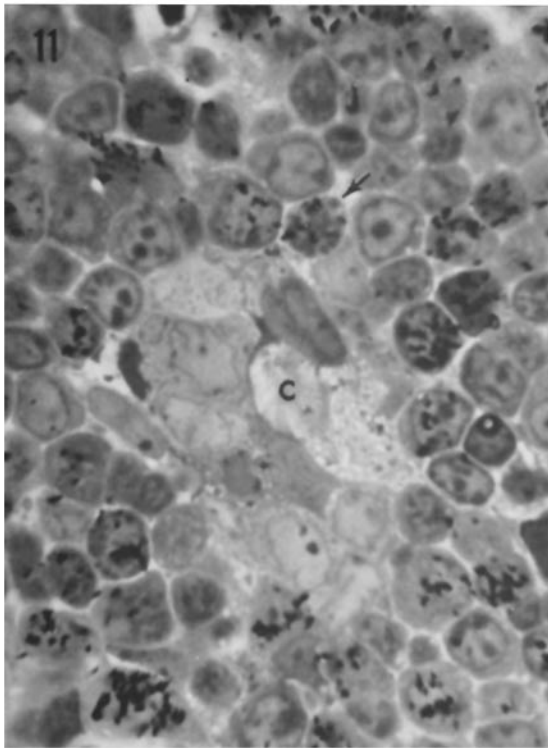
953

Fig. 15. Radioautograph of another corticomedullary venule from a 7-day-old control mouse killed 2 hr after an injection of thymidine-³H. The perivascular connective tissue enclosed by epithelial cells (arrows) also contains an arteriole (A). Lymphocytes, many of them labeled, lie within the venular lumen, within the endothelium (L), and in the perivascular space. × 1200.

Fig. 16. Radioautograph of the subcapsular cortex of a 5-day-old mouse killed 24 hr after injection of cortisol and 2 hr after injection of thymidine-³H. Although only a few lymphocytes remain, most of them pyknotic, regeneration has already begun as indicated by the high proportion of labeled lymphocytes, some of which appear pyknotic. (M, macrophages laden with cellular debris; arrow, mitotic figure.) × 1200.

Fig. 17. Radioautograph of medulla from a 3-day-old mouse killed 24 hr after injection of cortisol and 2 hr after injection of both thymidine-³H and ³⁵sulfate. Regeneration has already begun, as indicated by the many heavily labeled lymphocytes. The large cyst, typical of those found 24 hr after injection of cortisol, is surrounded by epithelial cells (nuclei marked E), some of which contain clustered vacuoles and basophilic granules (lower edge of cyst). However, there is little radioautographic evidence for incorporation of ³⁵sulfate by these cells. In contrast there is heavy label over cytoplasmic vacuoles in an isolated epithelial cell (arrow). × 1200.

Fig. 18. Radioautograph of medulla from a littermate of the mouse of Fig. 17, treated similarly. Labeled lymphocytes are not so numerous in this medulla. Epithelial cells lining the large cyst, in spite of prominent vacuoles and basophilic granules, show little evidence of having incorporated ³⁵sulfate. (E, ciliated epithelial cell.) × 1200.
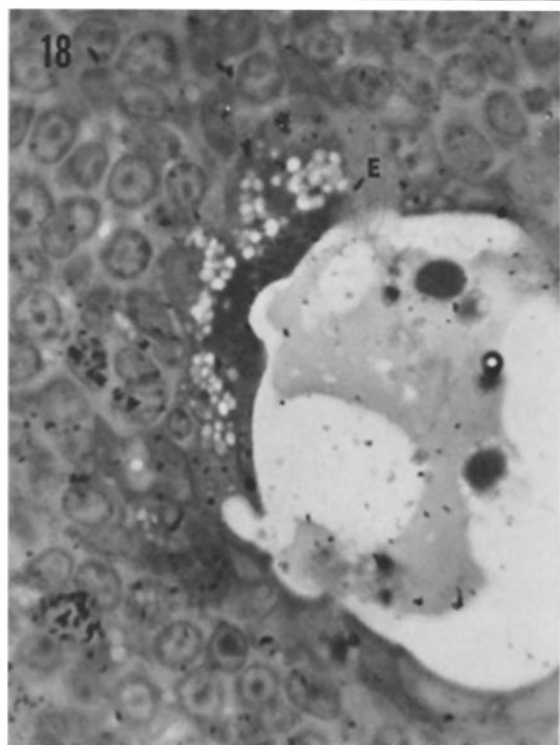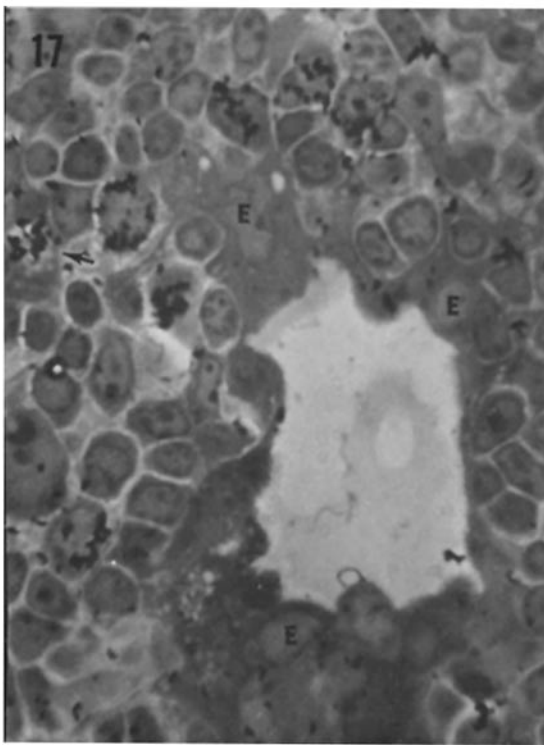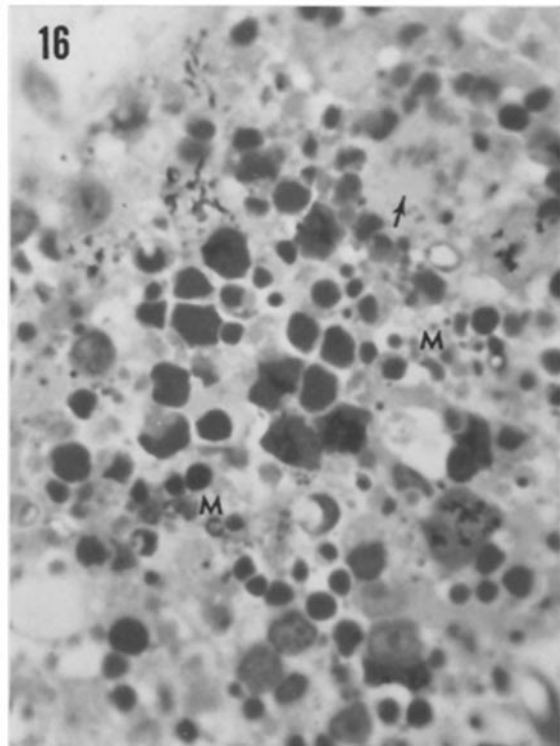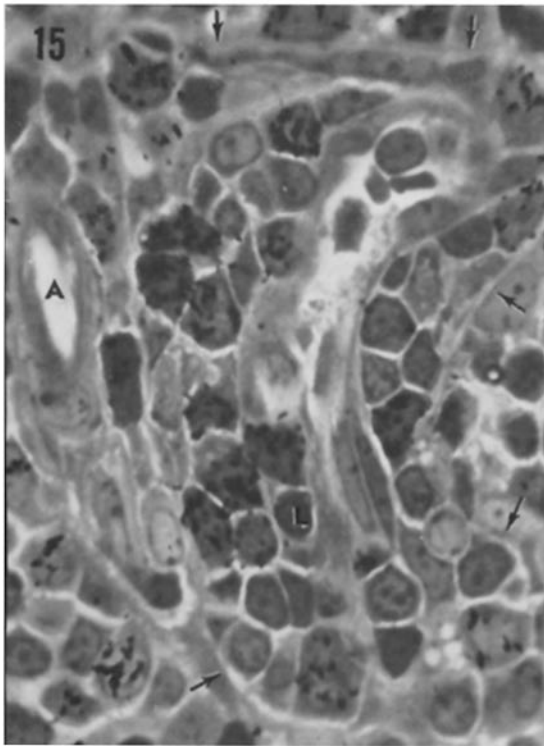
Fig. 19. Medulla from a 2-day-old mouse injected with cortisol on the day of birth. At this age regeneration began within 24 hr after injection. Mucoid vacuoles are unusually numerous in medullary epithelial cells (V) but basophilic granules (G) are no more common than in control mice. (E, incipient Hassall's corpuscle.) × 1200.

Fig. 20. Medulla from a littermate of the mouse of Fig. 19, treated similarly. Mucoid vacuoles are numerous (arrows) and there are lymphocytes in mitosis near the center of the field. × 1200.

Fig. 21. Radioautograph of medulla from an 8-day-old mouse killed 2 days after an injection of cortisol and 2 hr after injection of ^{35}sulfate. Incorporated sulfate is found in epithelial cytoplasm (E) and mucoid vacuoles (V), but not in the small ciliated cyst (C) or the adjacent colloid follicle (F). Mitotic lymphocytes are numerous (arrows). × 1200.

Fig. 22. Radioautograph of the subcapsular region from the same mouse as in Fig. 21. Incorporated ^{35}sulfate is widely dispersed in the cytoplasm of epithelial cells (E) that extend between the regenerating lymphocytes. The mitotic cell (arrow) probably is a lymphocyte, but mitosis of epithelial cells also occurs during regeneration. × 1200.