

THE FUNCTIONS OF THE THYMUS SYSTEM AND THE BURSA
SYSTEM IN THE CHICKEN*

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PLATES 11 TO 14

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In 1956 Glick et al. (1) described the role of the bursa of Fabricius, a hindgut lymphoid organ, in the development of humoral immunity in the chicken. With the subsequent definition of the pivotal role of the mammalian thymus in developmental immunobiology (2-8), the chicken aroused the interest of many investigators because it has a thymus as well as a bursa. A functional dissociation of the chicken immune system based on differences in thymic and bursal influences was originally suggested by Warner et al. (9-11). Although experimental support for this hypothesis was forthcoming from several laboratories (12-17), conflicting data were obtained in some of the studies of surgically and hormonally burssectomized and thymectomized animals. Basically unresolved were the details of the functional separation, the morphologic basis for the separation, and the degree of parallel, if any, of the development, organization, and function of the avian and mammalian lymphoid systems.

Because the lymphoid development of the chicken spleen and other peripheral lymphoid tissue seemed to be well underway at hatching, we felt that destruction of the peripheral lymphoid components combined with surgical removal of the thymus or bursa in the immediate posthatching period might provide less ambiguous models of immunologic deficiency. This effect was achieved, and on the basis of the initial studies with these models we concluded that the chicken lymphoid system is composed of two major cell systems (18, 19). The thymus is necessary for the development of a widespread cell population which consists mainly of small lymphocytes. The bursa of Fabricius appears to be the site of origin for a cell system represented in peripheral tissues by larger lymphocytes as seen in germinal centers and by plasma cells. The early work defined the

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immunologic defect of the bursectomized-irradiated chickens as 19S and 7S agammaglobulinemia with complete failure of specific antibody production. The thymectomized-irradiated chickens had depressed antibody production resembling that of neonatally thymectomized mice and rats.

The present studies are intended to present observations providing clear morphologic and functional definition of these two systems in the chicken. They show that the thymus and the system of lymphocytes dependent upon it play the same functional role in the chicken as in mammals: they are the effectors of delayed hypersensitivity and graft *versus* host reactions, and the major elements in homograft rejection. This cell system and the bursa-dependent system, each with its own specialized activity but capable of working together, execute the complex series of processes characterizing the immune responses of the chicken.

We believe, then, that the morphologic and functional dissociation of the immunologic system as defined in chickens exists in mammals, and submit that the chicken presents a particularly fortunate model in which to study differentiation, development, and function of the lymphoid tissues because in this species the two systems can be differentially manipulated.

Materials and Methods

Chickens.—The Ghostley strain (Ghostley Chicken Hatchery, Anoka, Minnesota) of non-inbred White Leghorn chickens was used in most experiments. The details of egg incubation, housing, and care were given in an earlier paper (20). Fertile California Gray (cross between White Leghorn and Barred Rock strains) eggs were also obtained from the same source for use in one experiment.

Surgical Procedures.—The methods of thymectomy and bursectomy were those used previously (17). All were performed on the 21st day of incubation, hereafter termed the day of hatching although some birds hatched earlier. The skin grafting technique was that of Cannon and Longmire (21). Skin from newly hatched chickens was always used. In wattle grafting the wattle of an adult chicken was excised and split in half; 1.5 cm squares of this tissue were grafted onto the backs of the experimental chickens, sealed along two edges by collodion *u.s.p.* and covered with Furacin[®] impregnated gauze taped into place.

X-Irradiation.—Irradiation was given the day after hatching and surgical manipulation if any. The dosage in most experiments was 650 roentgen in air at the surface. The conditions of irradiation were: 220 kv, 15 m amp with 0.25 mm copper and 1.0 mm aluminum filtration (half value layer of 0.89 mm Cu) at a dose rate of 48.3 r per min in air. The chicks were closely packed in ordinary egg cartons, placed on 15 cm of pressed wood to afford full scattering, and irradiated in a circular field of 744 cm² from a source 60 cm above the top surface of the egg cartons. Under these conditions 750 r is the LD₅₀/21 days for 1-day-old chickens. The irradiated birds in the wattle homograft and graft *versus* host assay experiments received 740 r. The conditions of irradiation were the same with the exception of the dosage rate (35.2 r/min), source distance (70 cm), and field size (1017 cm²).

Antigenic Stimulation.—10⁹ killed *Brucella abortus* organisms (United States Department of Agriculture, Ames, Iowa), 20 mg of crystallized bovine serum albumin (Armour Pharmaceutical Company, Kankakee, Illinois), and 10 mg keyhole limpet hemocyanin (prepared by centrifugation of plasma from keyhole limpets supplied by Pacific Biomarine Supply, Venice, California) were the antigen dosages used. They were administered in 0.15 M saline or complete Freund's adjuvant (100 mg killed *Mycobacterium tuberculosis* H37RV, kindly supplied by Eli

Lilly and Company, Indianapolis, in 10 ml of incomplete Freund's adjuvant, obtained from Difco Laboratories, Detroit), intra-abdominally or intravenously.

Diphtheria toxin was obtained from Eli Lilly and Company. Purified diphtheria toxoid was kindly supplied by Biologic Laboratories, Massachusetts Department of Public Health, Boston. Sensitization to diphtheria toxoid was accomplished by foot-pad injection of 1 Lf toxoid in complete Freund's adjuvant.

Antibody Determinations.—*Brucella* agglutinins were measured by a standard tube bacterial agglutination method beginning with a 1:10 dilution, and antibodies to BSA and hemocyanin by a tube hemagglutination technique using bis-diazotized benzidine linkage of antigen to rabbit erythrocytes (22, 23). Diphtheria antitoxin activity was assayed by the rabbit intracutaneous test (24).

Delayed Hypersensitivity.—Sensitized chickens were tested 7 days later for delayed allergy by intradermal wattle injection of 1 Lf diphtheria toxoid in 0.1 ml saline containing 1% normal chicken serum. The control wattle was injected with 0.1 ml serum-saline. Readings were performed at 5 and 24 hr by measuring the thickness of the wattle with Lange calipers (Cambridge Scientific Industries and Co., Cambridge, Maryland) especially constructed for measuring skin thickness in man (25); a difference of 0.4 mm or more in thickness at 24 hr between the experimental wattle and the control was considered indicative of significant response.

Graft Versus Host Assay (26, 27).—In these experiments 0.1 ml of heparinized peripheral blood from each experimental White Leghorn chicken was injected intravenously into each of eight 14-day-old California Gray chicken embryos. 5 days later the eggs were opened, each spleen weighed, and mean weight determined for each group.

Clearance of Colloidal Gold (28, 29).—A highly purified radioactive gold foil suspension Au^{198} , containing gold particles approximately 0.003μ in diameter, was obtained from Abbott Laboratories, Chicago. Each experimental chicken was injected in the wing vein with 0.5 ml of a suspension containing $5\mu\text{c}$ of radioactivity and approximately 1.25 mg of gold, and blood samples (0.6 ml) obtained by cardiac puncture at 0.5, 1.5, 2.5, 5, 10, 20, and 30 min. Gamma radioactivity was determined in a well scintillation counter and recorded as counts per minute.

Immuno-electrophoresis.—Microimmuno-electrophoresis of sera was performed by the method of Scheidegger (30). Antisera to whole chicken serum were produced by injecting whole chicken sera in complete Freund's adjuvant into the foot-pads of rabbits, followed by triweekly intramuscular injection of undiluted chicken sera for 3 wk. Antisera were harvested 1 wk later. Two antisera to chicken gamma globulin were used. One, kindly supplied by Dr. Bruce Glick and Dr. Fred McDuffy, Mississippi State University, State College, Mississippi, was prepared by immunizing rabbits with a chicken BSA-anti-BSA precipitate prepared in the presence of EDTA (31). The other was produced in large quantities by absorbing rabbit antisera to whole chicken sera with lyophilized sera from bursectomized-irradiated chickens lacking in detectable gamma globulin.

A modification of the technique of Yagi et al. (32) was used for radio-immuno-electrophoresis to identify hemocyanin antibody activity in chicken sera. The I^{131} -labeled hemocyanin was prepared by a modification of the methods of Talmage et al. (33) and McFarlane (34).

Sucrose Density Gradient Studies.—The method of Kunkel (35) was used. 0.2 ml undiluted whole chicken serum was centrifuged in a Spinco model L machine at 35,000 RPM for 15 hours. Drop samples were collected from the bottom of the tube. Folin-Ciocalteu color values of the samples were measured at $700\text{m}\mu$ in a Beckman DU spectrophotometer.

Hematology.—Total white blood cell counts were performed on wing vein blood at 7 wk of age using a Natt-Herrick stain (36) to identify the white cells among the nucleated chicken erythrocytes. Blood smears were made simultaneously and stained with Wright-Giemsa for differential white cell counting.

Autopsy.—Groups of chickens were sacrificed at 7 wk. Body and spleen weights were recorded. Thymus, spleen, bursa, or bursa area, and gut at the junction of the cecum and cecal

appendages were fixed in 10% formalin and absolute ethanol, cut, and stained with standard hematoxylin and eosin and methyl green-pyronin (37).

In all sacrificed animals that had been thymectomized or bursectomized a thorough search for remaining thymus or bursa was made. In addition tissue from the bursa site was removed from bursectomized chickens and serial sections prepared for histologic assessment of completeness of bursectomy.

Approximately 5% of the thymectomized birds had thymus tissue detectable in the gross at autopsy, consistent with our previous experience (17). Bursectomies were consistently complete, however, since no remnants were found either by gross inspection or extensive histologic examination.

RESULTS

Several models of immunologic disability in chickens are presented in the sections to follow. For convenience the groups are designated as follows: Bx, bursectomy on the day of hatching; Bx-X, bursectomy on the day of hatching with sublethal X-irradiation the next day; Tx, thymectomy on the day of hatch-

TABLE I
Effect of Thymectomy and Bursectomy on Skin Homograft Survival

Group	Graft survival at 10 days	Graft survival beyond 27 days
Control sham operated	7/17	2/17
Bursectomized	5/13	1/13
Thymectomized	6/11	2/11
Thymectomized and bursectomized	5/15	0/15

ing; Tx-X, thymectomy on the day of hatching with sublethal irradiation the next day; Bx-Tx, thymectomy and bursectomy on day of hatching; Bx-Tx-X, thymectomy and bursectomy on the day of hatching with sublethal irradiation the next day.

Thymus-Dependent Lymphoid System

As noted earlier, it is known that the thymus-dependent system of cells plays a role in specific antibody production: when it has been depleted by thymectomy and irradiation, specific antibody formation is deficient although the production of immunoglobulins continues at a normal rate (18, 19). In the sections to follow, the deficiency of "cellular immunity" in such chickens is analyzed.

Skin Homograft Rejection.—In the first experiment, 2-wk-old Bx, Tx, Bx-Tx, sham-Bx, and sham-Tx chickens received skin homografts from newly hatched donors on one side of the back and skin autografts on the other. Graft readings were begun on the 10th day. Autografts were successful in 96% of the experimental birds. Table I gives the results of this experiment: none of the surgical procedures had a significant effect on skin homograft survival.

In the second experiment, irradiation with 650 r was added to bursectomy and thymectomy in newly hatched chickens. In these groups grafting was delayed until 3 wk of age to allow recovery from the acute effects of irradiation. As in the first experiment newly hatched chickens of the same noninbred strain served as donors. Autografts were successful in 92% of the experimental birds. As shown in Table II, significant prolongation of graft survival was seen only in the Tx-X group; in 5 of the 12 animals grafts were intact at 27 days, trebling the normal survival time.

In another homograft experiment adult wattle grafts were employed. Here the irradiation dosage was increased to 740 r, again given on the day after hatching. The recipients were 5 wk old at time of grafting. In this instance collodion was not applied over the surface of the homograft and readings were begun on

TABLE II
Effect of Thymectomy-Irradiation and Bursectomy-Irradiation on Skin Homograft Survival

Group	Graft survival at 10 days	Graft survival beyond 27 days
Control non X-irradiated	0/10	0/10
Control X-irradiated	1/15*	0/15
Bursectomized and X-irradiated	0/15	0/15
Thymectomized and X-irradiated	5/12	5/12

* Although not completely rejected, this graft showed edema and pallor indicating active rejection at the time of examination.

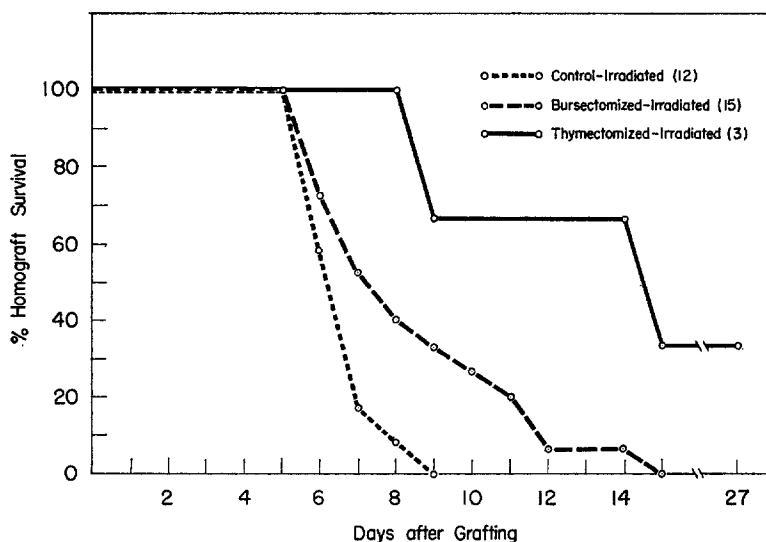
the 5th day after grafting. Again, although the group of Tx-X birds was very small, markedly prolonged homograft survival was seen only in these chickens (Text-fig. 1). Although Bx-X birds again did not tolerate homografts for prolonged periods, a difference in the rejection rate is seen when this group is compared to the controls. This difference in vigor of graft rejection was particularly striking on the 9th day after grafting when 5 of 15 Bx-X birds retained healthy appearing pink homografts while all 12 control birds exhibited black necrotic graft tissue.

It must be concluded from these observations that the thymus and its system of cells are those primarily responsible for skin homograft rejection. Nonetheless, the bursa and its system of cells seem to play a minor role in the skin homograft response in the chicken.

Delayed Hypersensitivity.—In this experiment 4-month-old thymectomized-irradiated chickens and control-irradiated chickens were sensitized with diphtheria toxoid in Freund's adjuvant by the foot-pad route, and tested 7 days later by injection of this antigen into the wattle. None of the birds had detectable circulating antibody to diphtheria toxin at this time. While all of the control

chickens had positive wattle reactions beginning after 5 hr and reaching maximal intensity by about 24 hr, only 2 of 14 Tx-X chickens had significant wattle reactions (Table III). Bursectomized-irradiated birds were not used in this experiment as few of this group survived long enough to grow testable wattles.

In separate experiments in which bursectomy was performed without the adjunct of irradiation, complete removal of the bursa of Fabricius at hatching did not appear to affect the capacity to develop delayed reactions to diphtheria toxoid (38).



TEXT-FIG. 1. Effect of thymectomy, bursectomy, and irradiation on wattle homograft survival. Thymectomy and bursectomy were performed on the day of hatching and 740 r of X-irradiation given the following day. Grafting of adult wattle homografts was performed 5 wk posthatching. Slight homograft prolongation was observed in bursectomized-irradiated chickens although marked prolongation was again observed only in the thymectomized-irradiated chickens.

In observing the Tx-X and control birds, it became apparent that the thymectomized chickens did not manifest the severe adjuvant reactions of the feet that characterized the control group. In fact, the chickens could easily be separated into experimental groups on the basis of size and severity of adjuvant reactions in their feet (Fig. 1).

It is apparent from these observations that the expression of delayed hypersensitivity to diphtheria toxoid depends on the thymus-dependent system of cells, as does the violent local cellular reaction observed after inoculation of killed tubercle bacilli in oil.

Graft versus host reactivity.—The model of graft *versus* host reactivity chosen

was the Simonsen assay in which peripheral blood from 3-wk-old chickens of the different experimental groups was injected intravenously into 14-day-old California Gray chick embryos. 5 days later the recipient embryos were sacrificed and their spleens weighed, the degree of splenic enlargement reflecting graft *versus* host reactivity. A mean was calculated for each donor from the spleen weights of the several surviving embryos receiving cells from that donor. Since highly inbred chickens were not available, control spleens from uninjected chick embryos at the same stage of incubation were employed in the experimental evaluations.

There were three experimental groups: Bx-X, Tx-X, and a control-irradiated group. The surgical procedures had been performed on the day of hatching, and 740 r of irradiation given the next day. As seen in Table IV, the Bx-X chickens

TABLE III
*Effect of Thymectomy and/or Irradiation on Delayed
Allergic Reactions to Diphtheria Toxoid*

Group	No. positive* total No.	Average wattle thickness in millimeters at 24 hr†	
		Control (normal saline)	1 Lf diphtheria toxoid
Control X-irradiated	10/10	1.50	3.18
Thymectomized and X-irradiated	2/14	1.51	1.79

* Test wattle 0.4 mm thicker than control.

† Chickens sensitized at 4 months of age by foot-pad injections of 1 Lf diphtheria toxoid in complete Freund's adjuvant and tested 7 days later.

were as competent as the controls with respect to capacity of their blood cells to induce graft *versus* host reactions. On the other hand, the Tx-X chickens showed significantly less capacity to express graft *versus* host reactivity than did either the Bx-X or control animals. Peripheral blood from some of the chickens in the thymectomized-irradiated group produced no white nodular lesions visible in the gross and no significant splenic enlargement, whereas blood from other chickens manipulated in the same way regularly produced some gross lesions and significantly larger mean spleen size than in control embryos.

These results indicate clearly that the thymus-dependent system of cells is essential for expression of graft *versus* host reactivity, but that even with 740 r of total body irradiation combined with thymectomy in the immediate post-hatching period, it is difficult to eliminate this cell system.

Peripheral White Blood Cells.—Total and differential white blood cell counts were performed on 7-wk-old Tx-X, Bx-X, Tx-Bx-X, and control chickens. The irradiation dosage had been 650 r. As shown in Table V, thymectomy and irradi-

ation results in a lowered blood lymphocyte level. Although large and small lymphocytes were not tabulated, decreased small lymphocytes appeared to account for most of this reduction in total lymphocytes, consistent with the observations of others in chickens thymectomized in the newly hatched period (10, 11, 16). Neither bursectomy-irradiation nor irradiation alone affected the level of circulating lymphocytes. A surprising effect was the substantial increase in the number of circulating heterophils in the Bx-X group. Since many of these agammaglobulinemic chickens later die of infection, this heterophilic leuko-

TABLE IV
*Effect of Thymectomy-Irradiation and Bursectomy-Irradiation on
Simonsen Assay of Graft Versus Host Reactivity*

White Leghorn donor group	No.	No. of California Gray test embryos	Mean spleen weight of recipient embryos*	Standard error mean
			mg	
Control X-irradiated.....	12	82	74.0	3.81
Bursectomized X-irradiated.....	14	96	78.5	4.05
Thymectomized X-irradiated.....	9	64	43.0†	3.65
—	—	21	9.3	0.71

* The graft *versus* host reaction was produced by injecting 0.1 ml of whole blood from each chicken into eight 14-day-old embryos. The spleen weights from the embryos surviving 5 days were used as the basis for calculating the mean for each experimental animal.

† The difference between the thymectomized-irradiated and control-irradiated chickens was highly significant. This difference is 5.89 times the standard deviation of the difference as estimated by the square root of the pooled variance based on the 242 experimental spleen weights included in the 3 groups of chickens. The difference between control-irradiated and bursectomy-irradiated groups was not significant. All groups showed significant increases in spleen weight over noninjected controls.

cytosis could be a response to unrecognized infection present at the time of study.

Lymphoid Tissue Structure.—Neonatal thymectomized mice and rats suffer a persistent deficit of small lymphocytes and a reduction in mass of spleen, lymph nodes, and certain intestinal lymphoid tissue (5, 6, 39). A relative deficit of small lymphocytes in the tissues of chickens thymectomized in the newly hatched period has also been observed (10, 11, 16). In the present experiments, we compared the lymphoid tissue morphology of the spleen and the cecum at the junction of the cecal appendages in intact, intact-irradiated, and thymectomized-irradiated chickens at 7 wk of age.

In both the spleen and cecum, the Tx-X group showed marked depletion of small lymphocytes compared to the essentially normal numbers in the X-irradiated animals examined. Neither group showed any apparent reduction in

numbers of germinal centers and plasma cells. Illustrated in Fig. 2 to 4 are the characteristic morphologic appearances of the spleen in these groups.

In our earlier experiments, thymectomy alone in the newly hatched period did not result in the striking depletion of the peripheral small lymphocyte population seen in the thymectomized-irradiated chickens (40).

Bursa-Dependent Lymphoid System

The initial experiments (18), of which these are an extension, defined the bursa-dependent lymphoid system morphologically as the germinal centers and plasma cells in the peripheral tissues and functionally as the immunoglobulin-

TABLE V
*Peripheral White Blood Cell Counts in Experimental Groups of Birds**

Group	No.	Mean No. total white blood cells	Mean No. lymphocytes	Mean No. heterophils
Controls	24	22,791	14,544	7,190
Control-Irradiated	19	19,526	13,446	5,231
Bursectomized-Irradiated	9	23,667	13,260	9,093
Thymectomized-Irradiated	13	16,154	9,145†	6,010
Bursectomized-Thymectomized-Irradiated	8	18,125	6,796†	10,057

* Total white blood cell counts were made by direct counting of white cells after vital staining with Natt-Herrick dye mixture. Differential counts were made on standard peripheral blood smears stained with the Wright-Giemsa combination.

† The differences between these mean lymphocyte counts and those of the other groups were highly significant, based on F ratios with 1 degree of freedom in the numerator and 45 degrees of freedom in the denominator. F ratios were 11.7 for the thymectomy-irradiation and control irradiation group, and 1.08 for the bursectomy-irradiation and control irradiation groups.

producing system. The agammaglobulinemic Bx-X chickens produced no antibody on primary stimulation with bovine serum albumin and *Brucella abortus*.

In the preceding sections we have presented evidence that Bx-X chickens have slightly impaired homograft immunity, relatively intact graft *versus* host reactivity in the Simonsen assay, and normal levels of total leukocytes and lymphocytes, with some increase in heterophils. In the following sections, we present a further definition of the bursa-dependent system.

Antibody Production.—A large group of chickens was bursectomized on the day of hatching; some of them were given 650 r of X-irradiation the next day together with a group of intact birds of the same age. At 40 days of age all were immunized with 20 mg of BSA and 10⁹ *Brucella abortus* cells. 9 days later all birds were bled, and 6 of each group were killed and autopsied.

Table VI gives the results of the antibody determinations. None of the Bx-X

animals produced detectable antibody to either antigen. As expected, most of the Bx chickens were also lacking antibody to the two antigens; however, 2 of 11 responded to both antigens, and 2 additional birds responded to *Brucella* but not to BSA. Irradiation alone did not produce lasting inhibition of capacity to produce circulating antibodies.

In an attempt to bring out latent antibody producing capability in the Bx-X chickens, intense antigenic stimulation was given by combining intramuscular injection of BSA and *Brucella abortus* organisms in complete Freund's adjuvant with multiple intravenous injections of these antigens in saline. Even after this intensive antigenic stimulation, we were unable to detect antibody formation in the bursectomized-irradiated chickens (Text-fig. 2). The irradiated control

TABLE VI
*Effect of Early Bursectomy and X-Irradiation on Antibody Response in Chickens**

Group	<i>Brucella abortus</i>			Bovine serum albumin		
	No. responding total No.	Mean antibody titer ($\log_2 + 1$)	Mean standard error	No. responding total No.	Mean antibody titer ($\log_2 + 1$)	Mean standard error
Bursectomized and X-irradiated	0/14	—	—	0/14	—	—
Bursectomized	4/12	2.75	0.81	2/11	2.36	0.97
Control X-irradiated	15/16	8.10	0.79	16/16	5.81	0.98

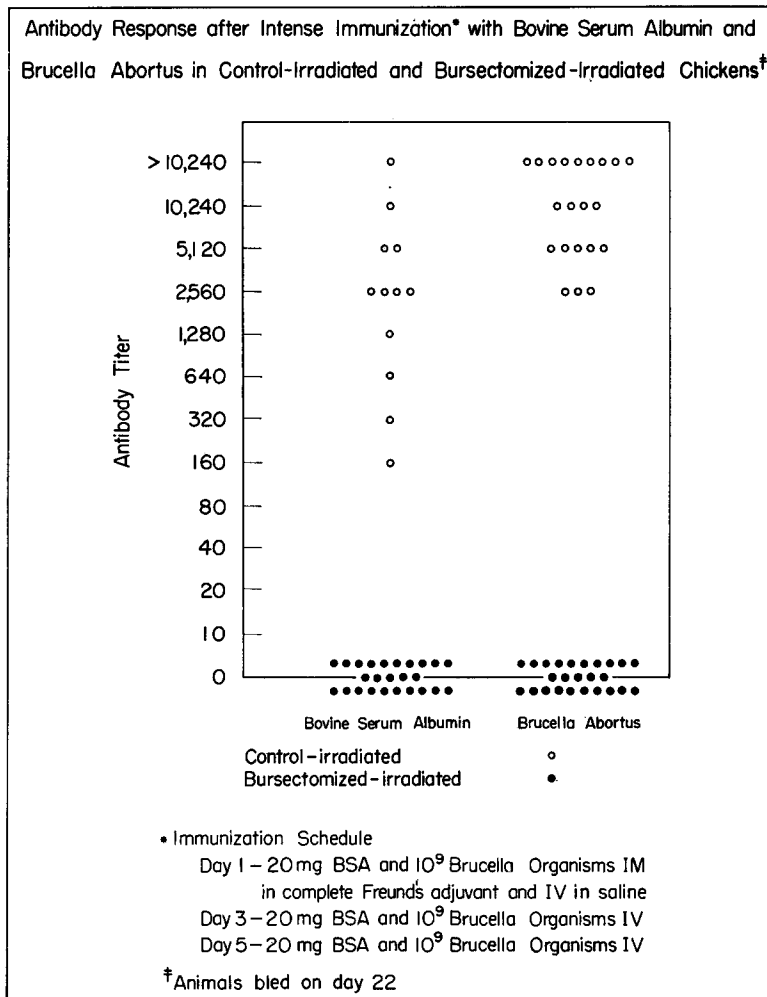
* The chickens were immunized intraabdominally at 40 days of age with 10^9 killed *Brucella abortus* organisms and 10 mg BSA. They were bled 9 days later.

chickens were very responsive to this method of immunization to both BSA and *Brucella*.

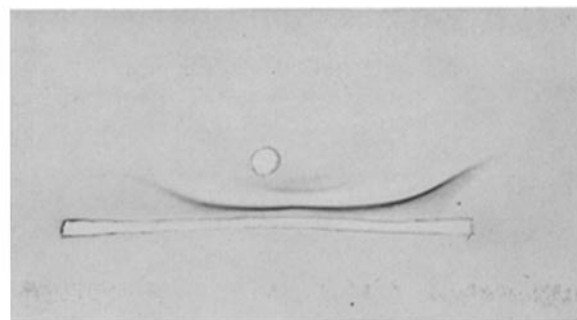
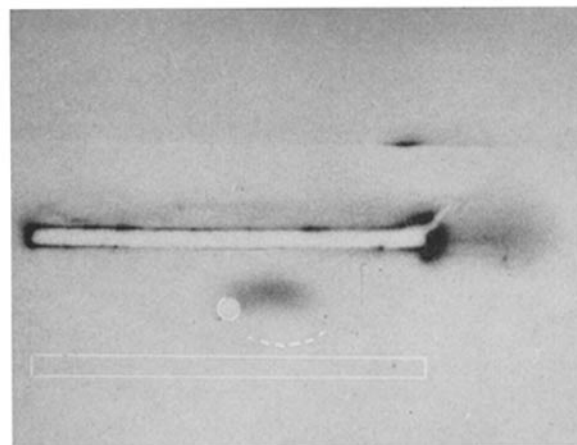
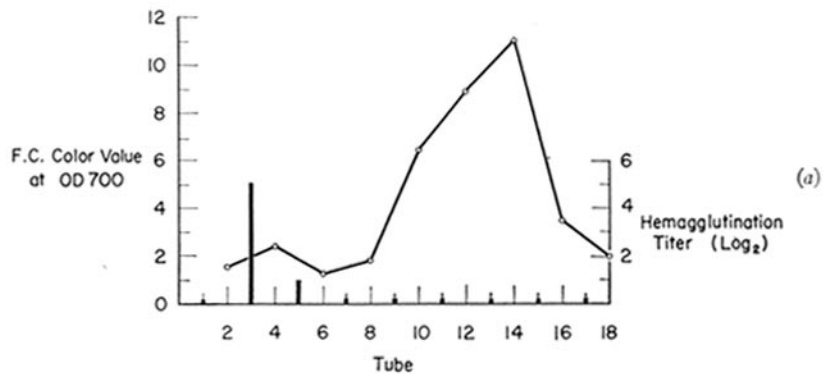
Lymphoid Tissue Structure.—As in our prior experiments with the Bx-X chickens (18) we found no germinal centers or plasma cells in sections of the spleen (Fig. 5). In addition, a search for germinal centers and plasma cells in the lymphoid area at the junction of the cecum and the cecal appendages was also unavailing in these animals. By contrast, both the Bx chickens and the control irradiated group regularly had germinal centers (Figs. 2 and 6) and plasma cells in the spleen and gut (Fig. 7). These were located with ease in material from both peripheral sites and appeared to be present in normal numbers although they were not counted.

Germinal centers are not recognizable until 2 to 3 wk after hatching and significant numbers of plasma cells appear after this age period in both bursectomized and intact chickens. Since bursectomized chickens go on to develop normal germinal centers and plasma cells, continued presence of the bursa is not necessary for the differentiation of germinal centers and plasma cells from their precursors in the early posthatching period, although it is necessary to adequate functional maturation.

Immunoglobulins.—In normal 7-wk-old chickens we identified two chicken immunoglobulin components by localization of hemocyanin antibody activity on radioimmuno-electrophoresis. In the early period following immunization, antibody to hemocyanin may be localized in an immunoelectrophoretic band morphologically similar to human gamma M globulin and in the lower fractions of a sucrose density gradient after ultracentrifugation (Text fig. 3). Later anti-



TEXT-FIG. 2. Antibody responses after intense stimulation of bursectomized-irradiated and control-irradiated chickens. As noted, the antigens were initially administered intramuscularly in Freund's adjuvant, followed on days 3 and 5 by intravenous injection of similar doses of BSA and *Brucella* in saline. High titers were attained in the controls by day 22, but the Bx-X birds remained completely unresponsive.

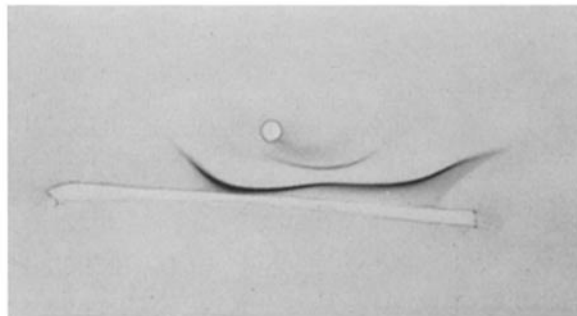
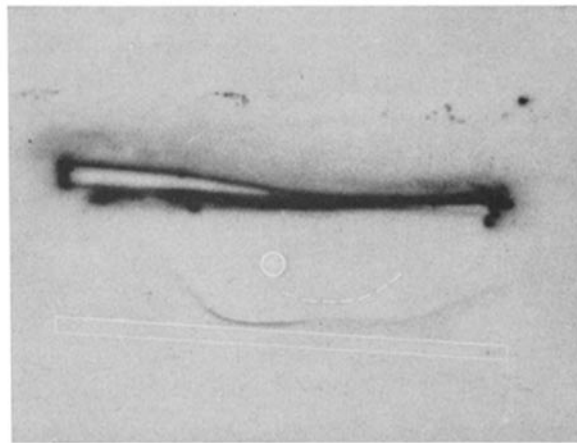
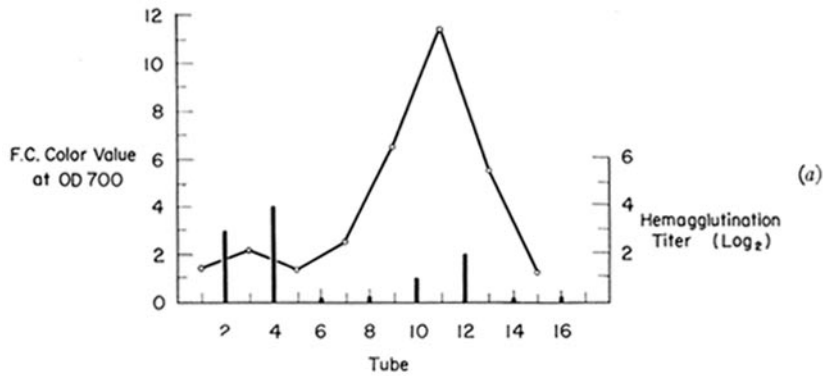


TEXT-FIG. 3. Analysis of antibody activity against keyhole limpet hemocyanin (KLH) in normal chicken (No. 55) serum 4 days after primary immunization with 10 mg KLH.

(a) Fractionation of serum by density gradient ultracentrifugation. Protein determination was performed by optical density measurement at $700\text{ m}\mu$ after addition of Folin-Ciocalteu (F.C.) reagent to even tube fractions. Log_2 titers of odd tube fractions for keyhole limpet hemocyanin hemagglutinating antibody are indicated by bars in the figure. Antibody activity was detected only in the lower fractions of the density gradient.

(b) Detection of KLH antibody activity by radioimmuno-electrophoresis. After electrophoresis of immune chicken serum, I^{131}KLH was placed in upper trough and rabbit antiserum to chicken gamma globulin was placed in lower trough. The dashed white line was added to identify a sharp band of radioactivity that might not otherwise be seen after reproduction and printing. This immunoglobulin band containing antibody activity is morphologically similar to human gamma M. Similar treatment of unimmunized chicken serum showed no localization of radioactivity from I^{131}KLH .

(c) Corresponding immunoelectrophoretic pattern of chicken serum reacted against rabbit antiserum vs. chicken gamma globulin.



TEXT-FIG. 4. Analysis of antibody activity against KLH in normal chicken (No. 55) serum 6 days after secondary immunization with 10 mg KLH.

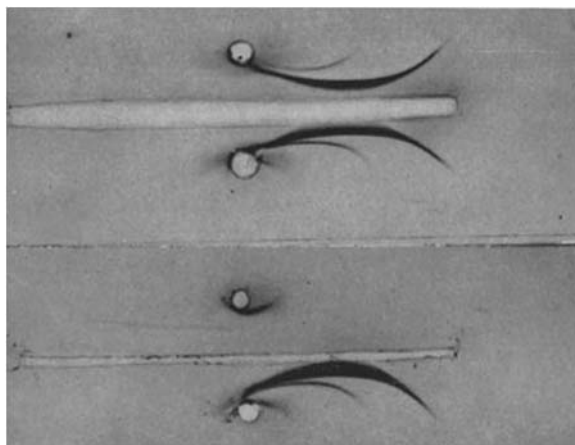
(a) Fractionation of serum by density gradient ultracentrifugation. Log₂ titers of odd tube fractions for KLH hemagglutinating antibody are represented by bars. Protein determinations were performed by optical density after addition of Folin-Ciocalteu (F. C.) reagent to even tube fractions. A bimodal distribution of antibody activity is seen indicating production of both heavy and light antibodies.

(b) Detection of KLH antibody activity by radioimmuno-electrophoresis. After electrophoresis of immune chicken serum, ¹³¹I-KLH was placed in upper trough and rabbit antiserum to chicken gamma globulin was placed in lower trough. The dashed white line was added to identify a sharp band of radioactivity that might not otherwise be seen after reproduction and printing. Antibody activity is now seen in both immunoglobulin bands, morphologically quite similar to human gamma G and gamma M immunoglobulins.

(c) Corresponding immunoelectrophoretic pattern of chicken sera reacted against rabbit antiserum vs. chicken gamma globulin.

hemocyanin antibody activity is also found in the immunoglobulin which is similar to human gamma G in behavior on immunoelectrophoresis and ultracentrifugal analysis (Text-fig. 4). These two immunoglobulins will be referred to as chicken gamma M and gamma G, as they have been by Dreesman et al. (41) who also identified in older chickens an immunoglobulin corresponding to human gamma A.

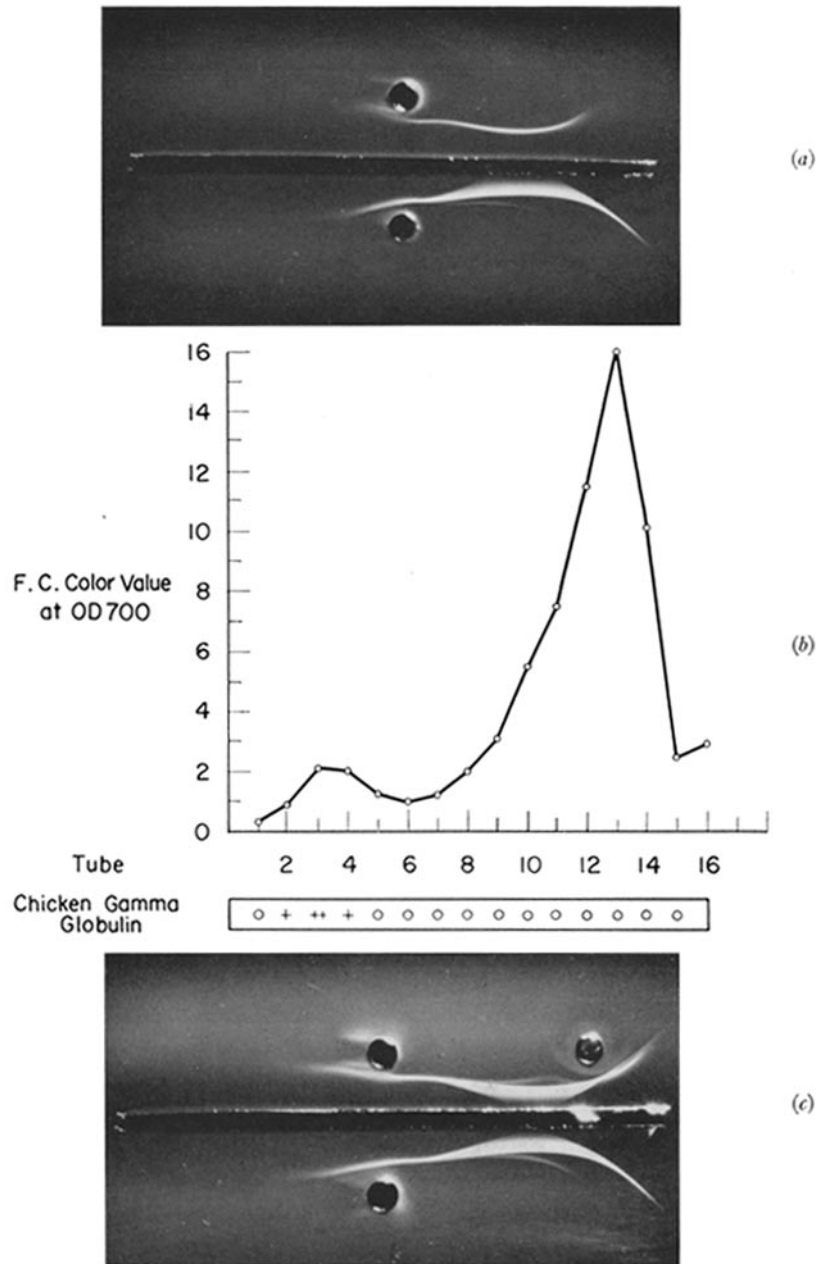
Representative immunoelectrophoretic patterns of 7-wk-old sera from Bx, Bx-X, and control chickens are illustrated in Text-fig. 5 and 6. For comparison



TEXT-FIG. 5. Immunoelectrophoretic analysis of chicken gamma globulins in experimental birds at 7 wk of age; thymectomized-irradiated chicken serum (top well), control chicken serum (second well), bursectomized-irradiated chicken serum (third well), and thymectomized-irradiated chicken serum (bottom well), developed against rabbit antiserum to chicken gamma globulin (troughs). There appear to be no significant differences with the exception of the virtually complete absence of the gamma globulin bands of the bursectomized-irradiated birds. By this criterion they are agammaglobulinemic.

the pattern of a Tx-X chicken of the same age is also shown. Both gamma M and gamma G globulins are regularly absent by this technique in birds subjected to bursectomy and irradiation immediately after hatching. By contrast, birds subjected to bursectomy alone frequently lack gamma G but regularly possess gamma M (Text-fig. 6), a finding similar to that presented by Ortega and Der (42). In our studies of the latter group, we found the gamma M globulin to be increased in most instances and often strikingly so. A few Bx birds had essentially normal levels of both gamma M and gamma G globulins. In control groups and in the Tx-X chickens, both immunoglobulins were regularly present in what appear to be normal amounts.

Thus we have defined two models of immunologic deficiency involving the bursa-dependent system: the Bx model involving greatly reduced capacity for



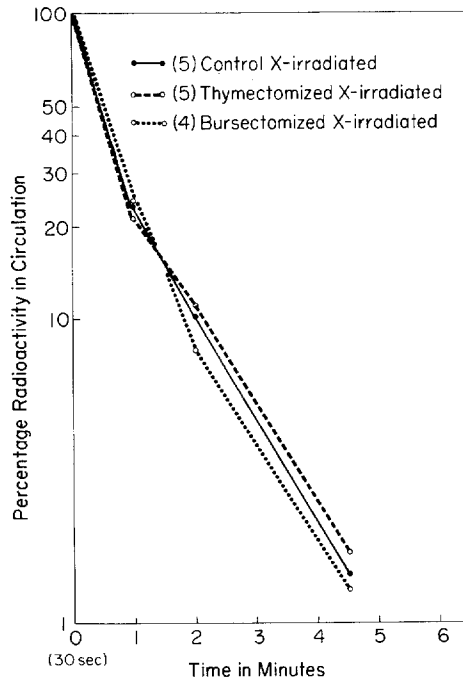
TEXT-FIG. 6. Analysis of gamma globulin in serum from 7-wk-old chicken 47 which was bursectomized at hatching.

(a) Immunoelectrophoretic pattern of bursectomized chicken serum (top well) compared with control chicken serum (bottom well). Antiserum to chicken gamma globulin in trough was prepared by absorbing rabbit antiserum to whole chicken sera with lyophilized agammaglobulinemic Bx-X serum. Note the single gamma band in the Bx serum.

(b) Sucrose density gradient analysis of the bursectomized chicken serum. Gamma globulin was detected only in the lower fractions of the density gradient, as was marker human rheumatoid factor activity in another experiment.

(c) Normal chicken serum was electrophoresed from both middle wells. The top right well was then filled with the bursectomized chicken serum and rabbit antiserum to chicken gamma globulin placed in the trough. Note the band of identity between bursectomized and normal 19S globulin and lack of identity with normal 7S gamma globulin.

specific antibody production, relatively normal development of germinal centers and plasma cells in the periphery, and, often, an immunoglobulin deficit involving greatly reduced 7S immunoglobulin production and, at times, greatly increased 19S immunoglobulin synthesis approaching that of some clinical dysgammaglobulinemias (43-45) and the Bx-X model involving complete failure of specific antibody production, uniform lack of germinal centers and plasma



TEXT-FIG. 7. Effect of early thymectomy, bursectomy, and irradiation on clearance of colloidal gold (Au^{198}) from the circulation in 1-month-old chickens. Neither thymectomy plus irradiation nor bursectomy plus irradiation result in diminished reticuloendothelial clearance of this particulate substance from the blood stream.

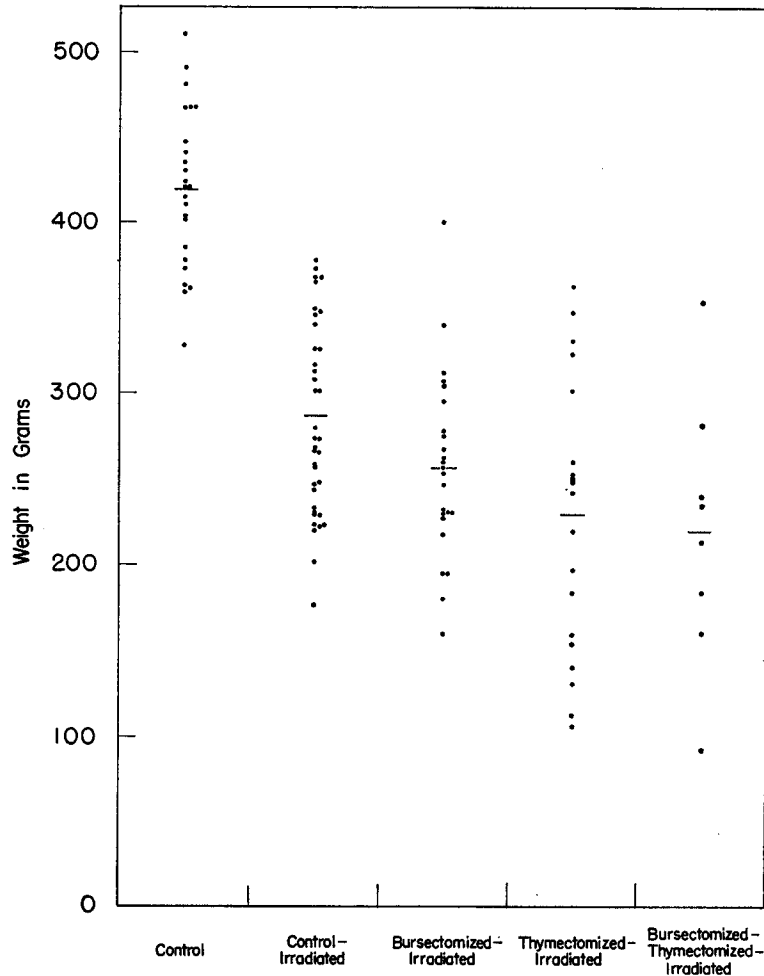
cells in the periphery, and 7S and 19S agammaglobulinemia by immunoelectrophoretic criteria.

Functional Evaluation of Reticuloendothelial System

The rate of clearance of colloidal gold is considered to reflect the capacity for clearance of particulate material from the circulation by the reticuloendothelial system (RES). This experiment was performed to test whether an extreme deficit of the thymus- or bursa-dependent system would affect this ability, particularly whether circulating antibody is necessary for *in vivo* phagocytosis

of particulate substances and soluble antigens, as it seems to be for some, if not all, bacteria (46).

Colloidal gold (Au^{198}) was injected intravenously into 1-month-old Tx-X, Bx-X, and control chickens. The mean percentage of radioactivity remaining in the circulation is plotted for each experimental group in Text-fig. 7 as a function



TEXT-FIG. 8. Effect of thymectomy, bursectomy, and irradiation on growth in chickens. Thymectomy and/or bursectomy was performed on the day of hatching and 650 r X-irradiation given the following day. Individual total body weights of experimental chickens at 7 wk of age are represented by dots in scattergram. Significant retardation in growth is observed in all irradiated groups. Thymectomy resulted in a further significant suppression of growth while bursectomy did not.

of time after injection. No difference in colloidal gold clearance is seen among the different groups. These results suggest that neither the thymus- nor the bursa-dependent system plays a direct role in this reticuloendothelial function. Further, the results show that neither immunoglobulins nor specific antibody seems to play a significant role in this function of the RES.

In further studies (47) it was shown that clearance from the circulation of radioiodine-labeled hemocyanin by Bx-X birds did not differ from that of controls.

*Body Growth Following Thymectomy, Bursectomy, and Irradiation
in the Immediate Posthatching Period*

Prior studies have indicated that neonatal thymectomy in mice regularly results in runting and ultimately wasting disease and early death. Studies with germfree animals indicate that these developments are dependent on availability of a microbial flora (48, 49). It was of interest then in these experiments to determine whether the forms of immunologic deficiency produced in the Bx-X and Tx-X chickens would influence their growth. Compared in Text-fig. 8 are the body weights at 7 wk of age of normal chickens, irradiated controls, and groups of Bx-X, Tx-X, and Bx-Tx-X animals. It will be seen in the figure that all of the groups of treated chickens grew less well than the unmanipulated controls. The lowest mean weight was observed in the Tx-X and Tx-Bx-X groups, and the differences between these groups and the irradiated controls are significant statistically. By contrast, the Bx-X group did not show significantly greater growth failure than did the control irradiated chickens. These conclusions were based on F ratios with one degree of freedom in the numerator and 84 in the denominator. The values for the F ratio were computed as 1.17 and 11.0 for the Bx-X and Tx-X groups respectively.

DISCUSSION

The observations presented in this paper are of interest for several reasons. Firstly, they provide substantial support for the concepts implicit in the discoveries of Glick et al. (1, 50, 51), Mueller et al. (52, 53) and Miller (4, 5), Good et al. (6, 7) and Waksman et al. (8, 39, 54) that both the bursa of Fabricius and the thymus are organs of fundamental importance in development of the lymphoid system and immunologic competence. In addition, they strongly support the contention of Warner and Szenberg (9-11), Aspinall et al. (12), Waksman et al. (8, 39, 54) and Janković et al. (14-16) that the immunologic functions collectively referred to as cellular immunity can be sharply dissociated from those immunologic functions resulting in formation of circulating antibody and gamma globulins. We have presented evidence herein that irradiation when combined with central lymphoid organ extirpation in the newly hatched chick brings the separate roles of the major central lymphoid organs of the chicken

into sharp focus. We have further shown that irradiation together with thymectomy in newly hatched chickens produces the same immunologic defects in these animals that neonatal thymectomy produces in mice. Chickens manipulated in this way are deficient in circulating lymphocytes, lack a substantial population of small lymphocytes in their peripheral lymphoid organs, e.g. spleen and cecal-appendiceal junction, are lacking in ability both to develop delayed allergic reactions and exhibit graft *versus* host reactions, and also are deficient in capacity to reject homografts of skin. In addition, other experiments (18, 19) show that such chickens are quantitatively deficient in capacity to form circulating antibodies against certain antigens, even though they possess normal or near normal amounts of the two immunoglobulins studied, 19S (gamma M) and 7S (gamma G). These animals further show growth failure and die early. Thus, in every parameter, the chicken deprived of its thymus when the lymphoid tissue in the periphery is deficient shows the same developmental defects and the same consequences of these developmental defects as does the mouse thymectomized in the neonatal period.

By contrast, chickens irradiated at hatching and subjected as well to complete extirpation of the bursa of Fabricius seem to develop the system of small lymphocytes in the peripheral lymphoid tissue in normal fashion. As would be appropriate, they reject skin homografts and show normal graft *versus* host reactions. They are, however, prevented from developing the two clearly definable immunoglobulins and are completely unable to form circulating antibodies even in the face of strong antigenic stimulation. As a basis for this immunologic defect we find failure of development of germinal follicles and plasma cells. No recognizable deficit in this latter system of cells is observed in chickens irradiated and thymectomized in the newly hatched period.

It seems most important to realize that we can now speak of the thymus and its system of dependent cells, and the bursa and its system of dependent cells, and can identify, morphologically at least, the major components of these two separate systems. It seems of further significance that each of the two systems can, as in these experiments, develop independently of the other and of the other's central component once differentiation has proceeded beyond a critical point.

Looked at in another way, these data indicate that at some point differentiation along two distinctly different pathways occurs within the lymphoid system and that the critical point seems to focus about two separate central lymphoid organs which in the chicken derive from polar positions in the gastrointestinal tract.

One of the most useful aspects of these studies is the experimental models which they provide for intact cellular immunity in the absence of significant immunoglobulin and antibody production, deficient cellular immune processes in the presence of normal ability to make immunoglobulins, and extreme

deficiency of all immunologic parameters. Further, these models may be related in a most meaningful way to genetically determined human diseases of immunologic deficiency. For example, in the Bruton type of agammaglobulinemia (55) the thymus develops and the thymic system of cells seems to develop and function normally (56), whereas germinal centers and plasma cells, together with immunoglobulins and ability to make circulating antibody, are lacking (55, 57-60). How strikingly similar this clinical disorder is to the experimental disorder produced by irradiation and complete extirpation of the bursa of Fabricius in the chicken! In another clinical syndrome, well defined by Allibone et al. (61) and Nezelof et al. (62) there is deficient thymic development and apparent lack of the thymic system of cells in the periphery, in the presence of germinal centers and plasma cells as well as immunoglobulins. Such patients are immunologically deficient and seem to be the closest clinical analogue of the neonatally thymectomized mouse and the thymectomized-irradiated chicken. The irradiated-thymectomized-bursectomized chicken, agammaglobulinemic, lymphopenic, and lacking both effective humoral and cellular immune responses, approaches the defect, inherited as a simple autosomal Mendelian recessive trait, and so well defined by the Swiss investigators (63-66). Such patients are lacking in all the responses of adaptive immunity. Further study of these clear-cut experimental models may well shed further light on the nature of these human diseases and provide guidelines for more effective management of such patients.

Interesting also in this light is our demonstration that bursectomy in newly hatched chickens, when not combined with irradiation, often leads to 7S agammaglobulinemia, just as described by Ortega and Der (42). In our chickens manipulated in this way, those that lack 7S gamma globulin regularly show greater than normal amounts of the higher molecular weight, higher charge density immunoglobulin. In other chickens subjected only to bursectomy at hatching, the 7S is formed and the increase in the heavier immunoglobulin does not occur. It may be that in the latter animals the bursa was not completely removed, although we have no evidence that this is the case. More likely, perhaps, is the possibility that birds showing this response to bursectomy had more advanced lymphoid development in the periphery when bursectomy was carried out. Whatever the basis for these observations, they suggest several explanations both for the experiments themselves and for the syndrome in man which has been labeled dysgammaglobulinemia (43-45). One possibility is that preventing normal formation of 7S immunoglobulins and antibodies removes an important basis for a negative feedback mechanism capable of shutting off 19S immunoglobulin formation (67, 68). Still another possibility is that the natural transition from a capacity to synthesize only 19S immunoglobulin to responsiveness which includes a 7S component might involve an inducible enzyme or a repressor-derepressor genetic mechanism activated during differentiation by a bursal hormone. That the 19S globulins can be formed in such chickens is

attributable to the fact that precursors of the germinal centers and plasma cells are already in the periphery at the time of bursectomy. A final possible explanation for the dysgammaglobulinemia of chickens bursectomized in the newly hatched period is that failure to develop a vigorous 7S response might leave an immunologic gap which indirectly, by failure to prevent or eliminate infection or infestation, could foster excessive antigenic stimulation, the animal responding according to its remaining potential, namely synthesis of 19S immunoglobulin in excess. The several possibilities are not mutually exclusive and more than one may be operating.

A central question raised by these data concerns the origin of the cells of the two systems. Evidence has been presented in mammals that cells of the thymic system may come from the thymus, but certainly the magnitude of such seeding is not as impressive as many might have anticipated (69-73). We do not present in this report direct evidence to support or reject the hypothesis of cellular distribution; however, the observations in the chicken that irradiation plus bursectomy eliminates development of a system of cells, whereas irradiation alone or bursectomy alone does not prevent such development, might be taken as an indirect argument for distribution of cells by the bursa. The argument is not conclusive, for a humoral action of the bursa such as has been recently demonstrated (74-76) as it has for the thymus (77-79) could permit differentiation of the cells of the bursal system from another source without a direct contribution from the central organ. The two bases for development of the two separate systems are, of course, not mutually exclusive.

A further important question raised by these observations is the question of how, if at all, these two systems of cells relate to one another, as well as whether they relate to the system of cells playing a major role in digestion and possibly preparation of antigen, the reticuloendothelial system (RES). We have presented in this report evidence that absence of the thymus and depletion of the thymic system of cells, or absence of the bursa and the bursal system of cells does not interfere with capacity of the RES to clear colloidal particles from the circulation. Whether or not the digestive and preparatory system of cells feeds information into either the thymus- or bursa-dependent systems needs further study, as does the question of whether these two cell systems must intercommunicate in mounting the full immunologic response and achieving its termination. That the thymus system in some way contributes to the function of the immunoglobulin-producing system is suggested by the observations that the irradiated-thymectomized chickens are quantitatively deficient as producers of antibody at least following stimulation with the antigens we have used. Similar observations have been made repeatedly in neonatally thymectomized mammals (5, 6, 8, 80-83). It certainly could be that the thymic system of cells responsible for the rapidly developing immune responses is working alone or together with the RES to achieve recognition of foreignness and de-

velopment of cellular immunity, and that information so derived is passed, by one of several possible means (84-86), to the specialized production system of cells where the majority of antibodies that reach the circulation are formed.

These observations pose the pressing question: what is the bursal equivalent in mammals and man? It is clear that the thymus and thymus system of cells in birds and mammals are essentially equivalent from the structural and functional points of view. It is equally clear that the peripheral lymphoid tissues of birds and mammals have similar structural organization. Mammals possess a system of small lymphocytes that is dependent upon the thymus. The germinal centers and plasma cells seem independent of thymus (8, 39, 80, 81, 87, 88) and immunoglobulin production is little affected even by neonatal thymectomy (8, 80, 81, 89). Both developmental and involutinal history, as well as the experiment of nature posed by Bruton type agammaglobulinemia, indicate that the mammalian tonsils and other lymphoepithelial tissue of the gastrointestinal tract, e.g. the Peyer's patches (90), may subserve the functions we and others have attributed to the bursa of Fabricius.

SUMMARY

The bursa of Fabricius and the thymus are "central lymphoid organs" in the chicken, essential to the ontogenetic development of adaptive immunity in that species. Surgical removal of one or both of these organs in the newly hatched chicken, followed by sublethal X-irradiation the next day, has permitted recognition of two morphologically distinct cell systems in the "peripheral lymphoid tissues" of the spleen, gut, and other organs, and clear definition of the separate functions of each cell system.

The thymus-dependent development is represented morphologically by the small lymphocytes of the circulation and the white pulp type of development in the tissues. As in mammals, the thymus-dependent tissues of the chicken are basic to the ontogenesis of cellular immunity: graft *versus* host reactions, responses of delayed hypersensitivity and homograft rejection; and play a less clearly defined role in the antibody response to at least some antigens. Thymectomized-irradiated chickens are deficient in all these responses, and grow more slowly than any of the other experimental groups. In these animals germinal centers, plasma cells, and capacity for immunoglobulin synthesis remain intact.

The bursa-dependent development is represented morphologically by the larger lymphocytes of the germinal centers and the plasma cells, and functionally by the immunoglobulins. Bursectomized-irradiated chickens are agammaglobulinemic and unable to produce detectable antibody despite intense, repeated stimulation with bovine serum albumin and *Brucella abortus* organisms. The thymus-dependent development in these animals seems to be normal; they have adequate numbers of lymphocytes in the circulation and

tissues, are able to reject skin homografts, though more slowly than usual, and to exercise graft *versus* host reactions. The short life span of these chickens has precluded adequate study of responses of delayed hypersensitivity.

There was no evidence of significant impairment of reticuloendothelial function in either the bursectomized-irradiated or the thymectomized-irradiated group, as judged by the clearance of colloidal gold and I^{131} -tagged keyhole limpet hemocyanin.

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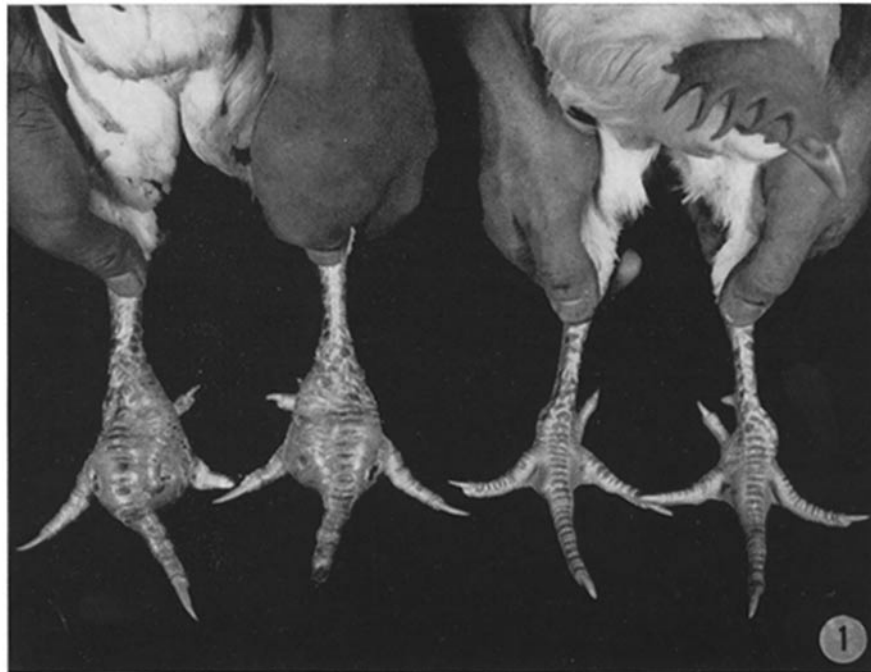
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EXPLANATION OF PLATES

PLATE 11

FIG. 1. Thymectomized-irradiated chickens, which had reduced delayed allergy to diphtheria toxoid, also had a greatly reduced response to the tuberculin-fortified adjuvant, as evidenced in the appearance of the feet. In this illustration the feet of a 4-month-old control chicken (left) 23 days after foot-pad injection of 1 Lf diphtheria toxoid in 1 ml complete Freund's adjuvant (10 mg *M. tuberculosis* H37RV per ml) are compared with the feet of a thymectomized-irradiated chicken (right) treated in the same manner.



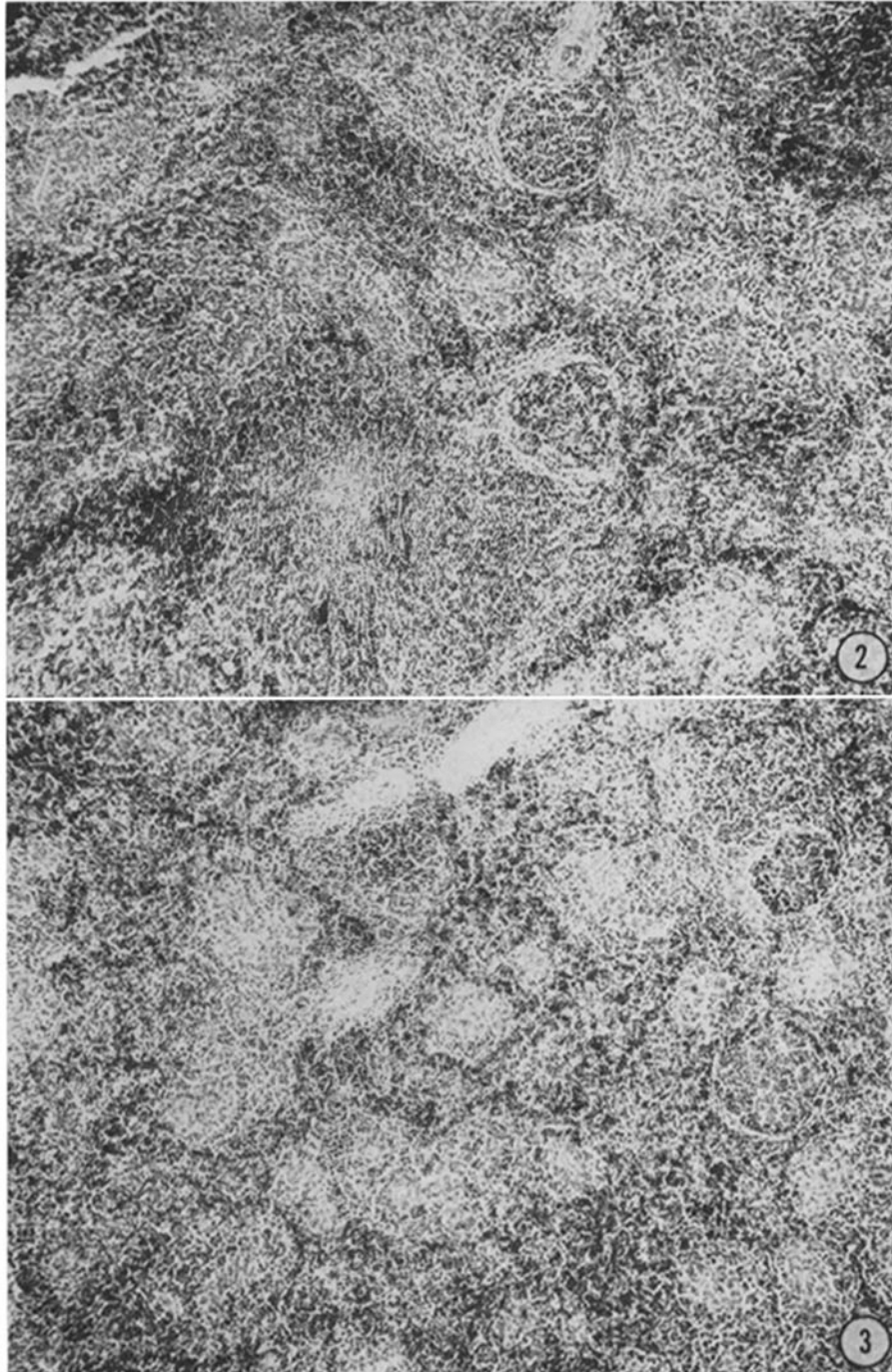
(Cooper et al.: Functions of thymus and bursa systems)

FIGS. 2 to 5. Spleen histology in 7-wk-old control, control-irradiated, thymectomized-irradiated, and burssectomized-irradiated chickens. Hematoxylin and eosin. $\times 100$.

PLATE 12

FIG. 2. Fig. 2 shows the distribution of thymus-dependent small lymphocytes in dark clusters and the bursa-dependent larger lymphocytes of the sharply circumscribed germinal centers in the spleen of a normal control chicken.

FIG. 3. Fig. 3 shows a spleen section of an irradiated but otherwise unmanipulated chicken. Both thymus-dependent and bursa-dependent components of the lymphoid system show full recovery from the effects of irradiation.

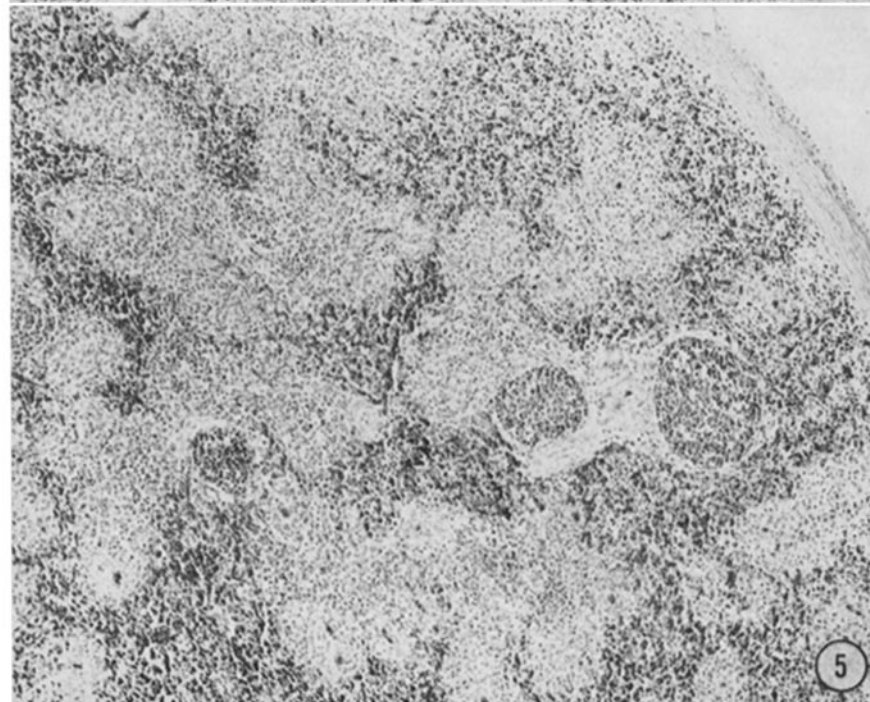


(Cooper et al.: Functions of thymus and bursa systems)

PLATE 13

FIG. 4. Fig. 4 shows a spleen section from a bursectomized-irradiated chicken. Though germinal centers are absent, the small lymphocyte population appears to be completely restored following irradiation.

FIG. 5. Fig. 5 shows the striking depletion of small lymphocytes in the thymectomized-irradiated chicken spleen. Note the presence of normal-appearing germinal centers. The darker staining cells outside the germinal centers are essentially all eosinophilic.

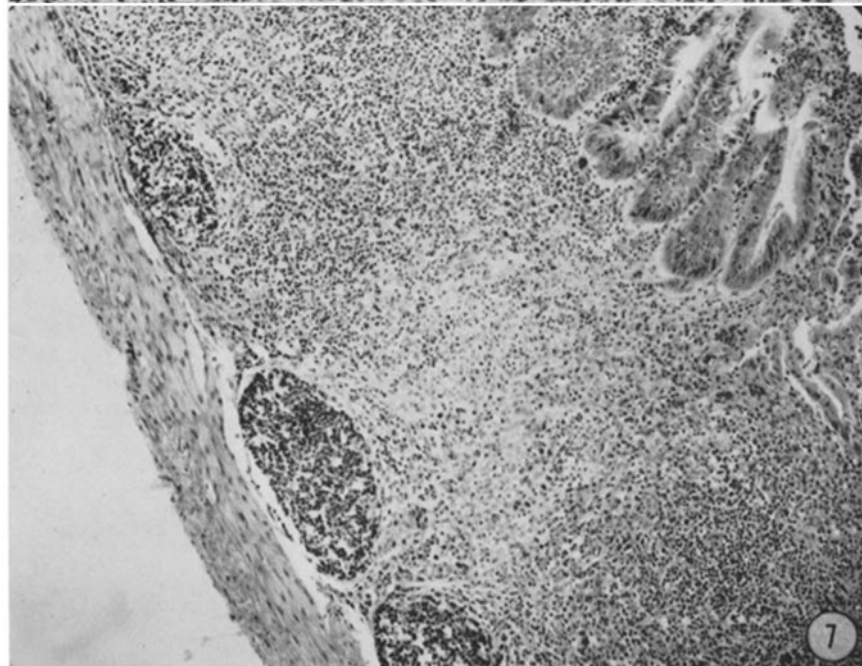
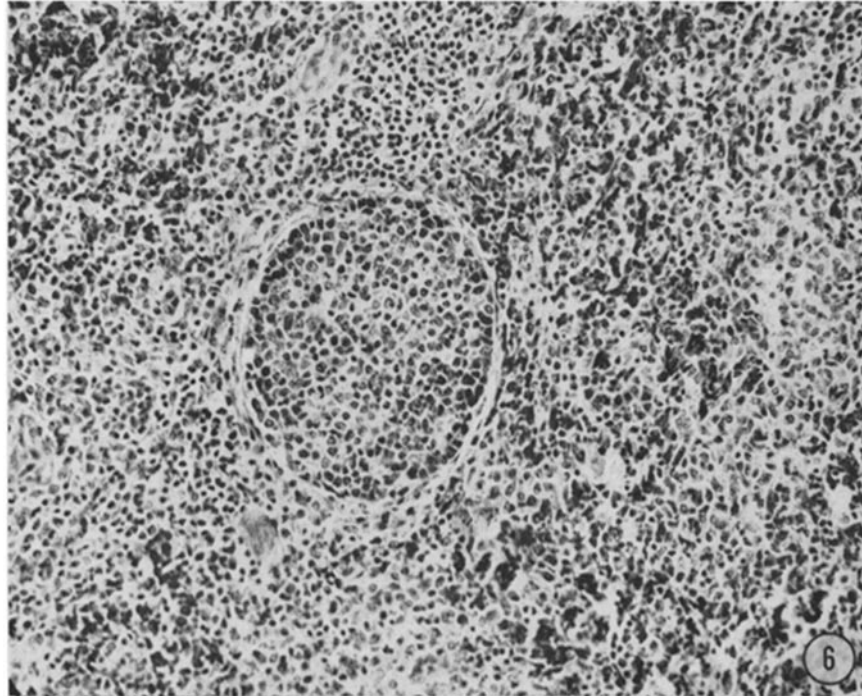


(Cooper et al.: Functions of thymus and bursa systems)

PLATE 14

FIG. 6. Spleen section from a 7-wk-old chicken (No. 47) bursectomized in the newly hatched period, lacking 7S gamma globulin on immunoelectrophoresis, and showing no detectable antibody response to BSA or *Brucella* organisms. The germinal center, representative of those seen in this animal and others of this Bx group, is normal in appearance. Hematoxylin and eosin. $\times 250$.

FIG. 7. This section shows normal germinal centers deep in the cecal lamina propria of the Bx chicken whose spleen histology is illustrated in Fig. 5. Plasma cells, not easily seen at these magnifications, also appeared normal in both the spleen and the cecum. Hematoxylin and eosin. $\times 100$.



(Cooper et al.: Functions of thymus and bursa systems)