from the protein found bound to the sulfated polymer prior to matrix formation.

The only metachromatic material which has been reported in the limb bud of the 3- to 3½-day chick was found in tiny granules in the golgi of vitally stained tissue(14). At 4½ days, this material had moved into the cytoplasm as a few large purple granules. Fixed material does not seem to show any metachromatic material at this time. This sequence of events conforms with that reported by Godman and Porter for differentiation of cartilage cells in the mouse(15). It is possible that polysaccharide-protein complex production commences very early in embryogenesis and that the product is stored in the golgi in packets so well covered that after fixation dyes cannot penetrate. This material may be released into the extracellular spaces at a much later time, either due to some stimulus or to the presence of some additional component.

Summary. Limb buds which had been allowed to take up radioactive sulfate were extracted and fractionated in the presence of crude non-radioactive polysaccharide-protein complex from 12-day chick embryos. The radioactive material was not soluble and did not become soluble after incubation for 18 hours at 37° in sodium hydroxide but did become readily soluble after digestion with papain. After treatment with sodium hydroxide and papain, the radioactive material was excluded by Sephadex G-50, but after treatment with hyaluronidase, the material was held up on the same column. Fractionation of the calcium salts of the radioactive material suggested the presence of chondroitin sulfates A, C, and a small amount of B.

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Absence of Antibody Plaque Forming Cells in Spleens of Thymectomized Mice Immunized with Sheep Erythrocytes.* (30073)

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Recent investigations in several laboratories have demonstrated that the thymus gland plays an important role in development of immunologic mechanisms(1-9). Removal of the thymus in early life, prior to maturation of immunologic competence, is associated with major disturbances in immune functions in experimental animals. Thymectomy at or near birth generally results in impairment of the ability to reject skin or tumor homografts(1-3). Early postnatal thymectomy of experimental animals also impedes or prevents development of delayed skin reactivity to serum protein antigens or

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to tuberculin(4,5), and impairs their capacity to form antibodies upon subsequent challenge injection with either particulate or soluble antigens(1-9).

The accepted criterion for suppressed immunologic competence of thymectomized animals has been the demonstration of the absence of an expected classic immune response, *e.g.*, failure to reject a homograft, failure to produce a graft *vs* host reaction, failure to show a positive skin reactivity following attempted sensitization, or failure to produce detectable circulating antibody following attempted immunization. There is very little information as to actual numbers and relative immunologic competence of individual antibody forming cells in thymectomized animals (1,2). For example, it is not known whether absence of circulating antibody in thymectomized animals after specific immunization is due to fewer antibody forming cells, each secreting normal amounts of antibody, or due to abnormally low concentrations of antibodies produced by relatively normal numbers of antibody forming cells.

This report is concerned with experiments designed to determine the relative presence or absence of antibody forming cells in spleens of mice thymectomized at birth and subsequently immunized with sheep erythrocytes. The localized hemolysis ("antibody plaque") procedure for detecting hemolysins secreted by individual lymphoid cells in agar gel was used for this purpose since this technique permits rapid estimation of the total number of antibody forming cells in a given suspension of cells(10,11). Estimation of the size of hemolytic zones in agar also permits approximation of the relative quantity of hemolysin secreted by each antibody forming cell(11,12).

Methods and materials. Litters of newborn NIH albino white mice were used for these experiments as follows: One-half of the animals in a litter was subjected to thymectomy within 24 hours of birth(13). The remaining mice in each litter were sham operated. At 6 weeks of age the surviving mice were injected intraperitoneally (I.P.) with 0.5 ml of a 50% suspension of freshly washed sheep erythrocytes suspended in saline. At close periodic intervals thereafter, individual mice from both groups were bled from the retro-orbital plexus to obtain blood for determination of hemolysins to sheep erythrocytes, using the micro loop technique with 0.025 ml volumes of serum dilutions and 0.05% washed sheep erythrocytes(12). Mice from each group were sacrificed periodically and spleens removed. The spleens were weighed and then "teased" with fine forceps and needles in sterile Medium 199 (Hanks' base), pH 7.2, to obtain a cell suspension(12,14). The cells were washed with sterile Medium 199, interspersed with serial centrifugation in the cold. Suspensions containing 10×10^6 to 5×10^5 viable nucleated lymphoid cells per ml were prepared. Trypan blue stain method was used for determining viability and a hemocytometer was used for cell counts.

An aliquot of each cell suspension was used for preparation of antibody plaque plates(10-12). In brief, 0.1 ml of a cell suspension was mixed with 2.0 ml of 0.7% melted Noble agar containing 0.1 ml of a 10% suspension of washed sheep erythrocytes. This mixture was poured over a base layer of solidified agar in a petri plate. Following one hour incubation at 37°C, guinea pig complement was added to each plate and the plates were again incubated for 30 minutes at 37°C. Zones of lysis appeared in the agar layer containing erythrocytes, indicating individual lymphoid cells secreting hemolysins(10-12). The number of plaques per plate was counted to determine the average number of hemolysin secreting cells in relation to the total number of viable lymphoid cells plated. Each spleen cell suspension was plated in duplicate or triplicate using several concentrations of cells.

Results. The level of serum hemolysins and the number of antibody plaques formed by spleen cell suspensions prepared from thymectomized or control 6-week-old NIH mice immunized 4 days previously with sheep erythrocytes are indicated in Table I. The animals had been either thymectomized or sham operated within 24 hours of birth. Histologic examinations following autopsy at completion of the experiments revealed no detectable thymus tissue remaining in the thymectomized mice used in this study. Thy-

TABLE I. Serum Hemolysin Titers and Antibody Plaque Formation by Spleen Cell Suspensions from 6 Week Old NIH Mice, Either Thymectomized or Sham Operated at Birth.*

Treatment at birth	No. of mice challenged	Mean hemolysin titer	Mean spleen wt (g)	Total No. spleen cells isolated per mouse (mean)	No. of spleen cells plated	No. of plaques per plate (mean)	Calculated No. of antibody forming cells/10^6 cells plated
				($\times 10^6$)			
Surgical thymectomy	17	1:31	0.103	17.2	2.1×10^6	105	53
					5.3×10^5	27	54
					6.8×10^4	4	59
Sham operated	23	1:428	0.296	94.5	2.4×10^6	TMTC†	—
					2.1×10^5	345	1649
					3.6×10^4	62	1722

* Each mouse injected intraperitoneally with 0.5 ml of a 50% suspension of sheep erythrocytes four days prior to sacrifice and testing.
† Too many to count.

mectomized animals which survived to 6 weeks of age and were challenged with sheep erythrocytes had a mean spleen weight of approximately 100 mg at sacrifice (Table I). Spleens from the sham operated mice were, on the average, 3 times larger. The thymectomized animals responded to immunization with sheep erythrocytes with a mean peak hemolysin titer of 1:31. The sham operated animals responded with a mean peak titer of 1:428 (Table I).

Agar plates containing spleen cells from either thymectomized or sham operated animals injected with sheep erythrocytes 4 days previously showed a marked difference in number of antibody plaques. There was an average of 53 to 59 antibody plaques per million viable lymphoid cells plated from thymectomized mice and approximately 1600 to 1700 antibody plaques per million viable lymphoid cells from the sham operated animals (Table I). Taking the size of the spleens in consideration, this constituted approximately a 200-fold difference in the average number of antibody plaque forming cells per total spleen cell suspension between the 2 groups of mice.

Results of a typical experiment in which experimental and control mice were immunized with sheep red blood cells and sacrificed at varying intervals thereafter is illustrated in Table II. Spleen weight, hemolysin titers, and the number of antibody plaque forming cells per million viable lymphoid cells from either thymectomized or sham operated mice sacrificed 1, 2, 4, 6, or 10 days after I.P. challenge injection with sheep erythrocytes are shown. Whereas sham operated animals had a readily demonstrable increase in spleen size following immunization, as well as a marked rise in hemolysin titer, the thymectomized animals had little change in spleen size, and only a slight rise in titer. Comparison of the number of hemolytic plaques obtained with spleen cell suspensions from both groups of animals showed a typical marked increase with spleen cells from control mice, and only a slight increase in number of antibody plaques with spleen cells from thymectomized animals. The time period for maximum antibody plaque formation was 4 days after immunization with both groups of mice.

The size of hemolytic plaques formed in agar by spleen cells from both thymectomized and control mice were approximately the same. The only discernible difference in plates with spleen cells from experimental and control mice was the total number of plaques. Ten to 20 percent of the plaques formed by spleen cells from both thymectomized and control mice were approximately 0.1 to 0.2 mm. The remainder of the plaques were smaller, regardless of the source of spleen cells.

Discussion. The results presented here demonstrate that surgical removal of the thymus of mice at birth results in a marked decrease in number of lymphoid cells capable of forming hemolytic plaques in agar following a

TABLE II. Serum Hemolysin Titers and Hemolytic Plaque Formation by Spleen Cell Suspensions from Individual Mice Thymectomized or Sham Operated at Birth and Immunized at 6 Weeks of Age with Sheep Erythrocytes.

Treatment at birth	Day after immunization*	Spleen wt (g)	Hemolysin titer	Spleen cells plated	Plaques counted on 3 plates	Plaque forming cells/10^6 cells plated
				($\times 10^6$)		
Surgical thymectomy	0	.106	<1:10	12.5	1,0,2	.08
	+ 1	.112	<1:10	13.4	3,0,5	.18
	+ 2	.105	<1:10	1.1	10,11,7	2.1
	+ 4	.126	1:10	1.3	43,39,52	34.2
	+ 6	.115	1:20	1.6	12,16,20	10.0
	+10	.101	1:10	1.3	7,5,6	4.6
Sham operated	0	.186	<1:10	9.5	2,3,6	.42
	+ 1	.210	1:20	10.5	25,23,35	2.6
	+ 2	.276	1:160	.30	42,38,59	1534.1
	+ 4	.364	1:640	.21	317,240,298	1425.0
	+ 6	.328	1:640	.40	88,130,179	542.5
	+10	.290	1:240	1.1	38,49,20	36.5

* Each mouse injected intraperitoneally with 0.5 ml of a 50% suspension of sheep erythrocytes and sacrificed on day indicated.

primary immunization with sheep erythrocytes. These findings indicate that the relative absence of circulating hemolysins in thymectomized mice apparently is not due merely to decreased antibody formation by normal numbers of antibody forming cells. If this were the case, then similar numbers of hemolytic plaques should have been obtained with spleen cell suspensions from both thymectomized and sham operated animals. The relative size of hemolytic plaques should have been the only difference.

In contrast to these findings, a marked difference in plaque size has been observed with spleen cells from mice exposed to whole body X-irradiation(15). Numerous small hemolytic plaques are often observed in agar plates with spleen cells from mice treated with 400 to 800 r 24 hours or less before immunization with red cells(15). Although it is possible that the marked diminution in plaque size with X-rayed mice could be due to differential formation of 7S or 19S antibody, it appears more likely that small sized plaques are indicative of lower quantities of antibody secreted by individual cells.

The use of the hemolysin plaque procedure in this study has permitted direct estimation of the number of lymphoid cells actively secreting hemolysins as well as an approximation of the relative amount of antibody formed for each plaque by lymphoid cells from control and thymectomized animals. Although the efficiency of the plaque technique for demonstrating antibody forming cells is not known, additional studies with this procedure should also be of value in investigation of antibody synthesis by lymphoid cells from thymectomized animals treated with various agents, including thymus extracts, which may be capable of restoring immunologic competence.

Summary. Surgical removal of the thymus of newborn NIH mice resulted in a marked suppression in subsequent ability to form circulating serum hemolysins to sheep erythrocytes. Spleen cell suspensions from young adult mice thymectomized at birth and immunized with sheep red blood cells were markedly less capable of forming hemolytic antibody plaques in agar gel than spleen cells from sham operated control mice. Although the number of antibody plaques formed by cells from thymectomized mice was markedly reduced, the size of individual plaques was generally similar to those formed by spleen cells from the control mice.

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Effect of Bilirubin on Binding of Thyroxine by Human Serum Albumin.* (30074)

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The binding of thyroxine to serum albumin can be attributed in part to the electrostatic interaction of anionic portions of the thyroxine molecule with cationic lysyl ϵ-amino groups on the protein(1). It was shown previously that various anionic compounds such as unesterified fatty acids, 2,4-dinitrophenol and salicylate have the ability to displace thyroxine from binding sites on serum albumin(2). Bilirubin is an anionic compound that appears to be bound principally to albumin while circulating in human blood(3). It seemed of interest to study the binding of thyroxine in the presence of bilirubin to note whether the latter compound is capable of displacing thyroxine from albumin.

Materials and methods. I^{131}-L-thyroxine was obtained from Abbott Laboratories, Oak Ridge. Nonradioactive L-thyroxine was purchased from Aldrich Chemical Co. Bilirubin was purchased from Mann Research Laboratories, Inc. Human serum albumin (Cohn Fr. V, Batch No. 1984) was obtained from the American Red Cross through the courtesy of Dr. James H. Pert.

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Equilibrium dialysis with I^{131}-L-thyroxine was used to study binding as described previously(1,2,4). All experiments were performed at 30° in 0.06 M potassium phosphate buffer, pH 7.4. Further details about the dialysis method are given in the legend to Fig. 1. The bilirubin solutions were made up fresh in 0.04 N NaOH before each experiment. The binding data were analyzed in the usual way(1,5) by plotting $\bar{v}$ *vs* $\bar{v}/(A)$ as shown in Fig. 1. The experimental data plotted in Fig. 1 are the means of duplicate determinations.

Results. Fig. 1 shows the effect of bilirubin on the binding of thyroxine by human serum albumin at pH 7.4 and 30°. Each intercept on the $\bar{v}/(A)$ axis in Fig. 1 (the $\sum n_i k_i$ value(1,5)) represents the overall affinity of the protein for thyroxine under the conditions of the particular experiment. It can be seen from Fig. 1 that thyroxine binding to albumin is reduced in the presence of bilirubin. For example, bilirubin, at a molar ratio to albumin of 1/1 reduces the $\sum n_i k_i$ value for thyroxine binding from 7.6×10^5 to 5.9×10^5, a reduction of 22% in the binding affinity of the protein for thyroxine. Table I gives the reduction in thyroxine binding produced by each of the concentra-