THE DISTRIBUTION OF S^{35}-LABELED BOVINE SERUM ALBUMIN IN NEWBORN AND IMMUNOLOGICALLY TOLERANT ADULT RABBITS*

By JUSTINE S. GARVEY, PH.D., DONALD V. EITZMAN,‡ M.D., AND RICHARD T. SMITH,§ M.D.

(From the Division of Chemistry and Chemical Engineering, The California Institute of Technology, Pasadena, and the Department of Pediatrics, University of Florida College of Medicine, Gainesville) ||

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Recent studies of immune tolerance, which have received extensive reviews (1, 2), have suggested (*a*) that the induction of the tolerant state requires that initial interaction with the antigen take place before or shortly after birth, and (*b*) that after induction, maintenance of the state depends upon the availability of additional increments of the antigen. Little however, is known about the special conditions of the perinatal period of life which are responsible for the induction of tolerance, rather than an immune response, following the injection of heterologous protein.

In the present studies the antigen used was bovine serum albumin, coupled with diazotized S-35 sulfanilic acid. This antigen was injected into rabbits within 1 day after birth as a dose of approximately 20 mg.; use of this amount of antigen induces the tolerant state for at least 3 to 4 months (3). In other studies young rabbits have been injected with bovine serum albumin (4, 5) or human serum albumin (6) for the purpose of observing the effect on the tolerant state of varying either the antigen dose or the age of the animal at time of initial injection with the antigen. Similar studies have involved the injection of bovine serum albumin into young chicks (7, 8) and into mice (9). The findings reported to date with a non-viable antigen have shown that the duration of the tolerant state is definitely dependent upon the dose of antigen.

Because of the emphasis on the role of antigen in tolerance, the present studies were undertaken to determine whether the neonatal or tolerant rabbit differs from the mature and immunologically competent animal, in the pattern of organ distribution of injected antigen, or in the intracellular distribution of

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antigen in one organ, the liver. Whereas, the data obtained provide evidence for the persistence of antigen after neonatal injection, they suggest strongly that the differences in response to antigen in neonatal or tolerant and mature animals cannot be explained by gross differences in organ distributions of injected antigen.

Materials and Methods

Preparation of S^{35}-BSA.—S^{35}-labeled sulfanilic acid was synthesized as described previously (10) by using a mixture of S^{35}-labeled sulfuric acid and unlabeled sulfuric acid to prepare aniline sulfate. The aniline sulfate was converted to sulfanilic acid by 8 to 10 hours of drying in a partial vacuum (8 to 10 mm. Hg) at a temperature of 200°C. The labeled acid was converted to the sodium salt, diazotized with nitrous acid, and coupled to BSA (Armour crystalline bovine serum albumin). The solution of sulfanilic azoprotein, S^{35}-BSA, was dialyzed exhaustively against 1 per cent NaCl until the dialysate showed a very low, but constant level of radioactivity. Before injection, the preparation was sterilized by Seitz filtration and assayed for sulfur and nitrogen. The sulfanilic-azo-BSA had an average of 10 sulfur groups per protein molecule and 10 microcuries S^{35} per mg. protein. The serological specificity of the protein was not altered greatly by the coupling as shown by a precipitin test with rabbit anti-native BSA. The amount of precipitate was greater than 90 per cent of that obtained with the anti-native BSA–BSA system.

Injection of Animals and Handling of Tissues.—Litters of New Zealand rabbits were injected with the appropriate amount of approximately 2 per cent S^{35}-BSA in 1 per cent NaCl either subcutaneously into two sites on the day of birth, or intraperitoneally, at various ages as indicated in individual experiments. Intravenous injections were made *via* the marginal ear vein. The infant rabbits remained with the doe until 3½ to 4 weeks of age, then they were separately caged. At the time of sacrifice, the rabbits were anesthetized with nembutal and exsanguinated. They were then perfused with cold 1 per cent saline, through the right ventricle, aorta, and portal vein until the tissues were essentially free of blood. The liver, spleen, kidneys, thymus, and lungs were removed, weighed, quickly frozen, and kept at −20°C. until the analyses were performed. Serum samples were similarly preserved. In some experiments the frozen organ samples were packed in dry ice for air shipment between laboratories.

Determinations for Antigen in Tissues and for Circulating Antibody.—Weighed aliquots of tissue were digested with concentrated nitric acid and the radioactivity was determined according to a method previously described (11). Earlier investigations (12, 13) provide the basis for assaying the radioactivity of the S^{35}-sulfanilic acid-azo group as a reliable measure of the amount of azoprotein in the tissues of adult animals. In these studies it is assumed that neonatal tissues can be similarly assayed.

Tests for antigen and antibody were performed using either capillary precipitation or interfacial techniques. Loss of antigen from the circulation was also followed by determining the radioactivity in samples of whole serum which were dried to constant weight rather than acid digested as were the samples of organs; otherwise, the technique of determining radioactivity was similar for residues of both dried serums and digested organ samples.

Fractionation of Liver Tissue.—After weighed aliquots of the liver were taken for determination of total radioactivity, the remaining liver tissue was frozen. The livers of the mature animals were pressed in the frozen state in a Carver laboratory press at 16,000 pounds per square inch. The neonatal livers were reduced to a brei by use of a Potter glass homogenizer; the laboratory press was not used because of the small weight of tissue. To the brei 6 volumes 0.25 M sucrose containing 5 per cent borate buffer (pH 8.4, $\mu = 0.17$) was added with thor-

ough mixing. Particulate fractions were separated as described previously (14), by differential centrifugation, washed with the sucrose buffer several times, and assayed for radioactivity. The supernatant fraction, essentially freed of microsomal material by centrifugation at 4°C. for 1 hour at 78,000 *g*, was dialyzed against 3 changes of borate buffer (pH 8.4, μ = 0.0017), lyophilized, and stored at −20°C. until used for the studies described below.

Starch Block Electrophoresis.—The supernate fraction was studied by starch block electrophoresis as described by Kunkel and Slater (15) with some modification, as will be noted. Potato starch, used to prepare the block, was washed thoroughly with 1 per cent NaCl, followed by washing with buffer. A thick slurry of the starch in buffer solution was poured into a lucite tray 1½ × 2 × 44 cm. The tray had been lined previously with thin polyethylene sheeting, and pieces of toweling, as "wicks," were fitted over the plastic sheeting at a distance of about 1 inch from each end and were allowed to extend about 3½ to 4 inches over each end of the tray. Excess moisture was removed from the block by blotting with filter paper. A cylindrical hole (1 × 1½ × 2 cm.) was made in the starch by using a metal cutter inserted at 13 cm. from the left end of the block. Some of the starch which was removed from the hole was replaced as a shallow layer in the bottom of the hole. A 300 mg. sample of the lyophilized liver fraction was mixed with 1 ml. buffer and with dry starch, previously washed with buffer. The sample, of putty-like consistency, was inserted into the hole. Some of the starch previously removed from the block, was used to fill the area of the sample to the level of the rest of the starch block. The polyethylene sheeting which surrounded the bottom and sides of the starch had been cut in a length to cover the block so it was drawn over the top surface of the block and firmly secured with cellophane tape. The block and buffer vessels were placed in a room cooled to 4°C. The buffer vessels were filled with buffer and the towel wicks, embedded at one end in the starch block, were immersed at the other end in buffer. After the block was equilibrated with buffer, the electrodes were connected and the flow of current was started. Voltage was maintained constant at 400 v. and the current was 13 to 15 ma. After 15 hours the current was turned "off" and the block was divided immediately into 1 cm. fractions, and these were eluted twice with 3 ml. volumes of 1 per cent NaCl. The combined eluates of each fraction were centrifuged to remove starch, and aliquots of each fraction were analyzed for (*a*) radioactivity by counting a sample dried to constant weight, (*b*) total phosphorus (16), and (*c*) protein by biuret determination (17).

Free Boundary Electrophoresis.—Supernatant fractions of liver were also observed in free boundary electrophoresis using a Perkin-Elmer apparatus. The protein concentration of samples (lyophilized liver supernatant fractions dissolved in buffer) was 2 per cent, and these samples were dialyzed in barbital buffer (pH 8.6, μ = 0.1) for 48 hours prior to electrophoresis in the same buffer at a current of 6 ma. Electrophoretic patterns of both the descending and ascending boundaries were obtained at ½ hour intervals for a total period of 2½ hours.

EXPERIMENTAL AND RESULTS

Comparison of Organ Distribution of S^{35}-BSA at Varying Intervals after Subcutaneous Injection at Birth.—The immunological unresponsiveness which follows neonatal antigen injection, is apparently induced at, or shortly after, the initial contact with antigen. An interesting question with regard to the injection of neonatal rabbits is: How does the immature animal handle antigen? This is an important question both because of the poor antibody response in young rabbits (18) and because of the induction of tolerance in early life. Since there is no available information on the fate of antigen in the tissues of

neonatal rabbits, the following experiment was performed with this purpose in mind.

Three litters of rabbits (5 in one litter, 4 each in the other two litters) were injected subcutaneously with 19 mg. S^{35}-BSA at birth, and 1 animal from each litter was sacrificed at 7 day

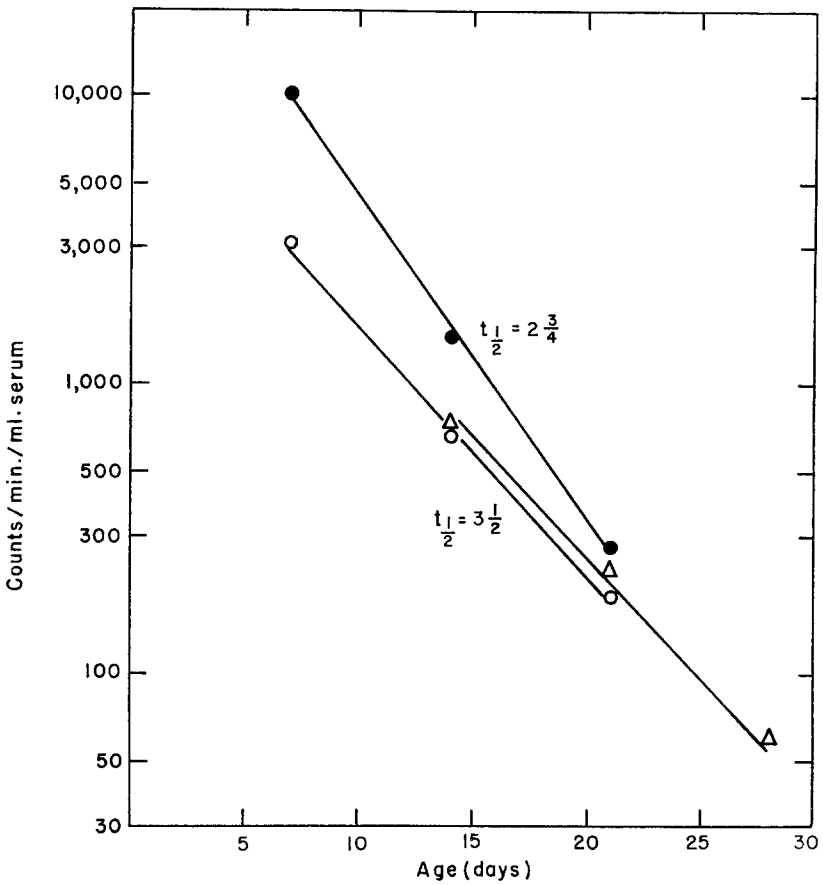

FIG. 1. The loss of radioactivity from the serum of rabbits, injected at birth with 19 mg. S^{35}-BSA and studied thereafter for a period of 7 to 28 days. Each curve represents a separate litter, (litter 2, O, litter 3, ●, and litter 4 △) and each point represents a single animal at the time of sacrifice.

intervals thereafter; the serum level of radioactivity and the organ distribution of radioactivity were determined.

Similar to data obtained for non-responsive animals (3, 19), the loss of antigen from the serum of these animals is exponential with no rapid phase (Fig. 1). The half-life values, uncorrected for growth of the animals average 3.1 days. No antibody was found in any of the sera taken at the time of sacrifice. The

TABLE I

*Organ Distribution of Radioactivity in Rabbits at Varying Intervals after Subcutaneous Injection of 19 mg. S^{35}-BSA at Birth**

Litter No. and average birth weight	Age at sacrifice	Weight at sacrifice	A, per cent of injected antigen in whole organ; B, specific activity (μg. antigen/gm.)				
			Liver	Spleen	Kidney	Lung	Thymus
	days	*gm.*					
2 (70 gm.)	7	164	A 0.57	0.0030	0.36	0.0370	0.0011
			B 14.44	4.14	31.92	3.73	0.60
	14	306	A 0.28	0.0015	0.26	0.0160	0.0008
			B 4.94	1.35	11.68	0.96	0.19
	21	478	A 0.14	0.0001	0.16	0.0026	0.0004
			B 1.24	0.04	5.74	0.12	0.06
	21	424	A 0.08	0.0018	0.14	0.0132	0.0003
			B 0.88	1.06	5.93	0.82	0.06
	28	460	A 0.10	0.0018	0.16	0.0112	0.0004
			B 0.69	1.08	5.74	0.86	0.10
3 (45 gm.)	7	80	A 0.55	0.0066	0.31	0.0338	0.0002
			B 32.30	20.52	46.74	6.50	0.87
	14	186	A 0.23	0.0013	0.29	0.0183	0.0011
			B 4.98	1.19	21.28	1.76	0.20
	21	406	A 0.13	0.0008	0.21	0.0075	0.0011
			B 1.41	0.40	9.02	0.46	0.20
	28	586	A 0.06	0.0016	0.16	0.0175	0.0008
			B 0.52	0.40	4.48	0.45	0.09
4 (48 gm.)	8	82	A 0.41	0.0007	0.43	0.0464	0.0002
			B 11.02	1.65	60.80	5.13	0.38
	15	304	A 0.25	0.0013	0.32	0.0236	0.0010
			B 3.57	1.01	15.68	1.43	0.23
	22	456	A 0.11	0.0016	0.25	0.0169	0.0006
			B 0.84	0.60	8.08	0.95	0.08
	29	414	A 0.07	0.0014	0.18	0.0188	0.0033
			B 0.55	0.80	4.09	0.95	0.38

* Litters of rabbits were injected subcutaneously into two sites with 19 mg. S^{35}-sulfanilic acid BSA. All animals were injected on the 1st day of life, then sacrificed, and perfused on the indicated day. Each value given represents the corrected radioactivity in the organ of a single animal.

changes in the various organs with time are shown for the three litters in Table I, and a plot has been made in Fig. 2 from the data obtained for one litter.

Table I shows apparent differences in the specific activity of antigen present in various organs. More of the antigen is localized in the liver and kidney than in the other tissues. Consideration of serial changes in specific activities in the various organs show that the loss from liver and kidney is nearly exponential as long as measured, but that after initial reduction in specific activity, the spleen,

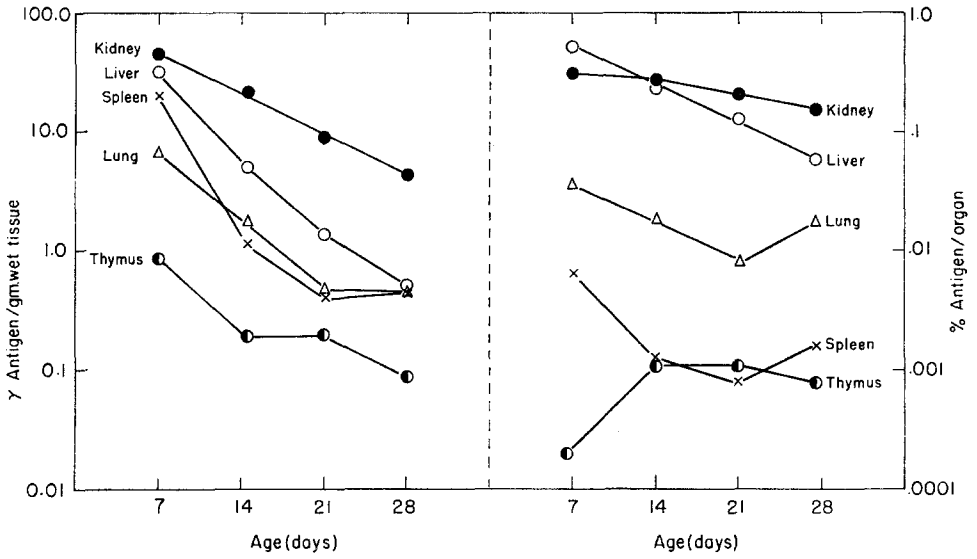

FIG. 2. Distribution of radioactivity in litter mates (litter 3) which received at birth 200 mg. of S^{35}-BSA per kg. body weight. The set of curves on the left are measurements of specific activity in five different tissues for a period of 7 to 28 days. The set of curves on the right represents percentages of S^{35}-BSA in the same five tissues.

lung, and thymus concentrations stabilize at about 3 weeks of age. Unfortunately data are not available for animals more than 4 weeks of age.

Table I shows the very rapid somatic growth which occurs during this period of life. The organs studied are also growing but at differing rates at this time; it is thus necessary to account individually for a dilution effect. The expression of per cent of injected dose in the whole organ incorporates this correction. Serial changes in the percentages of antigen, like the specific activity data (Fig. 2) also show an exponential loss of radioactivity from kidney and liver, and stabilization of the content of spleen, lung, and thymus.

Comparison of Organ Distribution of S^{35}-BSA in Rabbits at 3 Weeks after Intraperitoneal Injection at Birth, at 21 Days of Age, and at Maturity.—Following

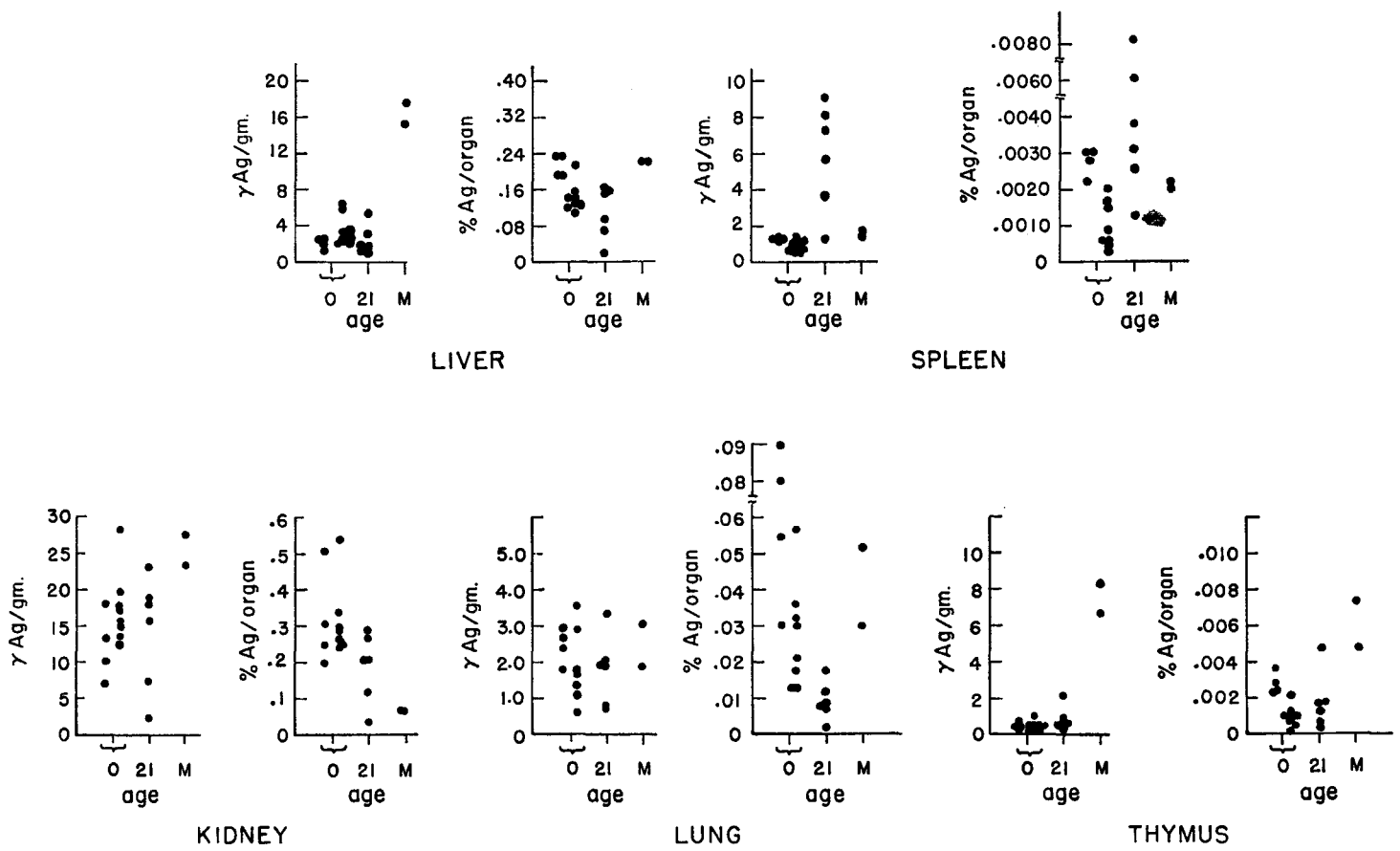

FIG. 3. Distribution diagrams for S^{35}-BSA in organs 3 weeks after injection. Both specific activity and percentage of the dose are indicated for each of five tissues on the ordinate. The abscissa is in arbitrary units of age at time of injection with *O* representing injection at birth of two litters, 5 and 6, with data given separately for the two litters; *21* representing injection at 21 days of age for litter 7 and *M* representing injection of 2 mature litter mates of age 3 to 5 months.

the serial observations in neonatal animals described above, it was of interest to compare the fate of S^{35}-BSA in rabbits during the period tolerance can be induced with animals in the transitional period when exposure results in antibody production in some rabbits, and with mature animals which respond regularly and vigorously to antigenic stimulus.

For this experiment, groups of rabbits were injected at birth (litters 5, 6), at 3 weeks of age (litter 7), and after maturity (2 litter mates, approximately 4 months old). The amount of antigen injected into the mature and 21 day groups was adjusted to body weight, about

TABLE II

*Distribution of Radioactivity in Organs of Rabbits of Varying Age 21 Days Following Intraperitoneal Injection of S^{35}-Labeled BSA**

Age when injected	No. in litter	Average weight at injection	Average amount injected	A, average per cent of injected dose in whole organ B, average specific activity (μg. antigen/gm.)				
				Liver	Spleen	Kidney	Lung	Thymus
		gm.	*mg.*					
Birth (litter 5)	8	71	19	A 0.182 B 3.11	0.0010 0.84	0.300 17.61	0.0280 1.80	0.0010 0.43
Birth (litter 6)	4	50	19	A 0.208 B 2.12	0.0025 1.29	0.318 12.22	0.0640 2.48	0.0028 0.38
21 days‡ (litter 7)	6	1090	207	A 0.110 B 2.30	0.0040 5.96	0.190 14.35	0.0110 1.91	0.0020 0.83
Mature§	2	3096	588	A 0.220 B 16.01	0.0020 1.60	0.070 25.46	0.0400 2.51	0.0060 7.53

* Litters of rabbits injected intraperitoneally at indicated time with S^{35}-labeled sulfanilic acid BSA, were sacrificed 21 days later and the organs perfused. No antibody was formed by animals in litters 5 or 6.

‡ Three of 6 of these animals had precipitating antibody at the time of sacrifice.

§ Both of these animals formed antibody.

200 mg. per kg. The newborn groups received 19 mg. each, which was approximately 250 mg. per kg. (litter 5) and 385 mg. per kg. (litter 6). Based upon the results of the first experiment, and the known response characteristics of rabbits injected with this particular antigen (10), it was elected to sacrifice all groups 3 weeks after injection.

The results are given as individual values in Fig. 3 and both as average values for specific activity and as average percentages of injected dose in each organ in Table II. Three out of 6 rabbits which were 21 days old when injected, and both mature animals had low levels of circulating antibody in their serum at the time of sacrifice; those injected at birth, as would be expected, had none. It will be seen in Fig. 3 that the individual values for the younger groups showed considerable scatter as compared with the mature animals.

Comparison of the organ distribution of radioactivity in the three groups reveals a striking uniformity in the proportion of the injected dose in the liver, except the specific activity in the mature liver which is distinctly higher than in the younger groups. In contrast the younger groups retained a higher

TABLE III

Organ Distribution of S^{35}-BSA in Normal and S^{35}-BSA-Tolerant Rabbits

No. and group of animals		Antibody present in serum, days after injection						A, per cent injected antigen in whole organ B, specific activity (μg. antigen/gm. wet weight)				
		2	4	6	9	13	21	Liver	Spleen	Kidney	Lung	Thymus
Tolerant* (received 20 mg. S^{35}-BSA at birth)	4-78	−	−	−	−	−	−	A 0.272 B 1.01	0.0079 1.33	0.116 2.6	0.0120 0.26	0.0012 0.07
	4-79	−	−	−	−	−	−	A 0.270 B 0.87	0.0054 0.90	0.135 3.1	0.0130 0.22	0.0011 0.08
	4-80	−	−	−	−	−	−	A 0.292 B 0.99	0.0040 0.91	0.151 3.7	0.0100 0.32	0.0015 0.09
Normal‡ (Littermates of above— no injection at birth)	4-81	−	−	−	−	+	+	A 0.346 B 0.99	0.0063 0.97	0.100 2.3	0.0070 0.13	0.0009 0.06
	4-82	−	−	−	+	+	+	A 0.238 B 0.92	0.0043 0.90	0.078 2.0	0.0065 0.15	0.0003 0.02
	4-83	−	−	−	+	+	+	A 0.221 B 0.86	0.0051 1.24	0.107 2.5	0.0046 0.16	0.0012 0.06
	4-84	−	−	−	−	+	+	A 0.224 B 1.01	0.0040 0.84	0.127 3.5	0.0065 0.23	0.0005 0.05

* Three animals in a litter of 7 rabbits received 20 mg. S^{35}-BSA i.p. at birth and 4 were uninjected. At 60 days of age all were given 19 mg. S^{35}-BSA per kg. body weight i.v., then bled at 2, 4, 6, 9, and 13 days for presence of circulating antigen or antibody, and sacrificed on the 21st day. Rabbits 4-78 and 4-79 were found to have exponential, non-immune type of loss of injected antigen from serum; No. 4-80 had a rather typical immune phase and hence could not be regarded as tolerant.

‡ Normal rabbits had rapid immune phase loss of circulating antigen at 6 to 9 days.

percentage of the injected dose in the kidney than did the mature animals. Both the kidney and the lung of the immature animals showed much individual variation in the proportion of injected radioactivity although the average specific activity for all groups was rather uniform. Two individuals of litter 6, injected at birth, had very high values for percentages of retention in the lung, thus elevating the average values above the other groups. The 21-day-old rabbits retained both high specific activity and a high proportion of the injected

material in their spleens. The thymus of the mature group appeared to retain more radioactivity, and to have a higher specific activity than the thymus of the other groups.

Comparison of over-all retention by litters 5 and 6, injected at birth, with comparable animals in the first experiment, in which injection was by the subcutaneous route suggests that a higher specific activity in all organs results from intraperitoneal injection, the route most commonly used for induction of tolerance. Whereas, some of the differences in antigen retention between groups could possibly be due to antibody production in the mature groups, no correlation is evident between the distribution of antigen in the animals of litter 7, (antigen-injected at 21 days) which produced antibody, and those of this group

TABLE IV

*Distribution of Radioactivity in Fractions of Perfused Liver of Tolerant and Immune Adult Rabbits 21 Days after Injection of S^{35}-BSA**

Group	No. in pool	Relative radioactivity in various fractions			
		Nuclei	Mitochondria	Microsomes	Supernatant
Tolerant‡ injected at birth	2	9.6	4.1	6.5	79.8
Litter mates§ of above; no neonatal injection	4	4.3	5.2	5.5	85.0
Normal adult rabbits	2	3.6	4.5	6.6	85.3

* For method of fractionation, see text.

‡ Tolerant as shown by exponential decrease of antigen in circulation.

§ Litter mates of tolerant group shown to have immunological decay of antigen from the circulation.

which did not. The only significant difference between litters 5 and 6, and litter 7, is in the high retention by the spleen and a lower per cent of retained antigen in the liver of the latter group. It is conceivable that there is an accumulation of complexes between S^{35}-BSA and antibody in the spleen. Likewise with the liver, the lower percentage of retention at 21 days might be associated with antibody production (20). Finally, over-all comparison of litters 5 and 6, and the mature animal, reveals a consistent difference only in antigen accumulation in the thymus of the mature animal. The significance of this observation however, is not known.

Comparison of Radioactivity in the Organs of S^{35}-BSA-Tolerant and Normal Rabbits.—The mechanisms responsible for the failure of an appropriate antigenic stimulus to induce antibody production in the tolerant animal, are not understood. It seemed possible that the neonatal injection which results in the tolerant state, while not in itself distributed uniquely in any demonstrable way, might alter the pattern of organ uptake of subsequently injected antigen.

Three animals of a litter of 7 rabbits were injected intraperitoneally on the day of birth with 20 mg. S^{35}-BSA, the other 4 provided uninjected litter mate controls. This amount of S^{35}-BSA has been shown to produce immune tolerance of at least 90 days duration in other experiments (3). At 60 days of age, both groups of rabbits were given a single intravenous injection of S^{35}-BSA, 19 mg. per kg. Serum specimens taken at 2, 4, 6, 9, 13, and 21 days were examined for the presence of circulating S^{35}-BSA and anti-S^{35}-BSA as precipitin. On the 21st day all animals were sacrificed, and the organs assayed for radioactivity. As will be described in the following section, this experiment provided the tissue for studying the distribution of activity in various cell fractions of liver.

The tolerant group, as expected, failed to produce circulating antibody and 2 of the 3 animals showed loss of antigen from the circulation, characteristic of tolerant animals. The normal controls produced antibody after a rapid immune type elimination of antigen from the serum.

Table III shows that there are no significant differences either in the specific activity or the proportion of radioactivity in the various organs of tolerant and normal animals. The slightly higher over-all values in the tolerant group are probably due to residual antigen from the neonatal injection, since the order of difference is that which would be expected from this source. If the tolerant group had in fact produced antibody which circulated after the second injection of antigen, then the percentage of antigen found in the liver would be expected from previous work (20) to be lower than indicated, and more similar to that of the normal group (excepting No. 4-81 which was significantly higher than other normal individuals).

Fractionation of Liver Tissue.—As no evidence of a significant difference in organ distribution of antigen between normal and tolerant animals was found, the distribution of radioactivity in cellular fractions of the liver was evaluated.

Liver brei, obtained as described previously, was pooled from tolerant animals 4–78 and 4–79 and from immune litter mates 4-81 to 4-84 (Table III). Similarly a pool of liver brei from two immunized adults (Table II) was prepared. These 3 pooled suspensions of pressed liver tissues were subsequently fractionated by differential centrifugation into subcellular fractions as described. Analysis of the fractions for radioactivity showed the distribution recorded in Table IV.

Nearly twice as much radioactivity was found in the nuclear fraction of tolerant as normal animals; however, no differences among the other fractions from the 3 pooled samples were observed. The largest amount of radioactivity was present in the supernatant fraction.

The supernatant fractions of tolerant and immune livers were preserved in the lyophilized state and an aliquot of this material was further fractionated by starch block electrophoresis. The eluates obtained by this electrophoretic fractionation were assayed for protein, radioactivity, and phosphorus. Distribution patterns resulting from these analyses are given in Fig. 4.

There are no clear-cut differences between the distributions of the materials analyzed for the two groups of animals. The phosphorus peak which has no

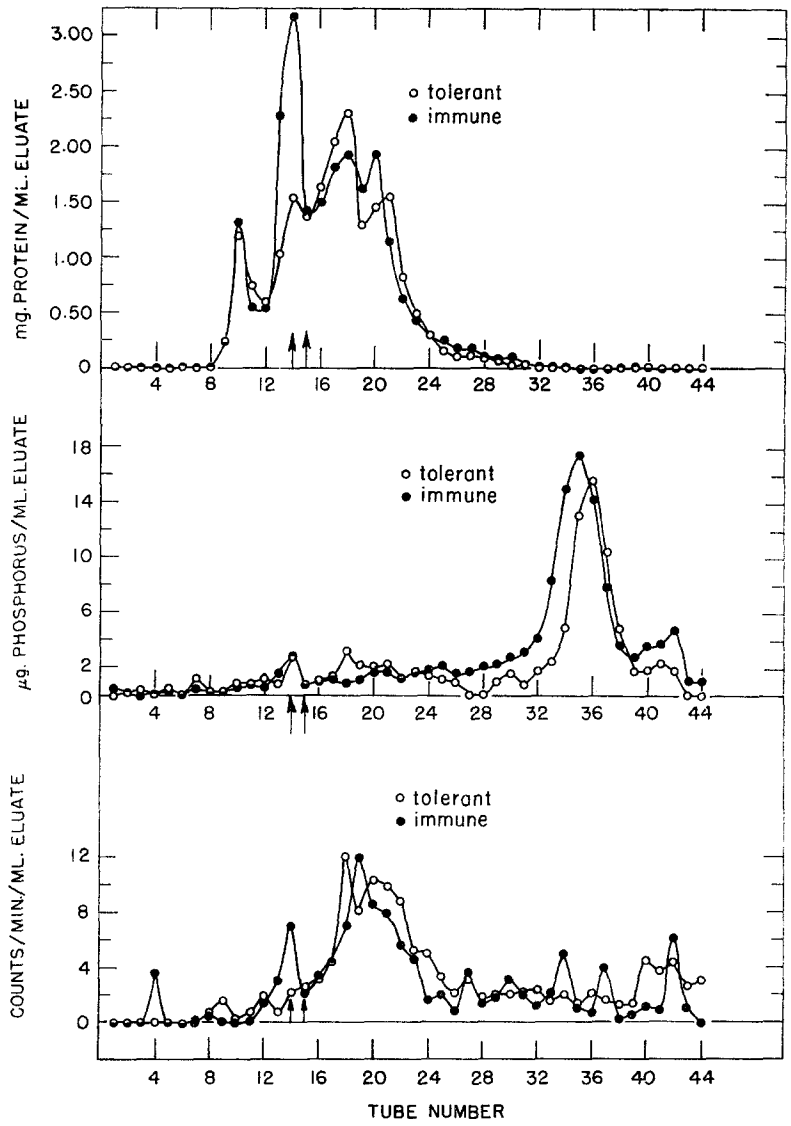

FIG. 4. Starch block electrophoresis of supernatant fraction from livers of tolerant and immune litter mates. Analyses of saline eluates from 44 separate fractions are indicated in separate curves for protein, total phosphorus, and radioactivity.

corresponding protein peak was found to have a maximal ultraviolet absorption at 2575 A, suggesting high nucleic acid content. An aliquot of the antigen, originally injected into the animals, was mixed with the liver supernatant fraction from normal liver tissue and assayed by identical technique, as a

control. The radioactivity in this control was located between the phosphorus and protein peaks. Thus it is evident that the radioactivity has become associated with tissue moieties during the 3 weeks following injection, but not differently, as detected by this method, in the immune and tolerant animals.

The liver supernatant fractions obtained from normal, uninjected rabbits at varying time in early life were analyzed by free boundary electrophoresis. The purpose of this study was to gain some information regarding any obvious gross differences in the composition of this fraction as the animal progressed toward maturity. Patterns are shown in Fig. 5, obtained after 90 minutes of electro-

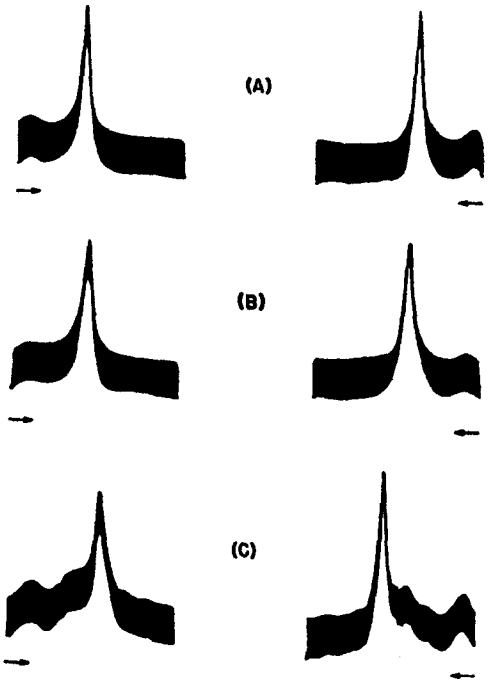

FIG. 5. Free boundary electrophoretic patterns of rabbit liver supernatant fraction from tissue obtained at different times after birth: (*A*) 7 days, (*B*) 21 days, (*C*) 28 days. In each of the 3 sets of patterns the descending boundary is to the left of the ascending boundary and all patterns were obtained after 90 minutes at 6 ma. in a barbital buffer of pH 8.6, 0.1 μ.

phoresis at 6 ma. in barbital buffer (pH 8.6, $\mu = 0.1$). The three sets of patterns represent fractions obtained from animals at 7, 21, and 28 days after birth. The patterns obtained at 28 days are consistently characteristic of a similarly prepared adult fraction (14) in both the number of components and also the mobilities. Occasionally the fraction from a 21 day old animal appeared very similar to an adult fraction but, more often, was similar to the fraction from a 7 day old animal which was the earliest age analyzed.

DISCUSSION

The present investigation was initiated in order to determine whether any great differences in the distribution of protein antigens in neonatal and mature animals and in normal and tolerant ones might account for the differences in the immunological response of these groups to antigenic exposure.

Disappearance of the antigen from the serum of infant rabbits, Fig. 1, is exponential and of similar slope to that found in newborn rabbits by other methods in a prior study (19). No "immune phase" of disappearance is observed and it is assumed that these animals would be unresponsive to reinjection of the antigen within an appropriate time interval as was demonstrated in other groups, Table III.

Over-all, the organ distribution of radioactivity indicates significant persistence of antigen in the tissues of the rabbit injected at birth, (Table I and Fig. 2). For at least a period of 4 weeks after injection, the localization was preferential in the liver and kidney and the loss occurred exponentially from these tissues. The degree of antigen persistence was lower in the other three tissues examined: spleen, lung, and thymus. In these tissues, the level of radioactivity appeared to become stabilized at about 3 weeks of age.

The differences in organ localization in immature as compared with adult rabbits (Table II and Fig. 3) appear to be minor differences. It is obvious, however, that 21-day-old animals accumulated a higher specific activity in the spleen than did either newborn or adult rabbits and mature animals were found to have more radioactivity in the thymus than did either of the other groups. These differences were apparent whether judged as specific activity or as the proportion of injected dose in the organ; the latter method for calculating antigen retention takes into consideration the relative growth of the organ involved. The other organs appeared to accumulate similar amounts of antigen in all groups when the retention was based on percentage as a means of accounting for growth. No satisfactory interpretation of the slight, though apparently significant differences in organ distribution in young and adult animals, which might clarify their fundamentally different response to injection of antigen, is apparent.

The obvious difference in specific activity of the adult liver tissue is an important consideration if a high concentration of antigen in some particular tissue is significant. It is well recognized that there are minimal doses of antigen required for antibody induction and below certain levels of antigenic stimulation, there is no antibody response. Extensive studies (10) with two soluble antigens, hemocyanin (KLH) and bovine serum albumin (BSA) have shown that the antibody response was better to the KLH which was retained in the liver several fold better than BSA upon primary stimulation. There are of course examples of foreign material being retained and antibody not being detected (21), yet retention is necessary. In the same studies as mentioned

above (10) with KLH and BSA, it was observed that the percentage retention of multiple injections was similar for both proteins but much less than from a primary injection. This finding led to other investigations which showed that the primary injection was lost from the liver during secondary immunization and that circulating titers could be related inversely to retention in the liver of the primary injection (20). Measurement of antigen retention and subsequent loss of this antigen describes in a very general manner the process of handling antigen during antibody formation. Whereas circulatory half-life values of different proteins (22) are very interesting, both as related to metabolism and presence of circulating antibody, they fail to indicate the antibody-forming capacity of the individual.

Reduced antibody production involves a mechanism which either prevents degradation of the foreign material into antigenic fragments and/or involves blockage of the material in tissue sites. That tissue must rid itself of antigen in order that antibody production be observed was similarly concluded by Johnson *et al.* (23). Among the tolerant and normal litter mates (Table III) there was 1 rabbit, No. 4-80, which gave circulatory clearance of antigen similar to an animal making an immune response; but, the animal had no circulating antibody and hence was tolerant, at least by definition.

Concerning the comparison of the distribution of S^{35}-BSA in the organs of normal and S^{35}-BSA-tolerant rabbits there was a failure to reveal a pattern of organ localization in the tolerant different from the normal counterpart. Only the slope of decrease in the concentration of S^{35}-BSA in the serum measured at intervals after injection into the two groups, and the appearance of antibody after an appropriate interval, distinguish the unresponsive from the normal group. The normal group cleared the antigen from the circulation rapidly starting on the 9th day after injection, whereas the tolerant group continued to show exponential type decay with no "immune phase" of rapid disappearance. The larger percentages in all tissues of the tolerant group compared with the normal are slight differences but are the levels expected to persist from the primary injection. There is no indication of an immune loss of antigen from tissues as predicted from previous work (20) if circulating antibody had been produced.

The resemblance between the pattern of gross organ localization in tolerant and normal animals extended to the distribution of radioactivity in the various subcellular liver fractions. The only difference evident was that twice as much antigen was found in the nuclear fraction of the tolerant as the normal group. Assessment of the significance of this observation will require further investigation.

The largest proportion of radioactivity was contained in the supernatant fraction. Attempts at further separation of this fraction by starch block electrophoresis revealed that essentially no difference in the mobility and association of the radioactivity with phosphorus-containing compounds could be discerned

by this technique. It is of interest that little of the radioactivity in either group had the same mobility as the similar amount of S^{35}-BSA added *in vitro* to normal liver supernatant fraction, which is of a mobility midway between the phosphorus and the protein peaks. By 3 weeks then, most of the antigen either was altered in mobility or associated with other proteins of a different mobility. These observations invite further elaboration.

No presently known hypothesis adequately accounts for available data concerning immunological unresponsiveness to a protein or more complex antigens. The importance of phylogenetic and structural similarity of the antigen to the host's own tissue components or products, the physical form of the antigen, persistence of the antigen, route of administration, age factors, and species variations, each has received emphasis from different investigators. The data presented here indicate only that antigen does persist in tissues of tolerant as well as normal animals, but fails to show any unique characteristic of the persisting antigen which explains inhibition of the immune response to antigen. Nor do the differences in distribution of antigen with age as revealed in this model explain the significance of the age factor. Critical location or distribution of a hypothetical small but significant proportion of the large excess antigen given could be overlooked by these methods, and the demonstrated distribution throughout the body in these experiments might possibly have no causative relation to the unresponsive state. These studies suggest that attention should now be turned to the examination of more subtle or indirect mechanisms.

One very obvious point of interest is to study the retained antigen for comparison with that isolated from adult tissues (10). Another study would involve an attempt to detect antibody other than precipitating, the latter being difficult to analyze in the young (18). Human infants at a very early age after immunization (24) with vaccines have produced antibody detectable in the S_{19} fraction of serum. This study emphasizes the need to search for an immune response in the young of other species by application of newer techniques of isolation and assay of antibody.

The free boundary electrophoretic patterns merely present the obvious fact of immaturity by showing the differences in one tissue fraction as a factor of aging. The time required for maturation coincides with the extent of time after birth when tolerance may be induced (3) and also with the age for attaining complete enzyme systems (25). It was reasonable to study this particular fraction since rabbits when injected for the first time as adults have shown very clear-cut gross differences in retention in the liver supernatant fraction and antibody production. For example, adult rabbits, receiving similar injections of antigen show less retention of the antigen in this fraction with increasing circulating antibody titer, whereas the occasional animal that fails to produce any detectable precipitating antibody to BSA stimulus retains the highest level of antigen detectable in this fraction (26). These findings beckon further investigation of this fraction particularly as regards the association of antigen

with soluble RNA (10). If, as indicated by other workers, soluble RNA has a unique role in protein synthesis (27), then studies of this fraction are indeed pertinent to any study of antibody formation.

SUMMARY

The fate of injected S^{35}-labeled sulfanilic acid-azoalbumin in the serum, various organs, and liver fractions was compared in the newborn and adult rabbit and in specifically unresponsive and normal adult rabbits. Exponential decay of the injected antigen without the usual immune phase of elimination was observed in newborn and unresponsive adult animals. Comparison of organ distribution of radioactivity in adults and animals injected at birth and 21 days of age showed persistence in the liver at least as long as 3 weeks in all groups (which was the time chosen for observation). The slight differences in spleen and thymus concentrations with age are of undetermined significance. Comparison of the organ distribution of antigen in unresponsive adult rabbits and in normal ones showed slight differences which were similar to those predicted from previous immunization of adults which gave low grade antibody response. There was a slight selective accumulation of antigen in the nuclear fraction of liver homogenates of unresponsive animals, but no other differences were observed.

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