THE CATABOLISM OF PROTEIN ANTIGENS IN THE NEWBORN AND MATURING RABBIT*

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The relative ease with which immunological tolerance to protein and transplantation antigens can be induced in the newborn animal as compared to the adult remains unexplained. Studies which revealed that the duration of tolerance was directly proportional to the dose of these antigens also showed an inverse relationship between the age of the animal and its susceptibility to induction of immune tolerance (1, 2). Following birth, a high percentage of animals could be rendered tolerant following introduction of these antigens. After this period, the same weight-graded dose of antigen, which formerly induced tolerance, now provoked an immune response.

Externally isotopic labeled foreign protein antigens have been employed previously in attempts to demonstrate significant differences in the metabolism, localization or retention of these compounds in the newborn and more mature animal (3–6). No differences found could be correlated definitely to the above mentioned susceptibility of the newborn to induction of specific tolerance. However, these experiments did not examine differences in the metabolism of the protein due to the addition of the hapten, nor did they account for differences in the rates of catabolism of various proteins in newborn and adult animals. Therefore, experiments were designed to compare in newborn and more mature rabbits the capacity to catabolize and to localize a heterologous and homologous azoalbumin and an S^{35} internally labeled homologous albumin. The rate of catabolism of the azoproteins was found to be slower in the newborn as compared to the maturing animal. Moreover, the addition of a hapten to homologous or heterologous protein determined the pattern of catabolism of the protein rather than the carrier protein itself.

Methods and Materials

Animals.—Rabbits used in these experiments were of hybrid New Zealand stock. The litters of newborn and 6-day-old rabbits were kept with the doe until the experiment was

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completed. 30-day-old animals had been weaned from the doe. All animals were offered standard rabbit chow and water *ad libitum*.

Internally labeled S^{35} rabbit serum albumin (IRSA) was prepared from the serum of newborn rabbits which had received high specific activity S^{35} L-methionine 8 hours previously. The albumin was isolated by the method of Schwert (7). The specific activity of the isolated protein was about 10,0000 CPM/mg protein at time of injection. Such preparations yielded a single line in agar gel when reacted with the appropriate antiserum by immunoelectrophoresis.

Lightly labeled S^{35} benezenesulfonate azoproteins, bovine serum albumin (SBSA), and rabbit serum albumin (SRSA), were prepared according to the method described by Garvey and Campbell (8) with the following modifications. High specific activity carrier-free sodium sulfate obtained from the Atomic Energy Commission, Oak Ridge, Tennessee, was evaporated to dryness and 0.5 ml of 1 N H_2SO_4 added. 0.0450 mg of redistilled aniline was added to the the $H_2S^{35}O_4$ in a vial and slowly heated to 150°C for 2 hours. The vial was then placed in an Abderhalden vacuum desiccator, and a vacuum of 10 mm Hg maintained over P_2O_5. The temperature of the reaction mixture was brought to 204°C with metacresol and the reaction allowed to continue for 6 hours. The S^{35} benzene sulfonate was dissolved in warmed deionized water and the pH adjusted to 8.0 with 1 N NaOH. The diazonium salt was formed at 4°C by the stepwise addition of 500.0 mg of sodium nitrite and 0.45 ml of 6 N HCl. The salt was then divided into 2 equal aliquots and added dropwise to 750 mg of BSA or RSA.[1] The pH of these reaction mixtures was kept as close as possible to pH 7.8 with 0.1 N NaOH. Coupling proceeded at 4.0°C for 5 hours after which the non-diazotized hapten was removed by dialysis against 0.14 M NaCl and 0.001 M benezenesulfonate for 12 hours. The protein solutions were subsequently passed through sephadex[2] G-50 and the protein-containing fractions lyophilized. 99.25 per cent of the radioactivity in the resultant eluate could be precipitated with 10 per cent TCA. The diazotized albumins contained on the average 2 to 3 hapten groups per molecule.

The labeled proteins were dissolved in 0.15 M NaCl and theirs concentration measured by the Biuret method (9). The specific activity of the protein at time of injection was approximately 6.0×10^3 CPM/mg protein.

Injection of the Proteins and Preparation of the Tissue for Radioassay.—Rabbits in each age group were injected subcutaneously in 2 sites along each flank with 8.0 mg/100 gm body weight of the azo proteins and 40.0 mg/100 gm body weight of the IRSA. The higher protein dose of the IRSA was necessary to provide sufficient radioactivity for accurate assay after injection. The urethra and rectum were sutured closed in each animal so that the contents of the bladder and colon could be collected intact. The rabbits were then returned to their nesting boxes. Reliable separation of feces from the mucosa was not possible so that the entire colon was taken with its contents and the total radioactivity reported as that contained in the feces. For the short term experiments, the animals were sacrificed 24 hours after injection by exsanguination and their tissues perfused with 0.15 M NaCl until the returning fluid appeared bloodless.

The organs were weighed and homogenized in 40 volumes of 0.01 M pH 4.0 acetate buffer per gm organ weight in a Waring blendor. Duplicate aliquots were removed for analysis. TCA precipitability was determined by adjusting the final concentration of each sample to 10 per cent TCA by volume and incubating the sample at 37°C for 1 hour, the supernatant fluid poured off and the precipitate resuspended in 10 per cent TCA and recentrifuged. This precipitate was assayed for radioactivity. All organs including skeletal muscle and the skin at the site of injection were taken for analysis. Assay of the concentration of S^{35} of the skin surrounding the site of injection never exceeded 2.0 per cent of the total injected radioactivity.

[1] BSA Pentex Inc., Kankakee, Illinois, lot 7; RSA, Pentex Inc. lot 1a.

[2] Pharmacia, Uppsala, Sweden.

Preparation of Carcasses for Analysis of Radioactivity.—The same weight-adjusted dose of albumins was injected into rabbits without suturing their excretory orifices. The animals were placed in 500.0 ml beakers and sacrificed 24 hours later with sodium pentobarbital anesthesia. The carcasses were transferred along with multiple rinses of the beaker and homogenized in 50.0 volumes of deionized water/100 gm body weight in a Waring blendor at 4.0°C. Duplicate aliquots were taken for analysis and for assay after precipitation of the sample with 10 per cent TCA as previously described.

Determination of the Rate of Loss of the S^{35} Albumins from the Circulating Blood Volume.—Rabbits in each age group were injected with the same weight-graded doses as described. The blood was removed at various intervals by intracardiac puncture and quickly mixed with 2.00 4.0 ml of 0.15 M NaCl with 0.1 mg of BSA to serve as a carrier. 1.0 ml aliquots were then removed for analysis of total and TCA-precipitable radioactivity. In the newborn and 6-day-old group, the total quantity of blood removed did not exceed 2.0 ml. The results are plotted as the counts per minute per total circulating blood volume (CPM/CBV). The CBV was estimated arbitrarily by multiplying the total body weight by 0.1 regardless of age.

Preparation of the Samples for Analysis of Radioactivity.—The extraction procedure used in these experiments was modified from that of Tarver (10). The sample was first digested with 2 volumes of Pirie's reagent by slowly heating a 75 × 100 mm combustion tube up to a temperature of 310.0°C for 36 hours. At the end of the combustion, the tops of the tube were gently flamed and the tubes allowed to cool. This procedure oxidized all tissue sulfur and S^{35} benzenesulfonate to sulfate and volatized the carbon, nitrogen, and phosphorus as their respective oxides. In this procedure, the sulfate is quantitatively retained as the cupric salt. 1.0 ml of concentrated HCl was added to each sample and the tubes slowly heated up to 100°C for 24 hours. The resultant powder was dissolved in 5.0 ml of 0.1 N HCl and a 1.0 ml aliquot taken for assay. Preliminary analysis of the total sulfate of these samples, using the method described by Wainer and Koch (11), showed that the total concentration of sulfate did not exceed 0.1 mg/sample. Therefore, the contribution of the tissue sulfur and benzenesulfonate in the samples was considered as zero.

3.0 and 4.0 mg of carrier sulfate was added and the solutions thoroughly mixed. The calculation of the theoretical yield of the precipitated sulfate therefore considered only the weight of the carrier sulfate added. 3.0 ml of 5 per cent solution of benzidine or barium chloride was added to precipitate the sulfate.

When benzidine was used, the precipitate was poured into a column fitted over a sintered glass filter held by a spring clamp.[3] The sintered glass was covered with Whatman No. 42 filter paper and was connected to a vacuum flask. With 20 mm Hg vacuum, a visible cake of benzidine sulfate could be seen after the fluid had passed through the column. The precipitate was washed with 95 per cent ethanol, the column was removed, and the cake transferred to an aluminum planchet and dried under an infrared lamp. The cakes prepared in this fashion had a surface area of 2.8 $cm.^2$

In later experiments, barium chloride was used to precipitate the sulfate. The barium sulfate precipitate was centrifuged at 600 *g* for 10 minutes and resuspended and washed with deionized water. The procedure was repeated three times. The precipitate was transferred to a weighed monel planchet of constant geometry and dried for 24 hours under an infrared lamp.

The samples were then weighed on a Mettler balance and counted 2 or 3 times for 5,000 or 10,000 counts in a gas flow counter using "Q" gas and a "mylar" window. The appropriate corrections were then made for recovery of the precipitated sulfate, sample dilution, self-absorption of the sample, isotope decay, and to a known carbon 14 standard.

This extraction procedure permitted 100 per cent recovery of the isotope from all tissue studied within a wide range of organ weights. The standard deviation of error of replicate

[3] MaCalaster Bucknell Corp., Cambridge, Massachusetts.

samples was less than 3.0 per cent and the counting efficiency using this procedure and apparatus was 7.8 per cent.

EXPERIMENTAL

Catabolism of Various S^{35}-Labeled Albumins in Newborn and 8-Day-Old Rabbits.—The total capacity of the newborn and 8-day-old rabbit to degrade the various S^{35} albumins into non-TCA–precipitable form was determined. 24 hours after subcutaneous injection of SBSA, SRSA, or IRSA, the animals were sacrificed and with their excreta homogenized whole in saline. Total and TCA-precipitable radioactivity from the resultant soluble material was determined. Recovery of the total radioactivity was 100 per cent. Since 99.25 per cent of the

TABLE I

TCA Precipitable S^{35} in Newborn and 8-Day-Old Rabbits 24 Hours after Injection of Albumins

Age of animal	No. in group	SBSA	SRSA	IRSA
		per cent	*per cent*	*per cent*
Newborn	2	61.5	69.2	76.3
		65.8	69.0	79.1
8 days	2	33.1	35.6	85.7
		32.7	39.6	87.2

Newborn and 8-day-old rabbits were injected subcutaneously with SBSA and SRSA (8.0 mg/gm body weight) and IRSA (40.0 mg/gm body weight) and placed in a 500.0 ml beaker for 24 hours. The animals were sacrificed and transferred with their excreta to a Waring Blendor where they were homogenized. Aliquots of the carcass homogenates were analyzed for total and TCA precipitable radioactivity. The results represent the per cent precipitable by 10 per cent TCA.

injected radioactivity was TCA-precipitable, TCA-precipitable radioactivity in the carcass after 24 hours indicated the proportion of S^{35} still protein bound. The results are shown in Table I.

Approximately two-thirds of the azoproteins were still precipitable by 10 per cent TCA 24 hours after they were injected into the newborn animals as compared to approximately one-third of the total amount present in this form in the 8-day-old animals. A small difference, about 6 per cent, was noted between the total amount and the TCA-precipitable S^{35} remaining in the newborn and 8-day-old animals injected with the IRSA.

The fate of the isotope, which is different in the biosynthetically labeled and azoproteins, influences the interpretation of these results. The S^{35} benzenesulfonate moiety of azoproteins has been shown not to be reincorporated into new protein after the carrier protein has been hydrolyzed but is rapidly excreted into the urine (8). Thus, it is valid to conclude, within limits of possibility of

preparing identically labeled BSA and RSA, that diazotization of both homologous and heterologous proteins alters their rate of catabolism equally. Further, their rate of catabolism was significantly higher at 8 days of age than at birth.

After hydrolysis, the S^{35} label of intrinsically labeled RSA may be reincorporated into tissue protein (12). This effect is minimal within 24 hours after injection (13). However, without comparable data upon the rates of reincorporation of reutilizable S^{35} amino acids in the newborn and maturing animal, the small differences seen between the 2 groups is in this experiment difficult to interpret and cannot be assumed to be significant.

The marked difference in the 24 hour degradation of intrinsically labeled and azoproteins is significant, suggesting that diazotization increases the catabolism of both homologous and heterologous proteins. In order to compare the fate of the heterologous and homologous proteins in greater detail, experiments were designed to examine their degradation in groups of newborn, 6- and 30-day-old animals.

Urinary and Fecal Excretion of Radioactivity 24 Hours after Injection of Newborn, 6-, and 30-day-old Animals with S^{35} Albumins.—The excretion of the S^{35} albumins was measured in the urine and feces of the newborn, 6-, and 30-day-old rabbits 24 hours after injection of the proteins. The results, shown in Table II, correlate well with the amount of TCA-soluble isotope present in the whole carcass of animals 24 hours after injection (Table I). 35 per cent of the activity in the azoprotein groups had been degraded to the TCA-soluble fraction in the newborn group. This value increased to about 65 per cent in the 8-day-old group.

The total radioactivity in the feces did not differ in the 2 groups injected with the azoproteins but appeared higher in the 6- and 30-day old animals than the newborns. TCA-precipitable radioactivity was small regardless of age group or protein injected. Fecal excretion of S^{35} in the IRSA group appeared to increase significantly between birth and 6 days. This is inconsistent with the data in Table I which indicated that the initial rate of degradation of the IRSA in the newborn animal was higher than the older groups. However, in this experiment, the total proportion of TCA-soluble S^{35} in the entire animal was not measured. Thus, differences in the rate or mode of excretion rather than the rate of catabolism might account for this discrepancy.

Comparison of Blood Clearance Rates of S^{35}-Labeled Albumins in Newborn, 6-, and 30-day-old Rabbits.—Blood clearance of the 3 albumins was assessed by 2 methods. The 1st method examined the total and TCA-precipitable radioactivity present in the circulating blood of animals injected 24 hours previously. The data in Table III indicate that there is delay in the initial clearance of both azo compounds in the newborn animals and that the clearances of both azoproteins were essentially equal. However, in contrast to the 6-, and 30-day-old groups in which the S^{35} activity in serum was nearly 100 per cent precipit-

able, only approximately 60 per cent of the S^{35} was in this form in the newborn group. These data suggest that the ability of the newborn rabbit to excrete the hydrolyzed benzenesulfonate moiety is impaired relative to the 6- or 30-day-old animal. These results are consistent with experiments which show that the renal clearing capacity of organic acids is low at birth (14, 15). Increasing ability to clear the azoproteins as the animal matures was shown by the faster

TABLE II

*Injected Radioactivity in Urine and Feces of Rabbits of Varying Age 24 Hours Following Injection of S^{35} Albumin**

Specimen examined	Age of rabbits								
	1 day			6 days			30 days		
	SBSA	SRSA	IRSA	SBSA	SRSA	IRSA	SBSA	SRSA	IRSA
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Injected radioactivity in urine	20.8	21.5	1.4	40.7	41.8	18.0	51.6	46.0	20.6
	34.0	24.5	1.6	42.2	42.7	21.3	51.6	42.0	18.9
	37.1	15.6	1.0	41.6	—	20.9	47.3	44.7	—
	24.1	20.6	—	—	—	—	—	—	—
Average	29.0	20.6	1.3	41.5	42.2	20.1	50.2	44.0	19.8
Injected radioactivity in feces	4.1	5.0	1.3	1.0	5.0	10.1	—	3.2	7.5
	1.4	4.0	0.8	2.4	4.0	8.0	—	3.0	4.8
	5.8	5.5	1.0	1.1	1.0	8.8	—	—	—
	5.8	5.7							
Average	4.3	5.1	1.0	1.5	3.3	9.0	—	3.1	6.7

* Rabbits were injected with 8.0 mg/100 gm body weight of the S^{35} SRSA or SBSA and 40.0 mg/100 gm body weight of the S^{35} IRSA and their urethrae and rectums sutured. 24 hours later, the animals were sacrificed and the bladder and colon contents assayed for S^{35} activity. The proportion of S^{35} radioactivity precipitable with TCA was less than 3.0 per cent in all the samples studied.

clearance of the proteins in the 30-day-old group as compared to the 6-day-old group. The most marked change, however, appeared in the period between the 1st and 6th day of life. A twofold difference was found between the circulating TCA precipitable radioactivity in the animals injected at birth and at 6 or 30 days of age.

The second method of determining plasma disappearance rates demonstrates the importance of examining the earliest days of life for metabolic phenomena which might be masked by more extended periods of study. Serial bleedings were taken at various intervals over a 10 day period after injection of groups

of rabbits at birth, 6 and 30 days of age with the same proteins. To account for the growth of the animals during the experiment, these data were expressed in terms of circulating blood volume. The slope of these clearance curves and the derived rates of degradation given as half-life values are shown in Table IV.

TABLE III

*Total Blood Content of Radioactivity of Rabbits of Varying Age 24 Hours after Injection of Various S^{35} Albumins**

Measurement	Age of rabbit								
	1 day			6 days			30 days		
	SBSA	SRSA	IRSA	SBSA	SRSA	IRSA	SBSA	SRSA	IRSA
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Injected radioactivity in estimated total blood volume	3.8	3.5	16.2	0.5	2.5	6.4	0.1	0.8	8.0
	3.7	3.5	11.6	2.1	1.9	6.4	0.7	0.9	9.0
	8.4	8.6	13.8	2.2	1.9	7.9	1.2	1.2	
	7.9	8.4							
Average	6.0	5.2	13.9	1.6	2.1	7.0	0.7	1.0	8.5
Radioactivity precipitable by TCA	62.1	64.6	96.8	94.3	97.2	101.0	102.3	92.5	103.4
	58.3	63.9	99.2	93.8	99.0	96.5	94.6	98.3	98.0
	59.0	61.0	101.2	96.2	93.1	100.2	98.2	99.1	
	57.6	53.7							
Average	59.3	60.8	99.0	94.8	96.4	99.2	98.4	96.6	100.7
Mean injected radioactivity precipitable by TCA	3.6	3.2	13.8	1.5	2.0	6.9	0.7	1.0	8.5

* Animals were injected with 8 mg/100 gm body weight of the S^{35} SBSA or SRSA and 40 mg/100 gm body weight of the S^{35} IRSA and their urethrae and rectums sutured. 24 hours later, the blood was removed by intracardiac puncture. Equal aliquots of whole blood content was calculated from the concentration measured by assuming a total blood volume of 10 per cent of body weight in each age group.

Both azoproteins were rapidly cleared from the circulation with a mean half-life of approximately 1.7 days in all age groups. Therefore, it appears that the rate of disappearance of the azoproteins was related to the addition of the benzenesulfonate and not to the carrier protein moiety. The same difference between the total and TCA precipitable radioactivity from days 1 to 5 was encountered in this type of experiment as in the first type used to study blood levels of the injected proteins. Between 35 to 42 per cent of the injected radioactivity was TCA-soluble 1 to 5 days after injection into the newborn animals.

In the 6- and 30-day-old group, only 0 to 5 per cent of the S^{35} was TCA-soluble. However, the slope of the values for TCA-precipitable radioactivity was essentially the same for all groups falling within the experimental error of the method. Data accumulated in this way, therefore, did not reveal the differences in catabolism of these azoproteins which had been shown by another method to exist between birth and 6 days of age.

No significant difference between the blood degradation rate of the IRSA in the three groups was demonstrated through either experimental design.[4] This finding is best interpreted as indicating either a higher rate of reincorporation or lower rate of excretion of the degradation products of IRSA during the

TABLE IV

*Average Half-Life in the Circulating Blood of S^{35} Albumins Injected into 0, 6-, and 30-Day-Old Rabbits**

Age of rabbit								
1 day (3 rabbits/group)			6 days (3 rabbits/group)			30 days (2 rabbits/group)		
SBSA	SRSA	IRSA	SBSA	SRSA	IRSA	SBSA	SRSA	IRSA
1.7	1.7	6.1	1.6	1.7	5.9	1.8	2.0	6.0

* Animals were injected with 8 mg/100 gm body weight of the S^{35} SBSA or SRSA and 40 mg/100 gm body weight of the S^{35} IRSA. Aliquots of blood were removed on days 1, 2, 3, 5, and 9 after injection and were analyzed for total and TCA precipitability. The $t_{1/2}$ is expressed in days and is derived from the 1st order equation $Kt = \text{Log}_e \text{CPM}_0/(\text{CPM}_0 - \text{CPM}_t)$. Results are expressed as the average of the values within each group and were calculated for the total CPM within the circulating blood by assuming a total blood volume of 10 per cent of body weight in each age group. The range of $t_{1/2}$ for each group was within 0.2 days of the average value.

first 24 hours of life rather than a slower rate of catabolism. Indeed, other workers have found that albumin turnover rates are higher in the newborn period than later in life (16, 4, 5).

Gastrointestinal Localization of Various Albumins in the Newborn, 6-, and 30-day-old Rabbit.—Evidence presented elsewhere supports an excretion–intestinal reabsorption cycle of native or near native albumins (17, 18). Therefore, decreased gastrointestinal hydrolysis of excreted protein in the newborn animal might increase the pool of circulating labeled protein over that in the

[4] Reincorporation of the S^{35} label in these experiments does not present the same problem that was encountered when the catabolism of the IRSA was investigated by the method of carcass analysis. Less than 1 per cent of the S^{35} of the amino acids of the IRSA is reutilized for plasma proteins so that the turnover of the isotope does not introduce an error greater than that encountered by the assay (16).

TABLE V

Total Radioactivity in the Stomach and Small Intestine and Their Contents of Rabbits of Varying Age 24 Hours Following Injection of Various S^{35} Albumins

		Age of rabbits								
		1 day			6 days			30 days		
		SBSA (4)	SRSA (4)	IRSA (2)	SBSA (4)	SRSA (4)	IRSA (2)	SBSA (2)	SRSA (2)	IRSA (2)
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Injected radioactivity in stomach	Average	0.6	0.7	1.3	0.4	0.5	0.7	0.3	0.1	0.5
	Range	(0.2–0.8)	(0.3–1.4)	(1.2–1.3)	(0.2–0.5)	(0.4–0.6)	(0.6–0.7)	(0.2–0.3)	(0.1–0.1)	(0.3–0.7)
Radioactivity precipitable by TCA	Average	3.5	1.5	6.1	0.0	0.4	6.2	1.3	1.2	10.7
Injected radioactivity in contents of stomach	Average	0.3	1.6	1.3	0.4	0.3	0.8	0.0	0.1	0.4
	Range	(0.1–0.5)	(0.3–4.7)	(1.2–1.3)	(0.2–0.5)	(0.2–0.4)	(0.6–1.0)	(0.0–0.0)	(0.1–0.1)	(0.4–0.4)
Radioactivity precipitable by TCA	Average	0.8	0.3	6.7	0.4	2.8	6.2	—	0.6	1.0
Injected radioactivity in small intestine	Average	4.0	4.2	2.9	1.3	2.8	8.2	0.8	1.7	7.7
	Range	(2.8–5.0)	(2.1–5.8)	(2.6–3.1)	(0.8–2.1)	(1.1–4.8)	(8.0–8.4)	(0.3–1.1)	(0.2–2.6)	(7.5–7.9)
Radioactivity precipitable by TCA	Average	31.4	24.4	34.0	11.5	10.4	12.1	—	10.2	—
Total injected radioactivity in contents of small intestine	Average	2.1	1.2	0.2	0.6	0.7	0.5	0.4	0.3	0.5
	Range	(1.3–2.4)	(0.2–2.6)	(0.1–0.2)	(0.5–0.7)	(0.4–0.9)	(0.4–0.6)	(0.3–0.6)	(0.2–0.3)	(0.3–0.7)
Precipitable by TCA	Average	35.8	33.6	40.0	1.3	0.5	0.5	0.6	0.6	4.2

Animals were injected with 8.0 mg/100 gm body weight of the S^{35} SBSA or SRSA and 40.0 mg/100 gm body weight of the S^{35} IRSA and their urethrae and rectums sutured. 24 hours later, the animals were sacrificed, perfused, and the lumens of the stomach or small intestine perfused with 0.145 M NaCl *in situ* and the contents rapidly collected and chilled. Homogenates of the organs and their contents were assayed for total and TCA-precipitable radioactivity. The results are expressed as the average with the range of values found. The number of animals in each group is shown in parenthesis under each column.

6- and 30-day-old animal. Data presented in Table III suggested that IRSA was less rapidly cleared from the circulation of 1-day-old animals than from the 6- or 30-day-old groups. Intestinal localization patterns revealed in the following experiments appear to support this hypothesis as shown by the results in Table V.

In the stomach and small intestine and their contents, SBSA and SRSA did

not distribute differently or differ significantly in TCA precipitability in any age group. The perfused stomach wall of the newborn animal contained about 1 per cent of the injected dose at the end of the 24 hour period but only one-half to one-tenth of this amount was found in the 6- and 30-day-old animals. Whereas an insignificant proportion of this amount was TCA-precipitable in the animals receiving the azoproteins, 6 to 10 per cent of the S^{35} found in the stomach wall and contents of animals receiving the IRSA was in a TCA-precipitable form.

The perfused small intestine of the newborn animals contained 3 to 4 per cent of all injected azoproteins or the IRSA. Whereas the azoprotein radioactivity was much lower in the 6 and 30 day groups, the IRSA content rose substantially over this period of development. A strikingly high proportion of radioactivity in the intestinal wall and contents was TCA-precipitable in the newborn group with both azoproteins and IRSA. The precipitability of protein in the intestinal wall was reduced fourfold in older groups, but nearly all TCA-precipitable activity disappeared from the intestinal contents in the older groups. No difference was seen between the concentrations of the azoproteins or IRSA in this group.

These data are consistent with others suggesting that the intestine has an important role in the metabolism of plasma proteins in the rabbit (17–19). The route by which the protein arrives in the intestinal contents is not revealed by these experiments. It could conceivably be secreted by the upper small intestine, pancreas or stomach and be reabsorbed in the jejunum,—a pattern of intestinal recirculation. On the other hand direct secretion by the intestinal mucosa is equally plausible. The data of Eitzman *et al.* (20) favor the secretory role of the stomach.

Localization of SBSA, SRSA and IRSA in various organs of the newborn, 6-, and 30-day-old rabbit.—In the experiments described above, perfused liver, spleen, kidneys, lungs, bladder, adrenals, and thymus were also taken for assay of total and TCA-precipitable radioactivity. The total radioactivity of the thymus, lung, and adrenals was always less than 0.1 per cent of the total injected dose. The perfused kidney, on the other hand, contained 1.5 to 3.0 per cent of the injected radioactivity but less than 2 per cent of this was TCA-precipitable and thus was presumed to represent contamination of the high specific activity urine (see Table II). Tables VI and VII thus represent data limited to the liver and spleen.

The liver contained the highest proportion of injected radioactivity of any organ assayed. Again, no difference in the fate of SBSA and SRSA could be detected but the animals which had received the azoproteins demonstrated consistently higher values of liver accumulation from the IRSA groups. The homologous azoproteins are selectively accumulated in the liver possibly because of the alteration of the protein during the diazotization process.

The liver of the newborn animals which received azoproteins contained a lower proportion of TCA-precipitable activity than the older groups as was found in the circulating blood. This probably reflects relative inability to excrete the benzenesulfonate. Therefore, the significantly lower accumulation of activity in the livers of newborn as compared to the maturing rabbits are prob-

TABLE VI

Injected Radioactivity in the Livers of Rabbits of Varying Age 24 Hours after Injection of Various S^{35} Albumins

Measurement	Age of rabbit								
	1 day			6 days			30 days		
	SBSA	SRSA	IRSA	SBSA	SRSA	IRSA	SBSA	SRSA	IRSA
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Injected radioactivity	9.3	7.0	5.1	13.7	13.0	1.8	11.3	11.7	1.7
	9.0	5.4	4.5	16.3	11.8	2.1	12.5	14.3	1.4
	9.5	7.3	5.0	13.8	13.2		11.8	13.1	
	9.3	6.9							
Average	9.2	6.9	4.7	14.6	12.7	2.0	11.9	13.0	1.6
Radioactivity precipitable by TCA	67.0	61.0	97.2	95.0	100.0	94.0	84.9	80.1	99.4
	63.0	66.0	88.3	88.2	80.1	100.0	93.7	83.9	96.3
	59.0	63.7	96.4	92.9	91.2		91.1	90.4	
	62.3	60.1							
Average	62.8	62.7	91.3	92.0	90.4		89.9	84.8	97.9
Total injected radioactivity precipitable by TCA	5.8	4.3	4.3	13.4	11.1	1.9	10.7	11.0	1.6

Animals were injected with 8.0 mg/100 gm body weight of the S^{35} SRSA or SBSA and 40.0 mg/100 gm body weight of the S^{35} IRSA and their urethrae and rectums sutured. 24 hours the animals were sacrificed, by exsanguination and perfused with 0.145 M NaCl. The liver homogenated was assayed for total and TCA precipitable S^{35} activity.

ably even greater than the values indicated by this experiment. In contrast, accumulation of TCA-precipitable activity in the livers of rabbits injected with IRSA was tenfold less than that of the azoproteins.

Because of the small size of the spleens in newborns, only the proportion of the total injected radioactivity in this organ was determined. The value is expressed as specific activity in Table VII since the weight of the spleen relative body weight changes markedly over the first 30 days of life.

The per cent total radioactivity of the azoproteins 24 hours after injection

was not significantly different in the 3 age groups despite the marked increase in size of the organ during this period (mean weight 0.02 gm weight at birth and 0.3 gm at 30 days). The specific activity of the spleen in the animals injected with the azoproteins decreased with age. This suggests that the spleen

TABLE VII

Injected Radioactivity in the Spleens of Rabbits of Varying Age, 24 Hours after Injection of S^{35} Albumins

Age group	SBSA		SRSA		IRSA	
	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Newborn	0.10	1.0	0.16	1.5	0.01	—
	0.12	1.2	0.15	0.7	0.03	—
	0.40	2.1	0.35	1.9	0.01	—
	0.18	1.91	0.20	2.0		
Average	0.20	1.55	0.22	1.53	0.01	—
6-day-old	0.25	1.1	0.15	0.7	0.00	—
	0.15	0.8	0.16	0.5	0.01	—
	0.30	0.6	0.30	1.6		
Average	0.35	0.84	0.21	0.90	0.01	—
30-day-old	0.10	0.02	0.11	0.02	0.01	—
	0.13	0.10	0.15	0.21	0.01	—
	0.24	0.46	0.10	0.31		
Average	0.16	0.19	0.12	0.12	0.01	—

Rabbits were injected with 8.0 mg/100 gm body weight of the S^{35} SBSA or SRSA and 40.0 mg/100 gm body weight of the S^{35} IRSA and their urethrae and rectums sutured. 24 hours later, the animals were sacrificed and their spleens analyzed for radioactivity. Since the spleen grew faster than body weight during this time, a significant difference between the groups is noted when the specific activity (CPM/mg tissue) is also plotted.

either has a finite capacity for clearing these proteins or that its role in metabolizing the injected dose and preparing it for excretion increases progressively over the period under study. The spleen accumulated little IRSA in any age group as compared with the azoproteins.

DISCUSSION

The physiological processes concerned with immunity develop very rapidly in the rabbit and human newborn. Induction of antibody synthesis in the newborn period has been shown to occur at almost the same rate as in the adult,

although the appearance of the 7s gamma-2 globulin antibody is apparently delayed in the newborn rabbit and human (21, 22).

Furthermore, the newborn rabbit can synthesize gamma globulin *de novo* at birth (23). It therefore appears that immune tolerance must be induced at a time when most, if not all of the capacities for gamma globulin synthesis are already developed. Attention was, therefore, directed to the preimmune phase of antigen catabolism during the newborn period of development.

The studies reported here show that the rate of degradation of heterologous and homologous azo albumins is markedly accelerated over that of native albumin. The close similarity in the deposition and excretion of the two azo proteins employed in these experiments indicates that the alteration induced by diazotization is the determinant of their metabolic fate, regardless of the ultimate immunological effect of the protein. In this case, tolerance of the hapten protein complex or carrier protein would be produced in either case by neonatal injection but injection into mature animals would result in antihapten antibody in both cases and, additionally, anti-BSA in animals injected with SBSA (24).

The small intestinal localization and the high fecal content of radioactivity in animals receiving the various albumins confirm the recently reported evidence for intestinal albumin catabolism (18–20). These data indicate that both heterologous and homologous proteins may be degraded in the gastrointestinal tract. The immunological significance of this catabolic route is unknown. It is interesting, however, that the group of proteolytic enzymes which require sulfhydryl group activation, whose activity is independent of divalent cations, and have relatively low pH optima, collectively known as cathepsins are present in the wall of the small intestine. These enzymes have been shown to degrade serum ablumin in such a way as to reveal new antigenic determinants (25). Therefore, the intestine may conceivably have some role in the preparation of antigen for immunological activity.

Deficiency in hydrolytic enzymes capable of degrading heterologous proteins might be predicted since many enzyme systems have been found to be undeveloped in the newborn period. Furthermore, maturation of enzyme activity to adult levels, occurs at different periods during development. Thus, the mature levels of beta galactosidase of of the small intestine of the rat appear during the last third of intrauterine life (26). Glucose-6 phosphatase activity in the kidney of mice at birth is quite low but reaches adult levels 24 hours after birth, whereas glucuronyl transferase activity in the human may not attain adult levels for several days after birth (27, 28). Complex systems of function such as the renal excretory function as measured by PAH excretion in the human may not mature until as late as the end of the first year of life (29). If it is assumed that several physiological processes participate in the development of an immune response, then it is probable that all systems do not mature at the same rate after birth. These experiments suggest that the total rate of catabolism of proteins, which are antigens in mature animals, is low during the first 24 hours of life in the rabbit, but that the mature level is probably attained by the 6th day of life. Studies to elucidate the developmental changes in enzyme systems having catheptic activity are now under way.

During the early phases of development, body weight is not necessarily related equally well to every biological function. For example, water metabolism is more closely

related to surface area in the maturing human (30). The deficient rate of catabolism of the azo-proteins would seem to be another example of a dosage schedule that is not correlated with body weight. Therefore, it is reasonable to question the assumption that the immunological capacity of the newborn animal is related to weight, the usual parameter for adjusting antigen dose in tolerance experiments.

It has now been established clearly that newborn animals are not unique in their susceptibility to induction of immune tolerance. Tolerance of protein antigens can be induced in adult rabbits by injection of large quantities of antigen which greatly exceed those on the basis of body weight which provoke this state in newborns (31). Another way to induce tolerance is through mechanisms which effectively reduce the number of lymphoid cells such as x-ray irradiation or 6-mercaptorpurine treatment (31, 32). These observations suggest that the critical dose of antigen may be related to the number of immunologically competent cells rather than body weight (33). Some indirect evidence can be cited to support such a hypothesis.

The amount of lymphoid tissue in the newborn rabbit is quite small. Little white pulp is present in the spleen and few lymphoid follicles are found in the lower gastrointestinal tract, peripheral lymph nodes or oropharynx (34). The body weight may treble in size by 30 days in the rabbit, while the spleen weight increases fifteenfold. The body weight at birth, therefore, may grossly overestimate the amount of lymphoid tissue as adjudged by the spleen in the animal.

Therefore, in these experiments as in all tolerance experiments in which amount injected was based upon weight, the amount of protein antigen administered to the newborn animal is very much higher per lymphoid cell than that received by the maturing animal. Furthermore, these studies suggest that protein is degraded at a slower rate and persists longer in the tissues during this period of life. The combination of undeveloped systems of catabolism and a paucity of immunologically competent cells, therefore, may provide the explanation for the ease with which immune tolerance can be induced in newborn animals when the dosage of antigen is based upon body weight.

SUMMARY

The rate of degradation, organ deposition, and blood clearance of SBSA, SRSA, and IRSA has been measured in the newborn, 6-, and 30-day-old rabbits. When the animals were injected with a weight-graded dose of the 3 proteins, differences in their catabolism in the newborns were demonstrable as compared to the 6-, and 30-day-old animals.

The capacity to degrade the azo compounds was shown to be incompletely developed at birth. At 6 days of age, however, the rabbits catabolized these proteins much at the same rate as the 30-day-old animals. Addition of the benzenesulfonate moiety determined the rate of degradation organ deposition and excretion rather than the carrier protein when the azo compounds were injected.

The biosynthetically labeled S^{35} rabbit serum albumin (IRSA) was catabolized at a slower rate than the azoproteins in all age groups. Very little differ-

ence in the metabolism and organ deposition of the IRSA was shown to exist between the newborn and maturing animals.

A dosage schedule, therefore, designed to test the immunological capacity of developing animals may not be valid when calculated upon body weight. The low level of activity of enzyme systems present at birth which degrade antigenic material may serve as an explanation as to why this period of development is so vulnerable to the induction of tolerance rather than immunity when compared to the adult.

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