URINARY EXCRETION OF FOREIGN ANTIGENS AND RNA FOLLOWING PRIMARY AND SECONDARY INJECTIONS OF ANTIGENS*,‡

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The following investigation was divided into two parts. The first was concerned with the rate and amount of excretion of soluble ^{35}S-labeled hemocyanin (KLH) and bovine serum albumin (BSA) following a single intravenous injection into normal rabbits and also with the properties of the excreted antigen material and its possible association with ribonucleic acid (RNA) or nucleotides. The second involved the release and excretion of ^{35}S-labeled material which remained in tissues, or extravascular spaces, at 7 days following the primary injection. This release was accomplished by injection of the unlabeled native protein antigen as described previously by Garvey and Campbell (1).

In a recent report by Garvey and Campbell (2) the loss of antigen material from hepatic tissue following a secondary injection of the native protein carrier was clearly demonstrated by radioautography. Although much of the released material was taken up by spleen and lymph nodes, some of it escaped through the kidneys. It should be pointed out that the radioautographic studies did not bear out the speculation presented by Campbell and Garvey (3, 4) that release of primary antigen resulted in uptake by adjacent hepatic cells and thus production of a "clone." However it is possible that clones of cells might be formed in lymphoid tissues by such a mechanism of release and transfer of antigen. The level may be too low for detection but still significant with respect to antibody formation.

It was expected that the present study of excreted material would give further information as to the nature of retained antigen and also provide material for future investigation of the biological properties of the released material.

Materials and Methods

Antigens.—Two soluble protein antigens were studied, namely, crystallized bovine serum albumin (BSA) obtained from Armour Pharmaceutical Co., Kankakee, Ill., and hemocyanin

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(KLH) isolated from the blood of giant keyhole limpets and purified as described by Campbell et al. (5). These two proteins were conjugated with the diazonium salt of sulfanilic acid-^{35}S, synthesized according to the method described in reference 5 *a*; these preparations of BSA-^{35}S and KLH-^{35}S were used as labeled preparations in primary injections. The initial specific radioactivity of both BSA-^{35}S and KLH-^{35}S was approximately 6 μc/mg of protein which represented a sufficient amount of ^{35}S (half-life of 87 days) for the antigens to be used about 9 months. BSA-^{35}S contained 20 azophenylsulfonate groups per molecule and KLH-^{35}S contained 30, the calculations being made from specific radioactivity measurements and average molecular weights obtained previously (6).

Animals.—Adult male rabbits weighing 3–3½ kg were selected, then allowed a period of 1 month to adapt to laboratory conditions before being given an initial injection of labeled antigen. They were maintained on a standard diet of food and water. The usual primary injection, either BSA-^{35}S or KLH-^{35}S, was 20 mg in a 1 ml volume of saline, and the secondary injection (either BSA or KLH to correspond to the carrier protein given initially) was 5 mg in a ½ ml volume of saline. All injections were made into the marginal ear vein. At least two to four animals were used for each category of data unless stated otherwise.

Collection of Urine.—In experiments reported before (7) rabbits were placed in metabolism cages and urine was collected at 24-hr intervals in a beaker containing a few milliliters of toluene. In the present experiments more effort was made to minimize possible degradation of excreted material by stimulating the rate of excretion, collecting urine at convenient short periods of 2–4 hr and adding ethanol immediately (see below) to the urine samples. Various diuretics that are used in the treatment of humans proved to be ineffective in prorated dosage for rabbits. The method chosen consisted of administering 500 ml of tap water into the stomach 2 hr after injection of antigen. This was done simply by immobilizing the animal in a supine position, inserting a catheter through the mouth, and allowing the water to flow into the stomach by gravity. In general, this procedure gave a relatively uniform pattern in the rate and volume of urinary excretion during the first 24 hr. During this period, 500–900 ml of urine was excreted at relatively regular 2–4 hr intervals beginning about 2 hr after introducing the 500 ml of water. Although the intragastric administration of 500 ml of water would be expected to be a fairly mild treatment, a few (20%) of the animals produced a trace of hemoglobin in the first sample of urine.[1] Consequently these samples were set aside and not pooled with later samples, all of which showed no trace of hemoglobin. Samples of urine were obtained as control material from all animals 24 hr prior to the initial injection of antigen.

Analytical Procedures.—Before attempting to detect and isolate antigen and nucleic acid materials that might be excreted in urine, certain analytical methods had to be selected. As far as antigen was concerned, only that given in the primary injection was of interest. Since the antigen used in the primary injection was the only material containing ^{35}S, radioactivity as determined by scintillation counting was used for the detection and estimation of antigen or antigen fragments. On the basis of previous studies by Garvey and Campbell (8) which indicated that the ^{35}S label remained attached to portions of the antigen after prolonged storage in rabbit liver, it was assumed that the label would be equally stable following excretion and indicative of the presence of antigen material.

Analytical procedures for the detection and estimation of RNA or its degradation products were more difficult and equivocal. Three general types of analyses were made, namely, the orcinol reaction for pentose sugar as standardized with purified yeast RNA (9), organic phosphorus (10), and UV absorption at 260 mμ. As might be expected, the data from all three methods became more significant as the fractionation of urine proceeded, allowing separation and elimination of extraneous material.

[1] Studies of these samples indicated that the rate of excretion and nature of the antigen material was the same as in samples free of hemoglobin.

Other miscellaneous analytical procedures involved estimation of protein concentration from nitrogen determination, biuret reaction (5 *b*), and refractometry. Molecular weights were estimated by thin-layer chromatography using Sephadex G-100 superfine developed with 0.05 M Tris-hydrochloride buffer (pH 7.5, containing 0.1 M KCl) as described by Andrews (11) and assuming molecular symmetry similar to the standards. Cytochrome, BSA, and synthetic polypeptides of known average molecular weight were used as reference standards.

Fractionation Procedures.—In general, procedures were fairly well standardized but underwent minor modifications as the investigation proceeded. The volume of each specimen of urine was carefully measured at the time of collection and quadruplicate 0.5 ml samples were removed for duplicate assays of total ^{35}S radioactivity (12) and of RNA material. In the initial experiments 10 ml samples of urine were separated into three fractions on the basis of solubility in ethanol at about 1°C. The first fractionation was made by mixing the whole urine with 2 volumes, i.e., 20 ml, of 95% ethanol. The precipitate was removed by centrifugation and the supernatant was further fractionated into a precipitate (F-3) and a supernatant (F-4) by an additional 8 volumes, i.e. 80 ml, of 95% ethanol. The supernatant (F-4) fraction was concentrated by flash evaporation at about 40°C and reconstituted to a known volume of 2.5–3.0 ml with distilled water. Likewise the precipitates from each alcohol precipitation were reconstituted in 2.5–3.0 ml water; F-3 was soluble in water but the precipitate obtained with 2 volumes of ethanol was incompletely soluble in water, resulting in two fractions, F-1 that was insoluble and F-2 that was soluble in water. Assays were made to determine ^{35}S radioactivity and RNA material in the various alcohol fractions. (See Table III.) These experiments indicated that the solubility properties of some of the ^{35}S material excreted during the first 12 hr after injection differed from that excreted during the second 12 hr, i.e., 12–24 hr after injection. However, since most of the ^{35}S occurred in the final alcohol-soluble fraction, i.e. in 10 volumes of 95% ethanol, even in the early stages of excretion, subsequent study was limited mostly to this fraction which was obtained in the following manner.

20 ml portions of urine were mixed immediately after collection with 10 volumes of 95% ethanol. After about 30 min at −20°C the precipitate was removed by centrifugation and the supernatant was concentrated to almost dryness by flash evaporation at 40°C. The residue was then dissolved in 6.0 ml of water and placed on a 2 x 4 cm column of Dowex resin (1-X8, Cl form, 200–400 mesh). Elutions were made first with 100 ml of water, followed by 200 ml 0.01 N HCl, then 250 ml 0.02 HCl and finally 30 ml 3.0 M sodium salicylate. The salicylate eluate that contained most of the ^{35}S material was treated with 90 ml of 1.02 N HCl to convert the salicylate to salicylic acid, which was subsequently extracted with three changes of 90 ml each of ethyl ether. In order to separate the NaCl from the antigen material, both of which remained in the aqueous phase, it was necessary to concentrate repeatedly by flash evaporation, using a large volume of absolute ethanol. For example, 10 volumes of absolute ethanol were added to the aqueous phase remaining from the ether extractions; this was evaporated until NaCl crystallized. Filtration on a sintered glass filter was used to remove the NaCl from the concentrate. The latter was again treated with 10 volumes of ethanol, evaporated and filtered as previously. The procedure of adding alcohol, evaporating and filtering to remove NaCl was repeated until such traces of NaCl as remained were soluble in a few ml of ethanol. The few milliliters of alcohol concentrate, in which no more than a trace of NaCl remained, was again concentrated almost to dryness and the resulting residue was redissolved in 3–4 ml absolute ethanol. Further fractionation was then made by paper chromatography using several solvent systems for comparing the resolution of radioactivity and UV absorption. These preliminary findings are presented in Fig. 1. However, the final analyses were based on elution from a 23 x 3 cm column of washed cellulose powder (Whatman Chromedia No. CF 11, formerly designated Ashless-Standard Grind) using a 3:1 isopropanol–water mixture for development. Washing of the cellulose was performed prior to packing the column and consisted of sequential mixing, settling, and decanting from large volumes of isopropanol, water, and ab-

solute ethanol. The column was packed under suction and dried in a nitrogen atmosphere. The sample was then placed on the column and developed with 150 ml of the isopropanol–water mixture. The eluate was collected in 0.5 ml fractions from each of which 20 μl was sampled for liquid scintillation counting. The fractions were combined into five final pools as a result

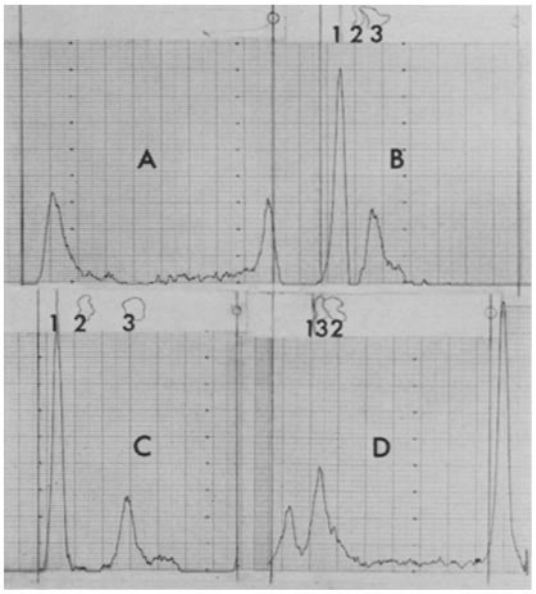

FIG. 1: Chromatograms of the 10 volume EtOH-soluble, Na salicylate fraction from a 24 hr sample of urine obtained after injection with BSA-^{35}S. Solvents used were *A*, EtOH (70), butanol (30); *B*, isopropanol (60), water (40); *C*, isopropanol (75), water (25); and *D*, EtOH (50), water (50). Peaks of radioactivity rise vertically with the grid lines of the graphed records obtained with a strip scanner used to detect radioactivity. The strips from the chromatograms are mounted to coincide with the radioactivity peaks as the scanning actually occurred. The point of sample application (circle) and resolution of components (spots outlined as obtained from scanning with a short wave UV 260 mμ absorption lamp) are indicated at the top of each respective graph of radioactivity. The component designated *1* was characterized as a fine line of yellow fluorescence; component *2* as a spot of blue fluorescence; and component *3* as a strongly-absorbing spot. It is to be noted that no resolution of components was observed with solvent *A*, and in the other three solvents, each of which resolved three components, the optimal resolution was with solvent system *C*. The high radioactivity peak to the extreme right of the assay of solvent *D* is a location marker.

of finding peaks of radioactivity and color changes that indicated probable differences in composition due to a varying number of diazonium groups per antigen fragment. Each pool of fractions was concentrated to a 2 ml volume. The following assays were made to characterize the composition of these pools that represented the final step in isolation: paper chromatography with development in 3:1 isopropanol-water and appropriate scanning to locate radioactivity and UV absorption; phosphorus determination (10), UV absorption at 260 mμ, and the orcinol reaction (9) to estimate the total content of nucleic acid; liquid scintillation counting to estimate total antigen as based on the specific radioactivity of the original injected

antigen; Sephadex thin-layer chromatographs (G-100 superfine) developed in Tris-HCl buffer, pH 7.5, (11) and scanned for radioactivity and UV absorption to determine the distance of migration compared with standards of known molecular weight that were assayed similarly.

When attention was directed to biological studies, larger samples were required for which procedures were modified accordingly.

RESULTS

Rate of Excretion of BSA-^{35}S and KLH-^{35}S Following Intravenous Injection into Normal Rabbits.—Four rabbits were injected with BSA-^{35}S and another group of four rabbits were injected with KLH-^{35}S, the amount of each antigen being 1 ml in a 2% protein concentration. Water was fed 2–3 hr later, after which time the animals were observed at least hourly so that voided urine could be collected and assays begun promptly. Considerable variation occurred in the amount of antigen excreted during the several hours that immediately followed the injection; this was true for values of individual rabbits given the same antigen and also for values of animals given one antigen compared with the other antigen. The latter finding is obvious in comparing the data above the horizontal line in Table I, where accumulative percentages are given for five intervals of

TABLE I

Rate of Antigen Excretion During 24 Hr Periods after Injection

Time	Average Accumulative Percentage	
	BSA-^{35}S	KLH-^{35}S
0–4 hr.	18	8
4–8 "	41	26
8–12 "	56	50
12–16 "	67	69
16–24 "	(71)*	(74)
2 days	88 (17)	87 (13)
3 "	92.0 (4.0)	91.6 (4.6)
4 "	93.8 (1.8)	93.9 (2.3)
5 "	95.0 (1.2)	95.2 (1.3)
6 "	95.4 (0.4)	95.7 (0.5)
7 "	95.5 (0.1)	95.9 (0.2)
	Reinjection	
	BSA	KLH
8 days	96.6 (1.1)	97.4 (1.5)
9 "	97.1 (0.5)	98.1 (0.7)
10 "	97.3 (0.2)	98.5 (0.4)

* The amount (in per cent) excreted daily is given in parentheses.

The latter finding is obvious in comparing the data above the horizontal line in Table I, where accumulative percentages are given for five intervals of

time during the first 24 hr following the injection of antigen. From 12 to 24 hr there is little difference between the two antigens whereas considerable difference existed earlier. At the end of the first 24 hr period more than 70% of the injected antigen was accounted for by the accumulative per cent of radioactivity. At

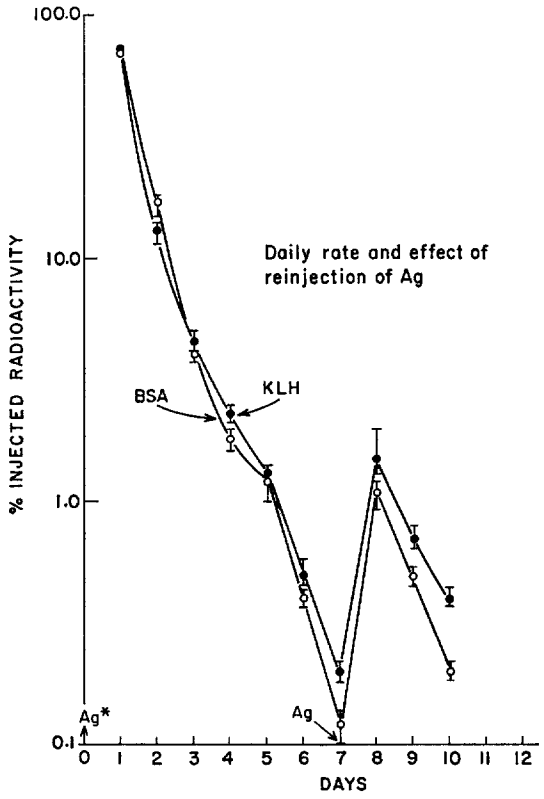

FIG. 2: Percentages of radioactivity excreted daily after primary injection of sulfanilate (^{35}S)-labeled antigen on day 0. On day 7 the *unlabeled* native protein that had been used originally as carrier was reinjected. The rise in excreted radioactivity that subsequently occurred is indicated as percentages for days 8–10.

each succeeding interval of 24 hr, i.e. day 2–8, the total per cent excreted in any one 24 hr period (value in parentheses) is added to the total per cent excreted previously to give the total per cent excreted from the time antigen was injected (value preceding the one in parentheses). If the latter figure is subtracted from 100%, the value obtained is the calculated amount retained at that particular time, e.g. the per cent of BSA-^{35}S retained 2 days following injection = 100 − 88 or 12%.

Rate of Excretion of a Labeled Primary Injection after Reinjection of Unlabeled Native Carrier Protein.—On day 7 from 0 time for the primary injection, 0.5 ml of 1% BSA was injected into two of the animals previously injected with BSA-^{35}S and a similar injection of KLH was made into two animals injected previously with KLH-^{35}S. The other two animals in each group were maintained as controls and given only water for diuresis. After the reinjection of the antigen, the rate of excretion of the retained labeled antigen was increased 7- to 11-fold or roughly one log (Table I). The controls, that were not reinjected, were observed for 20 days and showed a rather constant rate of excretion after day 7 that was about 0.1% daily of the initial injection of either antigen. The log increase that occurred in the rate of excretion, as a result of reinjection

TABLE II

Accumulative Mg RNA (Orcinol Test) Excreted during 24-Hr Periods

Antigen	Normal		Primary		Secondary
BSA	190	→	230	→	270
	206	→	225	→	257
KLH	188	→	237	→	278
	203	→	255	→	290

of specific antigen, is particularly obvious in Fig. 2 where values are plotted that are shown in parentheses in Table I. The points in Fig. 2 are for the total amount of label excreted in each successive day after the primary injection and also from days 8–10 that followed the reinjection of antigen. Reinjection of antigen into other rabbits was also tried at other time intervals, but the most pronounced increase in excretion of retained antigen occurred no later than 10 days after the initial injection.

Rate of Excretion of BSA-^{35}S and KLH-^{35}S Following Intravenous Injection of a Small Dose.—No detailed study was made of the relation of the amount of antigen injected to the amount excreted, but one such study involved injection of a dose that was $\frac{1}{100}$ of that used in the above study. Four rabbits received an injection dose that was equal in both protein and radioactivity to $\frac{1}{100}$ of that described previously (i.e. 0.2 mg instead of a dose of 20 mg) but given in the same volume of 1 ml. Two rabbits received BSA-^{35}S and two rabbits received KLH-^{35}S. At the end of the initial 24 hr period after the injection the amount excreted was $\frac{1}{100}$ of the amount following the larger dose. (Studies on the clearance of antigen from the blood stream, to be reported later, did not show such a direct correlation with the dose injected.)

Excretion of RNA.—In view of our previous studies that resulted in the isolation of antigen-RNA complexes from livers of immunized rabbits (7, 13),

TABLE III

Relative Concentration of ^{35}S and RNA in the Ethanol Fractions of Urine following Primary and Secondary Injection of Antigen

Time	Fraction	BSA				KLH			
		^{35}S		RNA		^{35}S		RNA	
		1st	2nd	1st	2nd	1st	2nd	1st	2nd
hr									
0–4	F-1	37	<1	7	<1	54	<1	<1	<1
	F-2	4	26	12	62	9	13	6	11
	F-3	8	30	6	4	6	41	14	37
	F-4	51	43	75	35	31	46	80	52
4–8	F-1	24	~2	<1	<1	32	<1	<1	<1
	F-2	12	13	4	22	6	9	1	5
	F-3	13	31	8	24	11	21	4	12
	F-4	51	55	88	54	51	70	95	83
8–12	F-1	23	~1	<1	<1	14	<1	<1	<1
	F-2	5	14	2	5	8	21	1	11
	F-3	6	24	8	6	6	38	3	23
	F-4	66	61	90	88	72	41	96	66
12–16	F-1	12	~2	5	<1	8	<1	<1	<1
	F-2	4	9	6	4	3	24	5	12
	F-3	8	38	10	10	8	31	7	18
	F-4	76	52	79	86	81	45	88	70
16–24	F-1	7	2	7	<1	<1	<1	<1	<1
	F-2	4	11	10	7	5	26	5	16
	F-3	5	36	17	19	8	34	4	43
	F-4	84	50	66	74	87	40	91	41
In 1% NaCl*	F-1	0				<1			
	F-2	82				3			
	F-3	9				92			
	F-4	9				4			
In normal urine‡	F-1	60		<1		69		1	
	F-2	25		15		22		16	
	F-3	2		37		2		37	
	F-4	13		48		7		47	

* 2 mg antigen added to 10 ml of 1% NaCl. No RNA added.

‡ 2 " " " " 10 " " urine. " " " .

F-1: precipitate with 2 vol. EtOH; insoluble in water.

F-2: " " 2 " " ; soluble " " .

F-3: " " 10 " " ; " " " .

F-4: supernatant " 10 " " ; " " " .

^{35}S: Per cent radioactivity relative to total cpm in fractions.

RNA: Per cent orcinol-positive material standardized with purified yeast RNA.

it seemed worthwhile to determine whether such an association of antigen and RNA existed in excreted material, particularly after the secondary response. In carrying out such a study it was necessary to apply analytical methods that had been used under other conditions than for detection of RNA or its breakdown products in urine since only minor attention had been given previously to the presence and detection of RNA in urine.

The orcinol reaction was used to determine RNA material in 24 hr pooled samples of urine before and after the primary injection of antigen, also before and after the secondary injection of unlabeled carrier protein. Separate pools were made from samples of four animals assayed previously for excreted radioactivity (Fig. 2 and Table I). As indicated in Table II, an increase in substances that gave an orcinol-positive reaction was detected after both the primary and

TABLE IV

Fractionation of Whole Urine with 10 vol Ethanol

24 hr pool after secondary injection.

Fraction	Net total	Ag	RNA	Ag:RNA
	cpm	*mg*	*mg*	
EtOH—insoluble	130,140	0.157	123	1:783
EtOH—soluble	147,000	0.177	146	1:825

secondary injections of antigen. Since the concentration of orcinol-positive material was essentially the same before both the primary (labeled) and secondary (unlabeled) injection of antigen, this value was considered as normal. The increase in the values obtained in the orcinol reaction was roughly proportional to the exponential rise in ^{35}S excreted during the 4–16 hr period following primary injection of antigen but thereafter, no consistent correlation was detected in ^{35}S and RNA.

Fractionation of Urine with Ethanol.—Samples were fractionated initially according to the procedure that yielded four fractions per sample, (see Materials and Methods) and the samples, representing various time intervals following primary injection, were the same as those studied in the top of Table I. All fractions were analyzed for ^{35}S and RNA (orcinol), for which the relative percentage values are given in Table III.

In general, the antigen material as well as the orcinol-positive material appeared to be present predominantly in the F-4 fraction which had an ethanol concentration of about 90%. There was also a definite trend for material to become more alcohol-soluble with time, e.g., in the 16–24 hr period as compared to the 0–4 hr period. Although it is of doubtful significance, the value for RNA seemed to peak at the 8–12 hr period.

When a similar fractionation was performed using urine that was collected

after the reinjection of the unlabeled carrier protein, (see Table I and Fig. 2) it was found that the radioactivity and RNA could not be so selectively concentrated in one fraction as had been the case with urine collected after the primary injection. Table III indicates this tendency toward a distribution among the fractions that were assayed after the secondary injection.

Another observation regarding the secondary injection indicated a somewhat equal distribution of antigen material and RNA in each of the two fractions obtained by treatment with 10 volumes ethanol (Table IV), the sample being 24-hr pooled urine, obtained after the reinjection of antigen (BSA). The findings in Table III that refer to secondary injection are similar to those in Table IV since an average of about 50% of the radioactivity was in F-4 after reinjec-

TABLE V

Fractionation of Alcohol-Soluble Fraction on Dowex-1 Resin

Eluant	Primary*		Secondary‡	
	% cpm	*% RNA*	*% cpm*	*% RNA*
H_2O	0.24	47	1.08	42
0.01 N HCl	1.38	16	10.03	12
0.02 N HCl	14.72	3	20.05	10
3 M $NaC_7H_5O_3$	63.40	21	52.66	18

* Applied cpm 2×10^6; RNA 12 mg.
‡ " " 45×10^6; " 12 ".

tion of BSA and the percentage of RNA material in F-4 remained at a similar relative percentage for approximately 8 hr following the reinjection of antigen.

Despite the generally recognized difference in the immunogenicity of BSA and KLH, it did not seem to be reflected in any differences in excretion values. Therefore, subsequent studies were limited to BSA-^{35}S.

Fractionation of the Alcohol-Soluble (F-4) Material.—Ethanol was evaporated from the F-4 material; this avoided excessive drying. The residue, dissolved in water, was added to a column of Dowex 1 resin. When the sample had penetrated the resin, -water, 0.01 N HCl, 0.02 N HCl, and 3 M sodium salicylate were added sequentially. Multiple fractions were collected initially to determine the volume for optimal elution with each solution; thereafter, a batch collection was made of each eluting solution. Each of the latter was concentrated and treated further if required, e.g., desalting of the sodium salicylate fraction. When assayed for radioactivity and RNA, the percentage of the amount applied was distributed as shown in Table V with at least an 80% recovery. The figures in Table V are a summary for specimens pooled in a 24 hr period after initial injection of BSA-^{35}S and also after a secondary injection of BSA.

Chromatography.—The bulk of the RNA material was eluted from the

Dowex resin free of radioactivity. The radioactivity was eluted with sodium salicylate and this eluant also contained a high percentage of RNA material. It was this latter fraction, containing more than 60% of applied radioactivity and approximately 20% of RNA material, that was further fractionated on a cellulose column that was eluted with 3:1 isopropanol-water mixture. The final isolated materials (Table VI) were made available in preparative amounts for

TABLE VI

Partition Chromatography of Na-Salicylate Fraction on Cellulose with Isopropanol–Water (3:1)

	Primary			Secondary		
Pool	Ag	RNA	Ratio	Ag	RNA	Ratio
	μg	μg		μg	μg	
1	17.3	5472	1:316	0.033	5160	
2	40.0	1000	1:25	0.024	2220	
3	1722.8	6360	1:4	10.440	10020	1:960
4	1765.8	9405	1:5	13.356	3402	1:255
5	2246.0	2650	1:1	14.883	3444	1:231
Total.....	5791.9	24887		38.736	24246	

TABLE VII

Calculated Recovery of Antigen

	Primary	Secondary
	% μg	% μg
Excreted in 24 hr	70 = 14000	1.1 = 220
EtOH—soluble	75 = 10500	55 = 120
$NaC_7H_5O_3$	65 = 6825	55 = 66
Cellulose	90 = 6143	90 = 59
Actual total recovery, μg	5792	39

further study. One such study was thin-layer chromatography used in an attempt to gain some idea of molecular size of the fractions. From such studies it may be concluded that the isolated material is still quite heterogeneous with a range of molecular weights under 5000. Because the characterization is not completed, the precise composition of the antigen-RNA components is uncertain. Meanwhile the measurement of radioactivity is used to estimate the amount of antigen material assuming that it has the characteristics of the antigen that had been used for injection and, similarly, purified yeast RNA is used as the reference standard for the orcinol determinations that were the basis for estimation of nucleic acid material. The resulting calculations led

to the ratios given in Table VI. When an estimate is made of the recovery of radioactivity, i.e. the initial injection, the figures in Table VII average approximately 30% for the primary injection and about 0.2% resulting from secondary injection of the unlabeled protein that resulted in washing out of the primary injection.

DISCUSSION

The present investigation points out once again than an intravenously injected soluble antigen is to a large extent excreted in the urine regardless of whether it is an antigen of fairly low molecular weight such as BSA or a high molecular weight antigen such as hemocyanin. Earlier studies by Ingraham (14) using mice and bovine-γ-globulin labeled with ^{35}S sulfanilate showed that about 75% was excreted in 24 hr. Our previous studies (6) using rabbits and similarly labeled hemocyanin gave essentially the same results. Other investigators using ^{131}I-labeled proteins noted the excretion of large amounts of ^{131}I but the significance has been questioned since much of the ^{131}I was in an inorganic form. [See the review by Campbell and Garvey (3) for more extensive discussion.] The surprising aspect demonstrated by the present study was the rapidity of excretion following a primary injection which was at least 50% in 12 hr. The question arises as to whether such rapid degradation takes place in the blood or in extravascular compartments. Since the loss of ^{35}S-labeled antigen from the circulation closely parallels the appearance in the urine, one can only speculate that the reaction takes place in the circulation. Also of significance was the finding that when the dose level of the primary injection was reduced to 1:100, the rate of excretion was essentially the same. This would suggest that an equilibrium is established between a proteolytic system and the foreign protein and is regulated by the concentration of the latter. However, studies showed that differences in the rate of loss from the circulation were not proportional to the amount injected. (These studies will be completed and reported later.) Until further studies are made one can only speculate as to whether degradation of antigen is the result of proteolytic enzymes in the serum or of intracellular digestion by circulating granulocytes (Speirs and Speirs, 15) and macrophages lining sinusoids, resulting in rapid breakdown and release of antigen fragments plus RNA. The fact that even after a primary injection, some of the antigen material is associated with RNA would suggest the possibility of at least some intracellular degradation.

In the present investigation no studies were made of the excretion rate of a secondary injection. The excretion and nature of the labeled antigen which remained in the tissues after the primary injection were of chief concern, so that in these experiments, the secondary injection consisted of the *unlabeled native protein carrier*. However, when in previous studies (6) attention was directed to the rate of excretion of the secondary injection, it was clearly demonstrated

that antigen disappears faster from the circulation and is excreted at a faster rate when it is injected into a sensitized animal than when it is injected into a normal animal.

There is no doubt that antigen material excreted in the urine is quite heterogeneous with respect to both physical and biological properties. This of course would be expected since the breakdown of the protein molecule would result in fragments representing different portions of the protein molecule as has been shown by Lepresle and his collaborators (16) and the demonstration of specific determinants on internal molecular structures by Ishizaka et al. (17). Heterogeneity of excreted antigen material was not only evident at any given time, but also with respect to time. This was particularly evident following the primary injection. For example, on the basis of alcohol solubility (Table III), the amount of ^{35}S material that was soluble in 10 volumes of ethanol at 4 hr was about 50% for BSA and 30% for KLH. After 16 hr about 80% of either antigen was soluble in the high concentration of alcohol. There is likewise some indication of heterogeneity of RNA material at the alcohol stage of fractionation, but this became more obvious with the fractionation on Dowex resin (Table V). It was by means of the latter fractionation that most of the RNA material, particularly all that was present as free bases and nucleosides (18) was separated (water and acid fractions) from nucleotides associated with antigen material (sodium salicylate fraction). The latter, when further fractionated on cellulose, resulted in a separation of materials varying in amounts of organic phosphorus and radioactivity. Bands of varying color on the column were assumed to indicate a separation of materials with varying degrees of azo linkage. More extensive characterization is required to determine what degree of heterogeneity in these final fractions can be attributed to antigen material and to nucloeotide material. Studies, to determine whether the nucleotides found after injection of antigen are characteristically different from those normally present, are under current investigation.

As might be expected from our previous studies (7), at least some of the antigen material retained in the liver following a primary injection was released in a condition similar to that obtained upon extraction from the liver. Previous studies (13) indicated that with increasing time the polynucleotides found associated with antigen fragments are of increased size. The present finding is an increased amount of orcinol-positive material in association with antigen material after secondary injection as compared with primary injection (Table VI). These results point to the need for precise characterization of the RNA-antigen material that would distinguish the extent of quantitative and qualitative changes influenced by injection of antigen. Also related to this discussion is a previous unpublished finding originating from studies that were based on a fractionation of whole urine into dialyzable and non-dialyzable material. A relative increase in the percentage of non-dialyzable radioactivity compared

with dialyzable radioactivity resulted with increasing time after a single injection of a radioactive antigen; i.e., at 1 day the percentage of non-dialyzable radioactivity was 1–2% of the total but with the gradual increase that occurred over a period of 10 days, the non-dialyzable radioactivity increased to more than 12% where the level either plateaued or showed random increases for the remaining period of the 20 days during which samples were collected and assayed.

Aside from providing information regarding the excretion of antigen material the investigation indicates the importance of giving more attention to urinary excretion of immunological by-products. The biological significance of excreted antigen material and of such material associated with RNA requires intensive investigation, but preliminary studies carried out in vitro indicate very significant immunogenic properties for some of these isolated materials.

The question arises in this, as well as in our previous investigations, as to the physical relationship of the antigen to the RNA which is isolated as unique components of liver tissue as well as urine. All that one can state at present is that such components are stable under mild conditions of salt precipitation, chromatography, electrophoresis, and analytical centrifugation. When dissociated (7), neither the RNA nor the antigen fragment has shown any stimulatory action in our work with respect to antibody formation. Numerous reports (19–22) have been made of the immunogenicity of nucleoprotein material isolated from various tissues of immunized animals but with the exception of Askonas and Rhodes (23), who related the immunogenicity to the antigen content of their RNA preparations, the presence of antigen together with the nucleic acid has received little attention. It seems logical at this point that there must be some binding, otherwise the method of phenolic extraction that has been commonly used to isolate nucleic acid would be expected to separate the two components; however, with the sensitivity provided by radioactivity, antigen material was detected in the RNA (13, 23).

SUMMARY

Two soluble antigens, BSA and KLH labeled with sulfanilate-^{35}S, when injected intravenously into normal animals, were excreted in the urine to over 70% in 24 hr. Over the next 6 days, 25% more was excreted after which time only a trace could be detected. Much of the antigen remaining from the primary injection appeared in the urine following a secondary injection of the *unlabeled* protein carrier at 7 days after primary injection.

The antigen material found in the urine was quite heterogeneous with respect to physical properties and much of it was associated with RNA material as shown by chromatographic analyses. The main difference between the labeled material released following the primary and secondary injection was the higher degree of association of antigen material with nucleotide material after second-

ary injection as compared with primary injection. Further study is needed to distinguish qualitative from quantitative changes of the components, antigen and nucleic acid, and also the nature of their association. Possible similarities were found for the RNA-antigen material released from tissue after secondary injection of *unlabeled* antigen, and the material that was isolated previously from liver.

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