THE RETENTION OF S^{35}-LABELLED BOVINE SERUM ALBUMIN IN NORMAL AND IMMUNIZED RABBIT LIVER TISSUE*

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In previous publications (1) and discussions (2) we have described the retention of antigen in normal and immunized rabbits. In all of these studies, S^{35} (*p*-azophenylsulfonate) labelled hemocyanin (from the Keyhole limpet) was used as the antigen. This material proved to be extremely useful for showing that significant amounts of a foreign soluble antigen, in an altered form, persisted in rabbit liver tissue for many months after intravenous injection. However, it became obvious that the original tagged hemocyanin was too heterogenous to allow one to determine the nature and extent of changes that occurred in the antigen after injection which is an important aspect of this problem. With this in mind the present investigation was made with crystallized bovine serum albumin which is relatively homogenous with respect to electrophoretic and sedimentation properties and had the additional advantage of having been extensively used in immunochemical studies. The work which is reported here is a continuation of the earlier studies, and methods are essentially the same as those described in our previous publications. There are the exceptions that physical characterization of antigen before and after injection was carried out in more quantitative detail, and a new method was devised for the isolation of antigen material from liver tissue. The results confirmed most of our previous findings, although there were some significant quantitative differences between the behavior of sulfur-labelled bovine serum albumin (S^{35}-BSA) and labelled hemocyanin (S^{35}-KLH).[1]

EXPERIMENTAL

Immunological Procedures.—Rabbits weighing 2 to 3 kilograms were given either a single intravenous injection of 50 mg. of S^{35}-BSA or 9 intravenous injections, 10 mg. each, on alternate days.

The amount of circulating antibody was determined in the usual manner by adding a

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[1] BSA indicates sulfur-labelled bovine serum albumin; KLH indicates sulfur-labelled hemocyanin; (this latter protein was abbreviated as SAH in previous publications).

constant amount of serum to varying amounts of non-radioactive S-BSA. The test antigen was prepared in the same manner as the S^{35}-BSA immunizing antigen so that it contained the same average number of azophenylsulfonate groups and exhibited the same physical properties. The precipitates were washed thoroughly after standing 48 hours at 4°C. and then analyzed by the Nessler technique as described by Lanni and Campbell (3). Antibody protein was calculated on the basis of point of maximum precipitation.

The antigenicity of retained or excreted antigen was determined by Schultz-Dale reactions, because only small amounts of antigen material were recovered. Preliminary studies with S-BSA showed that the original material was capable of inducing a high degree of sensitivity. These preliminary tests were with 300 to 400 gm. guinea pigs which were given a single dose of 20 to 40 mg. of S-BSA into the peritoneal cavity. 3 weeks later the animals were sacrificed and tests were made with strips of small intestine as described by Nicoll and Campbell (4). The Tyrode's solution was prepared according to Feigen and Campbell (5) and use was made of a tissue bath of the type described by Campbell and McCasland (6). Tests with antigen material obtained from liver were performed in the same manner except that the sensitizing dose had to be reduced to the equivalent of about 0.1 mg. of S^{35}-BSA owing to the small amount of material which was available.

Preparation of S^{35}-BSA.—Sulfanilic acid was synthesized by the "baking process" described in Groggins (7) using a mixture of S^{35}-labelled sulfuric acid and unlabelled sulfuric acid to sulfonate the aniline. The diazonium salt was prepared by a method described in Fierz-David and Blangey (8) and then coupled to crystallized bovine serum albumin (BSA).[2] During addition of diazonium salt to the protein, a pH of 8.0 to 8.5 was maintained by the careful addition of 0.5 N NaOH. The coupled protein was purified by repeated precipitation with ammonium sulfate at 50 per cent saturation at room temperature. The final precipitate was dissolved in pyrogen-free saline and dialyzed against 1.0 per cent NaCl containing 0.01 per cent merthiolate.

Properties of S-BSA.—The specific activity of S^{35}-BSA when freshly prepared, was of the order of 5 to 6 mc./gm. Calculations based on sulfur analysis of BSA and S-BSA,[3] gave a value of approximately 35 for the number of azophenylsulfonate groups per molecule of S-BSA.

The free boundary electrophoretic patterns of BSA and S-BSA are shown in Fig. 1. The difference in mobility of BSA (μ = 6.9) and S-BSA (μ = 9.8) is due at least in part, to the relatively large number of azophenylsulfonate groups which is highly ionized at pH 8.6. The S-BSA preparations appeared to have approximately the same degree of electrophoretic homogeneity as the original BSA.

Comparative sedimentation patterns of BSA and S-BSA obtained with a Spinco model E centrifuge are shown in Fig. 2. Sedimentation constants, based on determinations of several concentrations of antigen (0.3 to 1.5 per cent) and extrapolation to zero concentration with viscosity and temperature corrections were Sw20 = 4.2 for BSA and 3.9 for S-BSA. Molecular weight determinations, based on the Archibald method (9) gave 70,000 for BSA and 79,000 for S-BSA.[4] By essentially the same methods Klainer and Kegeles (10) have reported 70,300 ± 600, as an average value for three different concentrations of Armour BSA. A molecular weight of 79,000 would be 2,000 too great on the basis of the addition of 35 azophenylsulfonate groups to BSA of 70,000 molecular weight; however, this slight discrepancy is unimportant for the present investigation.

The serological properties of S-BSA, as determined by quantitative precipitation reactions against rabbit anti S-BSA showed the same degree of homogeneity as the BSA-anti BSA

[2] Supplied by Armour and Co.

[3] Sulfur analysis by Dr. A. Elek.

[4] We are greatly indebted to Dr. R. Srinivasan for the data and determinations for these values.

system. Precipitin reactions showed some cross-reaction between BSA and S-BSA. The anti S-BSA serums always contained a much greater amount of antibody for the haptenic group than for the BSA and the maximal concentration of antibody against the haptenic group developed much earlier. Under the experimental conditions used for the Schultz-Dale tests, no cross-reactions were obtained between BSA and the S-BSA (see Fig. 3).

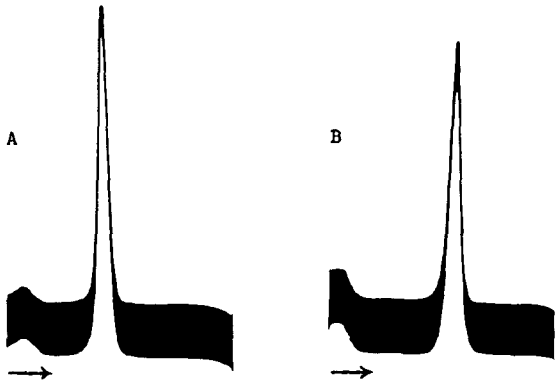

FIG. 1. Electrophoresis diagrams of (A) BSA and (B) S-BSA at 1 per cent protein concentration in barbital buffer at pH 8.6, $\mu = 0.1$. Migration was for 1 hour in the descending limb with the direction of migration indicated by an arrow.

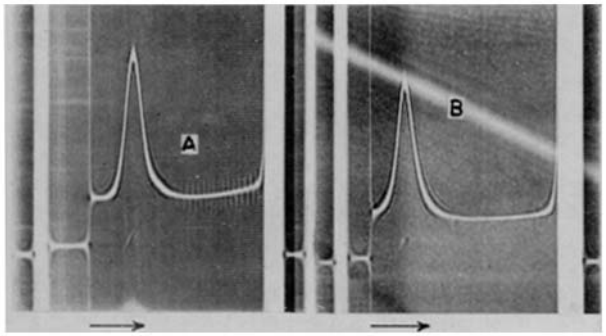

FIG. 2. Sedimentation patterns after 18 minutes for (A) BSA and 16 minutes for (B) S-BSA at 59,780 R.P.M. 1 per cent protein solutions were used with the diluent of 1 per cent NaCl being used to form a boundary in a synthetic boundary cell.

Assay of Radioactivity.—The method for determining radioactivity in liver tissue was described fully in one of our previous publications (1). A similar assay was made of urine for excretion data. The rabbits were kept in metabolism cages and the urine was collected in beakers containing toluene. An aliquot was clarified by centrifugation at 20,000 R.P.M. (average 25,000 *g*) for 1 hour, then was sampled in triplicate. The samples were evaporated to dryness at room temperature, then the air-dried residues were reduced to a constant weight with a heat lamp before counting. The antigen concentration was determined by comparing the radioactivity to a standard self-absorption curve obtained with normal urine (*i.e.* from a non-injected rabbit) to which was added known concentrations of the antigen.

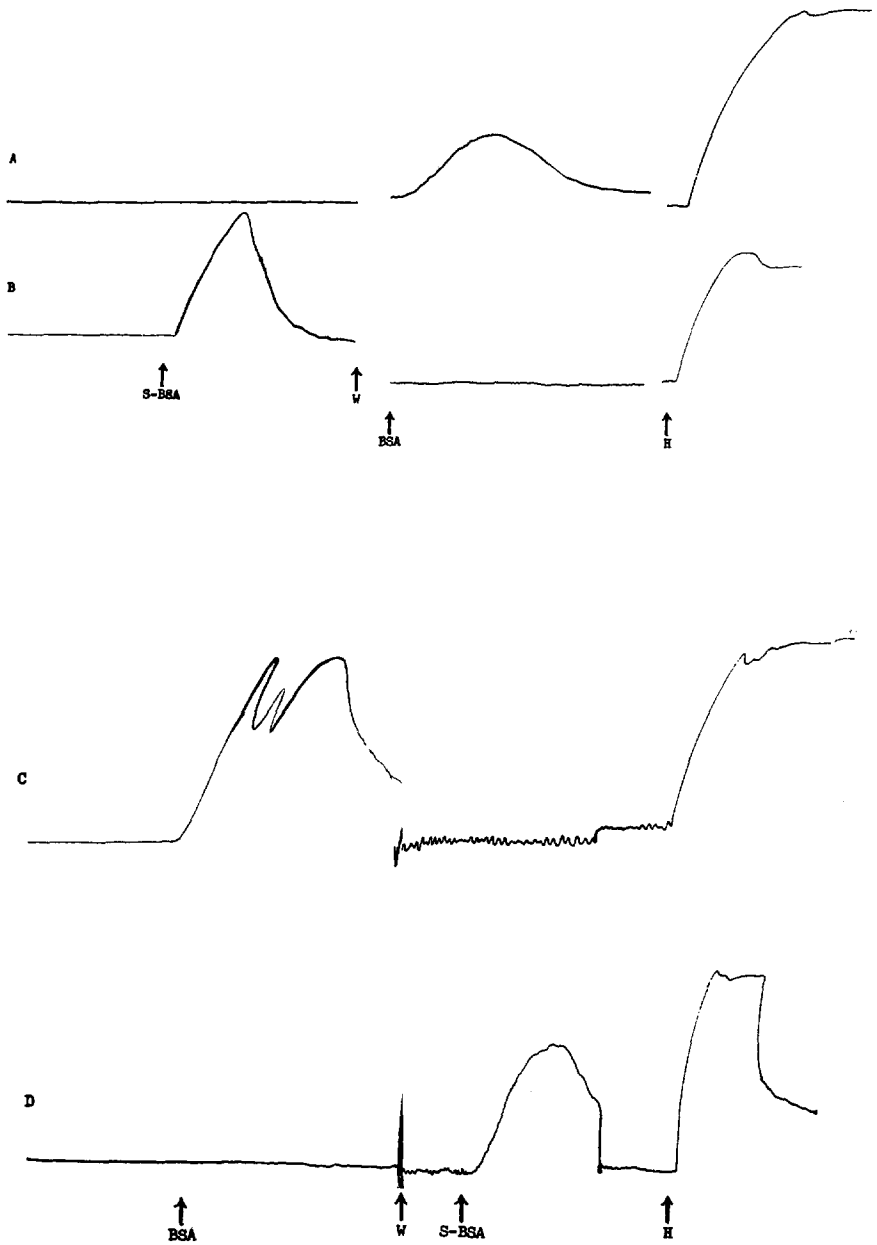

FIG. 3. Kymograph records of the specific reaction of BSA and S-BSA antigens following active sensitization of guinea pigs with 20 to 30 mg. protein. Muscle strips were 3 to 6 cm. from the cecum and the concentration of test antigen was 5 mg./150 ml. tissue bath. *W*, washing; *H*, 5 μg. histamine. (A), sensitized with 20 mg. BSA; (B), sensitized with 20 mg. S-BSA; (C), sensitized with 20 mg. BSA; (D), sensitized with 20 mg. S-BSA.

Isolation of S^{35}-BSA Antigen Material from Liver Tissue.—Livers were removed, immediately perfused with 0.9 per cent saline, and frozen at −30°C. They were then wrapped in heavy canvas and crushed in a press at 20,000 pounds pressure. The volume of tissue brei was measured and then mixed with 3 volumes of 0.25 M sucrose buffered at pH 8.4 with borate. This material was fractionated by centrifugation in a refrigerated Spinco centrifuge model L. After centrifugation at 30,000 R.P.M. (average 78,000 *g*) for 60 minutes, the supernatant contained 80 to 90 per cent of the total radioactivity of the liver. This solution was then dialyzed against 0.0015 μ borate buffer until free of sucrose. (No radioactive material was lost during this dialysis which was evidence that the haptenic group was still coupled to a large fragment of the original material.) After dialysis, the material was lyophilized to dryness and stored at 4°C. Care was taken to complete this entire operation in about 36 hours.

For further purification of the antigen, the lyophilized material was dissolved in water and centrifuged under refrigeration at 30,000 R.P.M. (average 78,000 *g*) for 30 minutes. A low density material which contained no radioactivity floated to the top and was removed by careful filtration. The cleared solution[5] was then passed through an ion-exchange column consisting of the chloride form of Dowex 2 resin. The resin was then washed thoroughly (with 0.9 per cent NaCl buffered at pH 8.4 with borate) until the wash solution failed to show a biuret reaction. The radioactivity of the liver extract was 95 per cent adsorbed to the resin. Elution of about 90 per cent of the radioactive material was then achieved by use of a 50 per cent aqueous solution of sodium salicylate. The solution of eluted material was dialyzed against 0.0015 μ borate buffer until free of salicylate, and concentrated by pervaporation at about 20°C.

The total amount of protein recovered from the resin was about 1.0 per cent of the amount present in the soluble liver extract. This material, contained about 70 per cent of the total amount of radioactivity in the liver tissue. It also formed a specific precipitate with anti S-BSA serum which contained all the radioactivity.

RESULTS

Retention of S^{35}-BSA in Liver Tissue.—The retention at various time intervals after single or multiple injections of antigen is given in Fig 4; each point in the graph is the average value for 3 to 5 rabbits. The amount of S^{35}-BSA retained in liver tissue 1 day after a single injection of 50 mg. of antigen was about 7.0 per cent of the total amount injected (as based on radioactivity). The amount retained then slowly decreased and at the termination of the present study (140 days) the amount of S^{35} remaining was about 0.023 per cent of the amount injected. The amount of S^{35} which was retained after a series of injections (9 injections of 10 mg. each) was less in terms of per cent injected as well as absolute amount. Of the 90 mg. injected, about 0.01 per cent was still present at 130 days after the last injection.

Fig. 4 also presents, for comparison, the data for S^{35}-labelled hemocyanin (S^{35}-KLH). Retention data for S^{35}-KLH have been given previously (1) but have been extended by additional data. Although the retention of S^{35}-KLH was somewhat higher than for the S^{35}-BSA, there was a similar difference between single and multiple injections. Fig. 5 is a log-log plot of the same

[5] This is essentially the same as the F-4 fraction described in reference 1.

data used in Fig. 4 in order to present all the data on a complete single plot. The significance of the slopes is not known at present.

The influence of multiple injections and the presence of antibody on the retention of antigen in liver tissue is being studied further at the present time. Preliminary data show that when animals are given an initial injection of S^{35}–labelled antigen and then a few subsequent injections of non-radioactive

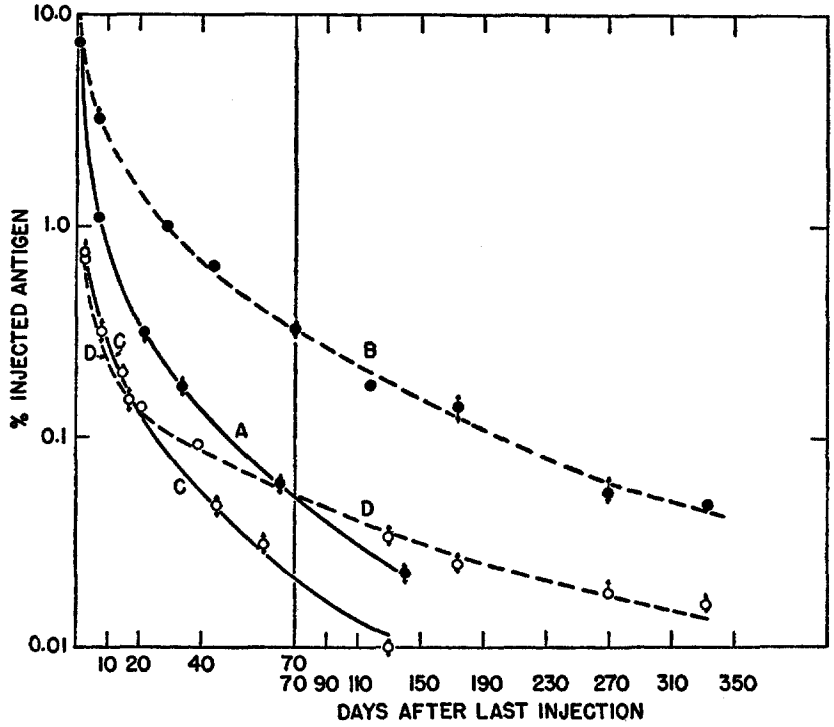

FIG. 4. Semilog plot of the retention in perfused liver tissue of a single injection of 50 mg. S^{35}-BSA (A); a single injection of 50 mg. S^{35}-KLH (B); multiple injections *i.e.* 9 of 10 mg. each of S^{35}-BSA (C); and multiple injections *i.e.* 9 of 10 each of S^{35}-KLH (D). The center of the circles indicates the mean of the distribution which is indicated by the arrows.

antigen, the radioactivity of the liver decreases much faster than when no subsequent injections are given. The decrease in radioactivity of the liver is accompanied by a corresponding increase in radioactivity of the urine.

Excretion of S^{35}-BSA in Urine.—Rabbits, given a single intravenous injection of 10 mg. S^{35}-BSA, were placed in metabolism cages and the urine was collected at 24 hour intervals for 10 days. Fig. 6 shows the excretion for 6 rabbits which received the S^{35}-BSA compared with 3 rabbits which received a similar dose of S^{35}-KLH antigen. The amount of S^{35} which was excreted at 48 hours plus the amount which remained in the liver, accounted for about 95

per cent of the amount injected. When plotted as cumulative 48 hour rather than 24 hour values, the variation in individuals of each group was small. Excreted radioactivity was to a very large extent (about 95 per cent) lost during dialysis against water. The material in urine which did not pass through the

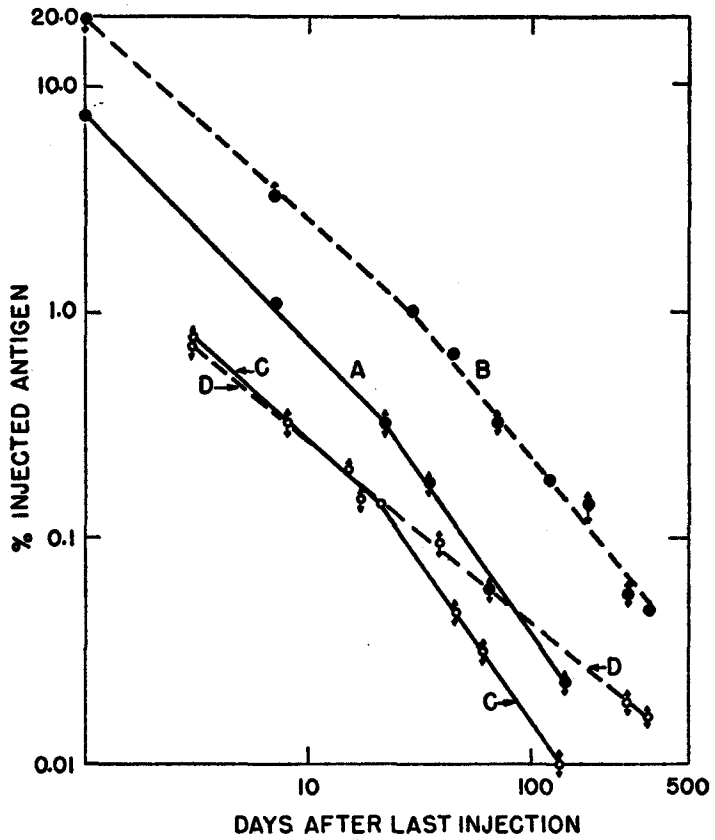

FIG. 5. Retention of single and multiple injections of S^{35}-BSA and S^{35}-KLH, using the same data as in Fig. 4 but as a log-log plot. The center of the circles indicates the mean of the distribution which is indicated by the arrows.

cellophane dialysis membrane reacted with antiserum to give a specific precipitate.

Characterization of Retained Antigen.—Particular attention was given to the partially purified material which was eluted from the Dowex 2 columns with respect to immunological, electrophoretic, and molecular size properties.

The antigenic properties were determined by injection into guinea pigs and subsequent tests for hypersensitivity by means of Schultz-Dale reactions. S^{35}-BSA preparations used for such tests were obtained from liver tissue about

21 days after injection of antigen. The exact amount of S^{35} antigen, and possible fragments, which was present in the solution used for immunization was difficult to determine, but on the basis of radioactivity of the original antigen, it was about 0.1 mg. 3 weeks after a single injection into the peritoneal cavity, the animals were sacrificed and tests were made on intestinal strips as described

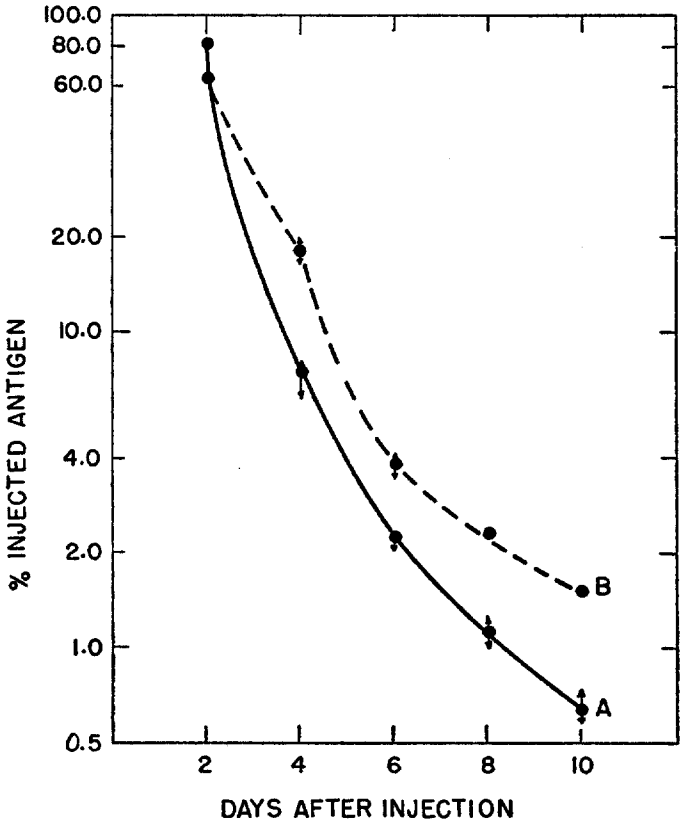

FIG. 6. A semilog plot of urine-excreted radioactivity when either 10 mg. of S^{35}-BSA or S^{35}—KLH was given intravenously. The center of the circles indicates the mean of the distribution which is indicated by the arrows.

above. Strips from animals sensitized with S^{35}-BSA liver extract eluate, failed to react when tested with BSA, but gave specific reactions when tested with S-BSA. A similar quantity, 0.1 mg., of the original S-BSA antigen, failed to sensitize guinea pigs. Owing to the limited amount of material available, it was impossible to carry out a complete serological characterization of the retained antigen. Qualitatively, however, S^{35}-BSA eluates gave positive ring tests with rabbit anti S-BSA, but not with anti BSA. No attempt was made to immunize rabbits with the S^{35}-BSA eluates.

Further fractionation of the eluate at half saturation with ammonium sulfate resulted in a concentration of at least 95 per cent of nucleic acid-like material[6] in the salt-soluble fraction together with 90 to 95 per cent of the

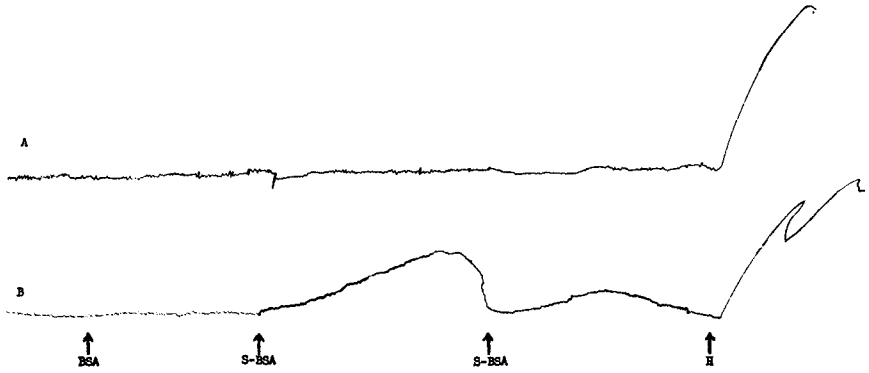

FIG. 7. A kymograph record of the Schultz-Dale responses in guinea pigs obtained with the salt fractions of the antigen material. Sensitization was with the equivalent of 0.1 mg. of antigen based on radioactivity. Test antigen added was 10 mg./150 ml. bath and histamine added was 5μg. (A), salt-insoluble fraction, indicating no sensitization; (B), salt-soluble fraction, demonstrating sensitization.

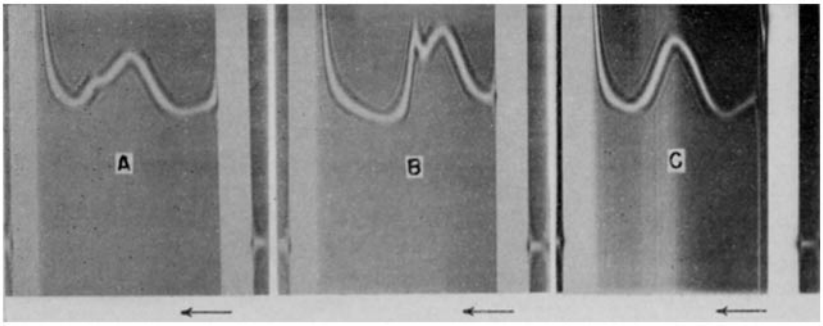

FIG. 8. Sedimentation boundaries of the salt-soluble fraction obtained from tissue removed 21 days after a single injection of 10 mg. S^{35}-BSA (A); salt-soluble fraction obtained from tissue removed 21 days after 9 injections totaling 90 mg. of S^{35}-BSA (B); and salt-insoluble fraction obtained from tissue removed 21 days after single injection of 10 mg. S^{35}-BSA (C).

Concentration of 0.5 per cent in 1 per cent NaCl + 0.0015 μ borate, after sedimentation at 59,780 for 56 minutes.

radioactivity originally present in the eluate. The Schultz-Dale reaction was repeated with the salt fractions of the eluate and remained positive only with the salt-soluble fraction (Fig. 7).

Sedimentation studies with a Spinco model E analytical centrifuge showed two components which were not resolved in the salt-soluble fraction and one

[6] An absorption maximum occurred at 2575 A.

component in the salt-insoluble (Fig. 8). For salt-soluble material isolated 3 weeks after a single injection the sedimentation constant of the shoulder component was higher (Sw20 = 4.3) than for similar material at 3 weeks after the last of 9 radioactive injections (Sw20 = 3.6). The main component of the salt-soluble and the single component in the salt-insoluble appeared to be similar; the Sw20 ranging from 2.3 to 2.5. Only one concentration (0.5 per cent) of these fractions was analyzed. The differences in radioactivity and nucleic acid between the salt-soluble and salt-insoluble fractions mentioned above appear to be attributed to the component with a larger S constant which was absent from the salt-insoluble fraction. A more complete characterization of the material is under way and will be described in a subsequent publication.

DISCUSSION

One of the first questions that arises in experiments dealing with tracers, such as described here, is whether the label remains combined with the protein portion after injection. The foregoing results, as well as those presented previously for S^{35}-KLH antigen (1), clearly indicate that, although the antigen may be altered and perhaps partially broken down in liver tissue, the S^{35}-label is definitely associated with material that is immunologically related to the original S^{35}-labelled antigen as shown by sensitization of guinea pigs and precipitin tests.

The foregoing investigations clearly indicated that bovine serum albumin was retained in some form in liver cells for many months after intravenous injection and confirmed our previous results on the retention of hemocyanin antigen. The rate of disappearance of antigen material from liver tissue became so slow after a few months that traces might be expected to persist for years. The amount of material which was retained after several months was quite small in terms of weight, but when expressed in terms of molecules, the number could approximate that of the total number of liver cells. For example, in the present study, the liver contained 0.01 per cent of injected antigen or 9 μg. (as based on radioactivity) at 130 days after a series of injections (Fig. 4). Calculations based on the radioactivity of the injected material which had a molecular weight of 78,000 would indicate that 8×10^{13} molecules still remained at this time. By extrapolation of the curves in Fig. 5 it is possible to obtain some idea of the amount which might be retained for longer periods of time. For example, after 3 years, the retention of S^{35}-BSA from a single injection would be 0.0015 per cent, equivalent to 0.75 μg. or 5.79×10^{12} molecules; the retention of S^{35}-BSA from multiple injections would be 0.0006 per cent, equivalent to 0.54 μg. or 4.16×10^{12} molecules; the retention of S^{35}-KLH from a single injection would be 0.0135 per cent, equivalent to 6.75 μg. or 5.22×10^{13} molecules and the retention of S^{35}-KLH from multiple injections would be 0.0064 per cent, equivalent to 5.76 μg. or 4.44×10^{13} molecules. Concerning the

kinetics of retention, one finds from the data that the reaction is not first order, especially in the period up to about 70 days after injection. Thereafter the rate of loss assumes unimolecular characteristics (Fig. 4), but the data do not permit a precise conclusion at present. When more is understood about antigen-antibody relationships in tissue it may be possible to derive some quantitative relationship between antigen retained and antibody produced.

The antigenic significance of the retained material was demonstrated by its ability to specifically sensitize guinea pigs to S-BSA. In fact, less of the retained antigen than of the original S-BSA antigen was required for sensitization. This suggests that the material retained by the liver is converted into a more active form and that the nature and localization of antigen after injection is an important factor in antibody production. The question as to whether retained antigen material in liver cells plays any role in antibody formation must await further investigation, but the fact that prolonged retention does occur must be seriously considered as a factor in antibody-forming mechanisms.

The loss of S^{35}-BSA from liver tissue was more rapid than the loss of S^{35}-KLH and this difference between the two antigens was reflected by a more rapid excretion of S^{35}-BSA. The abrupt change in the excretion curve for S^{35}-KLH at 4 days is of significance, since it may be indicative of the heterogeneity of the S^{35}-KLH antigen. Quantitative precipitin tests with the original antigen and antiserum which was obtained at about 7 days, always gave two points of maximum precipitation. Antiserum obtained about 20 days after injection gave only a single maximum. On the other hand, S-BSA gave a single precipitation maximum during all stages of antibody production and a continuous smooth excretion curve.

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SUMMARY

The S^{35}-label of S^{35}-BSA was detected in the liver tissue of rabbits to the extent of 0.02 per cent (10 μg or $\backsimeq 10^{14}$ molecules) of the injected material at 140 days after injection.

The rate of loss of antigen at the termination of the experiment was of such an order that significant amounts would be expected to persist for at least several years.

Data are reported which extend the retention data previously reported on S^{35}-labelled hemocyanin. They indicate that amounts of the order of 0.05 per cent (25 μg.) of antigen material persist at 330 days after injection.

All of the radioactivity of material retained in the liver tissue 6 weeks after injection was immunologically related to the original S^{35}-BSA antigen.

Preliminary studies are reported which indicate that the retained antigen is bound to ribonucleic acid.

A new method is described for the isolation of *p*-azophenylsulfonate bovine serum albumin from tissue extracts by means of a Dowex 2 adsorbent.

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