THE DISTRIBUTION AND STORAGE OF BLUE ANTIGENIC AZOPROTEINS IN THE TISSUES OF MICE

By HEINZ KRUSE and PHILIP D. McMASTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Plates 32 to 34

(Received for publication, May 13, 1949)

Several years ago Landsteiner and his colleagues (1–5) showed that azoproteins are antigenic, and soon after Heidelberger, with Kendall (6–8) and with Soo Hoo (9) produced a red azoprotein. Following these findings Sabin (10) and also Smetana and Johnson (11, 12) employed the product as a traceable protein for immunological and metabolic studies.

It became desirable in this laboratory to use a colored dye-protein for investigations of the physiology of the minute blood and lymphatic vessels. The linked protein of Heidelberger and Kendall did not possess sufficient tinctorial value for these purposes. Consequently, to obtain a more readily traceable protein it seemed wise to prepare a blue azoprotein from a brilliantly blue dye, in such a manner that there would be no free dye associated with it, or so little as not to produce, after injection into the body, any visible blue which could be mistaken for the azoprotein. Several blue dye–azoproteins were eventually obtained which, as a group, will be designated here by the initials AP.

The present paper describes the preparation of two of the AP and various tests made upon one of them to determine whether such a compound can be freed from unlinked dye. The fate of the dye-tagged azoproteins, following their introduction into the bodies of mice, will also be considered in relation to the general problem of the employment of tagged antigens.

Methods

To obtain the first of the AP, T-1824, a blue azo dye, was prepared in salt-free form by the method of Hartwell and Fieser (13). It was next diazotized and then coupled to the proteins of whole horse or rabbit serum, or to rabbit serum albumins, or to egg albumin. Diazotization which yielded a mono-azolinkage was considered preferable to tetrazotization and a resulting bis-azo linkage, since only half as many active groups of the protein are blocked by the former procedure and the immunological and physiological properties of the original protein should be altered as little as possible.

Preparation of Crude Azoprotein

Diazotization.—6 cc. of N/1 HCl was added to 1 millimol (960 mg.) of the dye, T-1824, dissolved in 30 cc. of water. After chilling the mixture to 10°C. a similarly cooled reagent, 1.1 cc. of N/1 sodium nitrite diluted to 4 cc. with water, was added, with stirring, and kept for 20 minutes while the temperature was maintained at approximately the same level. An

additional 0.15 cc. of the sodium nitrite in 1 cc. of chilled water was put in and the mixture allowed to stand for 10 minutes with only occasional stirring.

The excess of mineral acid was added to suppress intermolecular coupling of the dye. Under these circumstances it was not necessary to test for the acidity of the mixture since Congo red paper was always rendered blue. The sodium nitrite, too, was employed in slight excess, about 25 per cent more than the calculated amount necessary to yield mono-azo linkage. Although the excess undoubtedly caused tetrazotization of some of the dye, it helped to bring the reaction to completion more speedily and made up for the inevitable loss, by spontaneous oxidation, of some of the nitrous acid.

Coupling.—As already mentioned, the diazotized dye was sometimes coupled to whole horse or whole rabbit serum proteins and at times to rabbit serum albumin. The albumin was prepared by Howe's method (14), and completely freed from sodium sulfate by dialysis at 4°C. For coupling these proteins either 60 cc. of serum, made up to 100 cc. with water, or 100 cc. of a 5 to 6 per cent solution of albumin, was cooled to 8-10°C. in an ice bath and made alkaline with the addition of 12 cc. of similarly cooled N/1 sodium carbonate solution. The chilled, diazotized dye solution was then added, with vigorous stirring, to one or the other of these protein solutions. After standing at least 2 hours, or overnight at 8–10°C., the mixture was carefully brought to pH 7.0–7.6 with N/1 HCl, and concentrated slightly by dialysis. The resulting crude azoprotein solution contained about 3 per cent of protein and 0.7 per cent of dye. Since much uncoupled dye was present it required purification.

Purification of the Crude Dye-Azoprotein by Repeated Precipitations with Alcohol

Preliminary tests showed, in the following manner, that purification could be obtained by repeated precipitations with alcohol at the proper concentration. For example, various concentrations of alcohol were mixed with two test solutions, the first, to be called *plain dye solution*, an aqueous solution of T-1824, having the same tinctorial value as the crude azoprotein solution, and the second, to be termed the *dye-serum mixture*, consisting of 4.0 parts of whole serum and 6.0 parts of water, together with enough dye to give the final mixture the same color value as the crude azoprotein solution. When the final concentrations of alcohol ranged between 75 and 80 per cent, all dye in the plain dye solution remained dissolved, but, by contrast, the dye-serum mixture yielded a precipitate, since serum proteins are completely precipitated by alcohol in this concentration range. The precipitate was slightly colored, but when taken up quickly in water and reprecipitated the color became less. Three or four repetitions of the procedure freed practically all the dye from the protein with which it had been mixed. The final precipitate was almost white and yielded a clear colorless solution with water. In these procedures, as in all subsequent ones of a similar nature, a predetermined amount of 95 per cent alcohol was poured into the aqueous solutions containing the protein, using just enough to yield the desired final alcohol concentration. In this way the protein solution did not come into contact with higher concentrations of alcohol; the chances of denaturation were lessened, and any unlinked free dye, which would be precipitated by alcohol in concentrations higher than 80 per cent, was not brought down with the protein that was to be freed from it.

The separation of the admixed, but unlinked, dye in the crude azoprotein solution was accomplished in the same way. Successive precipitations, generally five or six, in 76 to 80 per cent alcohol, were carried out until the supernatants contained only the faintest traces of visible color. The final, almost black precipitates, when dissolved in water, gave clear, deep blue solutions.

To reduce the time of exposure of the azoprotein to alcohol, thereby avoiding as much as possible the likelihood of denaturation, almost instantaneous precipitation was brought about by the addition of 0.25 to 2.0 cc. of saturated NaCl solution. Centrifugations for only 3 min

utes, at 4000 R.P.M., sufficed to separate the alcoholic supernatants from the precipitates, and the latter were then quickly redissolved in water. The best results were obtained when the procedure was carried out in small lots which could be handled speedily. While it was not found necessary to work with chilled reagents, nevertheless precipitation at refrigerator temperature should be less injurious to the protein, and this precaution was found absolutely necessary when the protein used for coupling was a globulin.

Next, the final precipitates were dissolved in sterile physiological salt solution, pooled, filtered through cotton, and dialyzed in the ice chest for 2 or 3 days, against water which was changed two or three times each day. When all traces of alcohol had been removed the viscose tubing containing the dialysate was hung in a stream of cold air until the contents reached the desired concentration. Dialysis was then repeated against physiological saline solution containing 2 cc. of N/1 NaOH per 4 liters. Finally the material was either filtered or centrifuged, and sealed in small tubes. To some specimens merthiolate 1: 5000 was added as a preservative.

Obviously, the material prepared and purified as described would be of little use for physiological, pathological, or immunological research if free dye remained with it which might become detached from the preparation in sufficient quantity to become visible after injection into the body. Consequently it seemed advisable to attempt to purify one of the crude azoproteins by other means and to determine the ratio of dye to protein in the resulting product. If the ratio appeared to be similar to that of the same material obtained by alcohol precipitation the fact would indicate that a relatively pure product had been yielded by both methods.

It seemed probable that chromatographic separation of the free dye from the azoprotein might be effective if a suitable solvent could be found to carry the latter through columns of paper pulp while the former became adsorbed upon them.

Purification of the Dye-Azoprotein by Chromatography

Preliminary tests were made with a variety of solvents and the two test solutions, the dye-serum mixture and the aqueous dye solution. In chromatographic columns of paper pulp, 12 per cent aqueous sodium sulfate served best since it removed all the dye from the plain dye solution and yielded almost complete separation of dye from protein in the dye-serum mixture. Accordingly this solvent was next employed to study the separation of free dye from the crude azoprotein solution. Four test solutions were employed:—a crude dye-azoprotein solution, formed by coupling T-1824 to horse serum proteins (crude T-AP-H), a mixture of the same solution with sufficient dye added to it to increase the color content by 20 per cent, the dye-serum mixture, and finally, the plain dye solution. Identical chromatographic columns 25 cm. long and 10.5 mm. in diameter, each containing 5 gm. of paper pulp, were charged with 1 cc. of one of the test solutions in 20 cc. of 12 per cent sodium sulfate solution. All the columns were developed with the sulfate solution in the usual way.

None of the free dye in the plain dye solution and only 2 per cent of the color of the dye-serum mixture came through their respective columns of pulp. On the other hand, 51 per cent of the color of the crude azoprotein solution came through and, further, the mixture of the crude azoprotein solution to which 20 per cent of free dye solution had been added yielded an interesting finding. 49.9 per cent of its color appeared in the developer, that is to say, practically the same amount of color as that yielded by the crude azoprotein solution without the addition of free dye. Clearly the pulp took out all the free dye purposely added to this solution as well as that already present in it. Since not quite all the free dye was taken out of the dye-serum mixture, calculations (here omitted for brevity) indicated that 600 to 700 gm. of pulp would be required to purify 100 cc. of the crude azoprotein solution.

Relative Purity of the Products Obtained by Chromatography and by Precipitation with Alcohol

It became a matter of interest to compare the relative purity of the dye-azoprotein prepared by chromatography with that obtained by precipitation with alcohol.

TABLE I

Milligrams of Dye and of Protein in the Crude Dye-Azoprotein Solution as Such and in Samples Passed through Each of Four Paper Pulp Columns in a Serial Chromatogram Carried out until the Dye-Protein Ratio Reached Constancy

	Dye	Protein	Mol equivalent (prosthetic groups) calculated for total serum proteins	Mol equivalent as found by alcohol precipitation method
1	2	3	4	5
Crude azoprotein solution.	6.40	30.7	21.8	
Samples	*mg.*	*mg.*		
I	3.76	23.8	15.8	
II	2.62	19.6	13.9	
III	1.90	15.2	13.0	
IV	1.23	9.7	13.2	13.0

The figures presented in columns 2 and 3 show a great loss of material. This loss is apparent rather than real since only the first portions of the effluents from each tube were used for analysis. If each sample had been collected quantitatively, after adding much highly concentrated sodium sulfate to develop the pulp column, it would have become too dilute for the Kjehldahl determination of its nitrogen.

Accordingly a sample of pure azoprotein was prepared by chromatography, as will be described, and its dye-to-protein ratio was compared with that of the product obtained by precipitation with alcohol.

Four columns of paper pulp were made in the usual manner. 1.0 cc. of the crude azoprotein solution (crude T-AP-H) made up to 20 cc. with 12 per cent sodium sulfate solution, was poured through the first column and developed with several 10 cc. batches of the same solvent. Interest was centered only in the dye-protein ratio and not in the total dye or protein content of the effluent fluids. After taking out a sample for analysis the collected fluid was poured successively through the other three columns, and a sample of the effluent from each was saved for further study.

Table I and Chart 1 give the results of the test. The first column, which contained 1.3 gm. of pulp, reduced the mol equivalent of dye in the crude azoprotein solution from 21.8 to approximately 15.8. The 3.0 gm. of pulp in the second column brought the mol equivalent down to less than 13.9, and the third column, with 2.0 gm. of pulp, reduced it to 13.0 which was unaffected by the additional 3.0 gm. of pulp in the fourth column, since a mol equivalent of 13.2 was found in this sample. The curve indicates that 6.3 gm. of pulp, or even somewhat less, was needed to remove free dye from 1.0 cc. of the solution. It is clear from the table that an approximately constant ratio of dye to protein was reached in effluents III and IV indicating that no change was to be expected on further passages through paper pulp. Column 5 shows the mol equivalent of a sample of the same crude dye-azoprotein solution purified by precipitation with alcohol.

Since the mol equivalents of the azoprotein obtained both by chromatography and by alcohol precipitation of the crude solution showed excellent agreement, although the procedures differed widely, it can be assumed that the final products were similar and relatively pure.

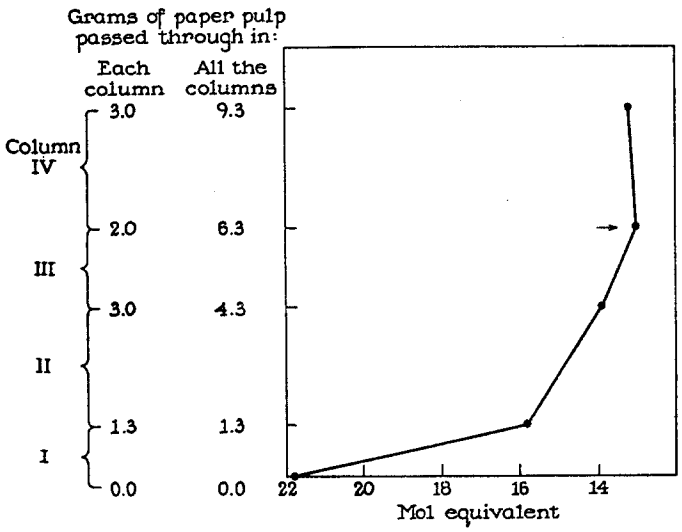


CHART 1. Grams of paper pulp required to obtain pure azoprotein from the crude azoprotein solution. The chart is plotted from the data of Table I. It makes plain the number of grams of paper pulp required to obtain, from 1 cc. of the crude dye-azoprotein solution, which contains 0.7 per cent of dye either permanently linked or free, a product having a constant molar ratio of dye to protein.

After passage through the third column (see text), the dye-protein ratio reached an equilibrium which did not change significantly in the fourth column. The arrow on the curve indicates that equilibrium was reached after passage through 6.3 gm. of paper pulp.

Difference in the Diffusion Rate of the Color of the Dye-Azoprotein and That of a Dye-Serum Mixture

Before employing the AP for physiological experiments it seemed desirable to determine whether its physicochemical behavior differed from that of a dye and serum mixture. Diffusion experiments seemed to offer a means to study the point.

In several series of tests the diffusion of a purified T-AP-H solution through 5 or 10 per cent agar was compared with that of the dye-serum mixture and the plain dye solution. The visible spread of color of the three test solutions through 10 per cent agar is shown in Chart 2 for various periods up to 98 hours. For the first 5 to 10 hours the color spread of the azoprotein and the dye-serum mixture was similar and slower than that of the plain aqueous dye solution. Thereafter, while the color spread of the azoprotein continued to be slow, the color of the dye-serum mixture began to move more rapidly and approached that of the aqueous dye solution, suggesting that, at first, the protein of the dye-serum mixture had retarded the spread of color.

This finding is in agreement with the observations of Rawson (15) and Allen and Orahovats (16) who have shown that the dye T-1824, when mixed with a solution of protein attaches itself to the latter by a cation linkage, and migrates through an electric field more slowly than the molecules of free dye in aqueous solution. The dye, in the dye-serum mixture, being in association with the protein by cation-anion linkage, will diffuse no faster than the protein itself as long as the bond is undisturbed. If, however, there is introduced into this dye-protein system, some protein hydrolysate containing anions attached to smaller, more diffusible molecules that are arranged in the same configuration as those present in the protein molecules of the dye-serum mixture,—peptone molecules, for instance—one might be able to disturb the dye-protein bond, to dissociate

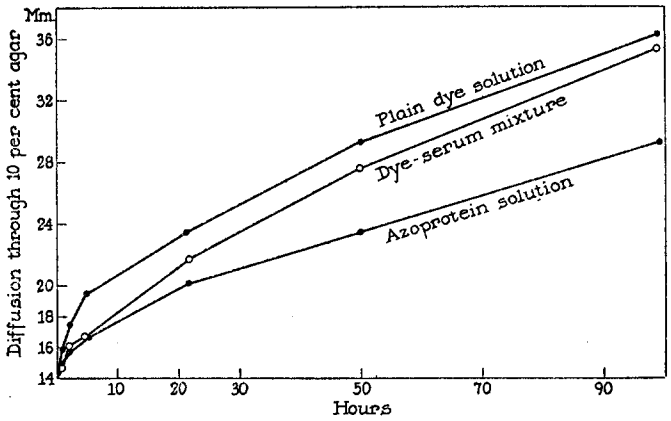


CHART 2. The rates of spread of visible color of the plain dye solution, the dye-serum mixture, and the azoprotein solution respectively through 10 per cent agar. For explanation, see text.

the dye from the protein molecules and transfer it to the faster moving protein hydrolysate. Then the rate of diffusion of color should be quite different from that of the dye that is firmly coupled to protein, as in the pure AP.

Accordingly, the preceding experiment was repeated using nutrient peptone agar. Chart 3 shows the diffusion curves obtained. By contrast with Chart 2, the color spread of the dye-serum mixture and of the plain dye, were similar in the first few hours. The movement of the former was not retarded by the protein, even initially, as in the first tests. On the other hand the diffusion rate of the color of the AP was that which one would expect from a slowly moving protein.

An AP Prepared with a Very Diffusible Dye.—For reasons that will appear below it became imperative to prepare an AP from an exceedingly diffusible dye, one so diffusible that it would escape from the body rapidly it if should

become separated from the protein to which it had been linked. The dye, echtsäure-blau B, Color Index No. 733, molecular weight 575, was found suitable, and azoproteins were formed from it using the methods outlined above. The most useful of these compounds (to be termed E-AP-G) was formed by linking the dye to bovine γ-globulin, fraction II.[1] It was readily purified by repeated isoelectric precipitation without risk of denaturation.

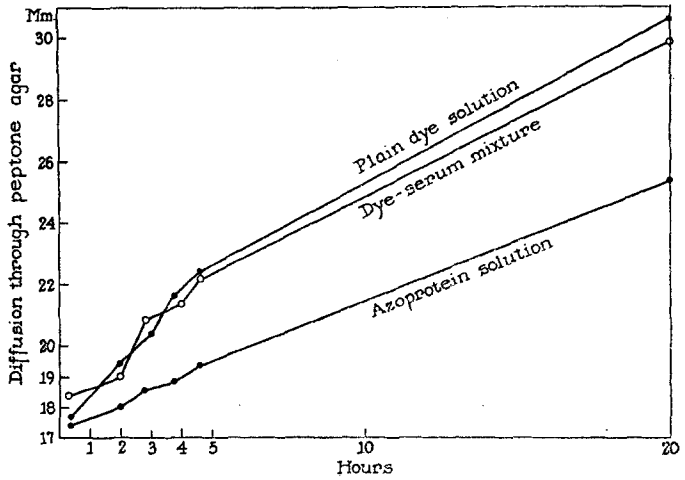


CHART 3. The rates of spread of visible color of the plain dye solution, the dye-serum mixture, and the azoprotein solution respectively through peptone agar. (See text.)

Physiological Tests

The various AP were well tolerated when given to laboratory animals, intravenously, intraperitoneally, subcutaneously, or intradermally. For example, 30 gm. mice appeared unharmed by intravenous injections of as much as 0.4 cc. of the AP solutions, containing nearly 1 gm. of azoprotein per kilo of body weight. As little as 0.05 cc. of these solutions rendered the animals blue.

It became clear in these earliest tests that the AP was taken up by the tissues in one form or another; the skin of the animals became brilliantly blue and retained its color for days. Gross and microscopic examinations of fresh and fixed tissues, prepared as described further on, showed that, after an intravenous injection of AP, blue matter appeared with great rapidity,—in as short a time as 3 to 5 minutes,—in the Kupffer cells of the liver, and after a few more minutes in reticulo-endothelial cells elsewhere in the body. More will be said of this, too, below.

The azoproteins proved to be highly antigenic, as had already been inferred they would be, from the work of Landsteiner and his colleagues (1–5). Mice

[1]Kindly supplied by Mr. Lawrence L. Lachat of Armour and Company.

injected either with the AP solutions or the proteins from which they were derived were readily thrown into anaphylactic shock by subsequent injections of the corresponding AP. The finding seemed to offer an excellent opportunity to study the whereabouts of colored antigenic material during and after anaphylactic shock and during the processes of immunization. The remainder of this paper will be devoted to a consideration of experiments planned to determine whether or not the AP can be used as tracer antigens. There follows a brief description of the localization of the antigenic material shortly after its introduction into the blood stream.

The Problem of Tracer Materials

Colored azoproteins have been used before as tracer materials for special problems (10–12, 45, 46) and so too, recently, have radioactive substances (17–19). However in studies of this sort neither the visible presence of color nor the demonstration of radioactive material in various tissues can be taken as proof that the intact antigenic material itself, as initially injected, has been localized in the tissues in which it seems to appear. Before one can assume that the whereabouts of an injected antigen has really been traced, several possibilities must be considered concerning its fate after injection.

In the case of colored azoproteins, the appearance of colored matter within certain cells of the body, even if present there only a few minutes after an injection, may indicate one of several happenings. While still in the blood the dye may be split off very rapidly from the protein, and only the former may enter the cells. The dye-protein may be taken up intact and immediately broken down, leaving only the dye within the cells. The protein portion of an AP may be partially broken down in the blood stream, allowing the cells to engulf only a modified protein, or, the protein portion may be changed intracellularly, after the AP has been engulfed.

Despite these possibilities it has seemed reasonable to believe that, since phagocytic cells can and do take up dyes (of molecular weight 500 to 900), and, at the other extreme, bacteria, India ink, and other relatively large particles, there should be no reason why these or other cells cannot take up the various AP intact, since the latter lie between these extremes having molecular weights of approximately 90,000. Accordingly, experiments were begun to learn something about the fate of AP at various periods after injection into the bodies of normal mice.

The Choice of a Suitable Azoprotein

It seemed possible to avoid some of the difficulties mentioned by a proper choice of a blue dye from which to prepare AP. Certain extremely diffusible dyes are not degraded within the organism, and form no loose or firm combinations with proteins. After injection into the blood stream of animals they do not become stored intracellu-

larly in granules, and they remain in the body only for very short periods of time. A dye of this sort, if capable of being diazotized and coupled to proteins, should form an antigenic AP that could be used to advantage to determine whether intracellular colored material found in the body after its injection into the blood stream was simply retained dye or some sort of dye-protein. Should the highly diffusible dye become split off from the protein coupled to it while it was still in the blood stream, that is to say before the AP had been engulfed by the cells, no colored granules would appear in the tissues. The dye might be taken into the cells, appear there for an hour or so as a transient coloration, and escape in the urine and bile. Should it become split off from the protein after the AP had entered the cells, its color would fade away in a few minutes as it passed out of them. By contrast the take-up of any blue material would indicate the presence of the AP since the retention of color would mean that the dye-protein linkage had not been broken, and that some sort of dye-protein, even if not the original one injected, remained within the cells.

After many trials, the blue dye, echt-säure-blau, was found to meet these requirements, and, as has been outlined above, the dye-azoprotein, E-AP-G, was obtained by coupling it with bovine γ-globulin. In a series of *in vitro* tests, this dye was added in various amounts to horse serum and to 5 per cent solutions of the γ-globulin. The dye formed no firm or loose combinations with the proteins, and the latter could always be precipitated from the dye in a colorless form.

The Behavior of the Dye, Echt-Säure-Blau, Following Intravenous Injection into Mice.—Aqueous solutions of echt-säure-blau, 0.7 per cent, containing the same amount of the dye as the E-AP-G solutions, when injected into mice in the same amounts in which it was employed later in combination with protein colored the animals visibly blue. Other mice received 2 to 5 times as much dye. All were killed, under ether or pentobarbital anesthesia, 5, 10, 15, or 30 minutes later, or at hourly intervals for 5 hours, or after 1 or 2 days. After blood specimens had been obtained by severing the great vessels in the thorax, the skin, mucous membranes, and viscera were examined *in situ* under the microscope. Fresh and frozen sections were made of liver, spleen, kidney, and mesenteric and peripheral lymph nodes, and blocks of tissue were immediately fixed in Carnoy's fluid and cleared by the Spateholz method (20).

The dye escaped rapidly from the blood, coloring the tissues diffusely blue. At no time, in any of the mice, did it appear in cells of any tissues in granular form or in vacuoles. The plasma of animals injected up to 1 hour before they were bled was brilliantly blue; fading occurred later and no blue could be seen after the 2nd hour. The bile and urine soon became intensely blue, and the skin, lymph nodes, and viscera appeared deeply and diffusely blue. However the elimination of the dye through the liver and kidneys was so rapid that all examinations made after the 3rd hour failed to disclose any blue coloration of the plasma or the tissues, either in the cells or outside of them.

The tests showed that the dye which had been present within the cells in a diffuse form never was stored as granules, and further, that it was so diffusible as to disappear from cells within 1 or 2 hours. It is clear from this that an azoprotein made from this dye should have the desired advantages outlined above.

The Behavior of a Mixture of Echt-Säure-Blau and Foreign Protein, When Injected into the Blood Stream of Mice.—Next, mice were injected with mixtures of an aqueous solution of the

dye and a 5 per cent γ-globulin solution, so made up that they contained either the same amount of dye as the E-AP-G, or three times as much. The findings were like those just described. The animals, killed at the same time intervals as in the preceding experiment, merely became diffusely blue, and then lost all color within 3 hours. No blue granules appeared in the cells at any time, and the plasma was colored for only an hour during which the bile, urine, and feces became heavily tinged. Clearly the dye did not become linked to the globulin, or to the plasma proteins, or to the cell proteins, and it was not held within the body.

Wholly different were the findings when similar tests were carried out with the echt-säure-blau azoglobulin (E-AP-G).

The Behavior of Echt-Säure-Blau Azoglobulin, E-AP-G, Following Intravenous Injection into Mice.—The E-AP-G solution, containing about 0.7 per cent of dye and 3 per cent of protein, was injected into the tail veins of scores of mice, in amounts ranging between 0.05 and 0.2 cc. per 30 gm. of body weight. Under these circumstances the amount of dye given as dye-protein was either the same or less than that used as plain dye in the preceding experiments. To study the fate of the injected AP, in the first few minutes, some mice were anesthetized with nembutal or ether and various viscera or peripheral tissues were exposed for microscopic inspection during and immediately after the injections. Individual animals were killed after 3, 5, 10, 15, 30, and 45 minutes and blood and tissue specimens were taken as will be outlined below. Other unanesthetized mice were injected with the AP and examined later, under anesthesia, at intervals ranging from a few hours to several months.

In all instances the living organs were examined under a binocular microscope, at magnifications ranging from 25 to 200 times. Next, the mice were bled from the great vessels in the thorax. Fresh contact impressions of the cut surfaces of various organs were mounted unstained, in a droplet of Locke's or Tyrode's solution under a cover slip ringed with paraffin. Some were fixed and stained with alcoholic eosin or safranin which allowed the blue azoprotein to stand out clearly, or with Giemsa stain which, of course, masked the AP. Thin, free-hand sections, stained and unstained, were mounted in saline or glycerine and studied immediately. Specimens of skin from the flanks and pieces of the walls of hollow viscera removed from the body, were examined in the same way, in the fresh state and again after fixation or clearing, as will now be outlined.

Since the azoprotein is insoluble in 80 per cent alcohol and higher concentrations, tissue blocks for subsequent sectioning were immersed either in alcohol or in Carnoy's fluid. Both permanently fixed the AP *in situ*. Unstained paraffin sections, at no time brought into contact with alcohol of less than 80 per cent concentration, were dehydrated in stronger alcohols and cleared in xyol in the usual manner. Many sections were counterstained with alcoholic eosin or safranin, and others were stained in the usual way with hematoxylin and eosin. The latter procedure, necessitating the use of dilute alcohols and aqueous solutions, dissolved out AP, and sections so prepared were used only for comparison with the unstained ones.

Finally other blocks of tissue were cleared by the Spateholz method (20) after fixation in Carnoy's fluid. In these the distribution of the fixed AP could be studied to great advantage, since it retained its color well and stood out in sharp contrast to the cleared tissues.

Findings.—Following intravenous injections of E-AP-G no color appeared in either the bile or urine. In both fresh and fixed tissues, obtained only 3 to 5 minutes after an intravenous injection of E-AP-G, some blue matter could be seen, as coarse granules, or in vacuoles, in the Kupffer cells of the liver, and in reticulo-endothelial cells elsewhere in the body. The characteristics of the storage of the injected material will be described at the end of the paper, after showing that stored material was

actually the antigenic protein, for 2 days at least after its take-up by the cells of the liver. However, it is necessary for clarity to anticipate and to emphasize here that the character of the distribution and stroage of all AP preparations was similar, regardless of which dye was coupled to what protein. All produced a *Speicherung* of the reticulo-endothelial system like that which follows an intravenous injection of vital dyes of large molecule, or fine particulate matter (21–26). Tissues examined after 3 to 30 minutes, or at hourly intervals up to 5 hours showed progressively more blue, the color lying intracellularly in sharply defined granules. In animals killed after 24 hours far more blue was found within the R-E cells, the maximum take-up, after injecting the usual amount of E-AP-G—0.15 cc. of the solution per 30 gm. of body weight—occurred between the 36th and 48th hours. At this time the visible blue of the plasma disappeared. The finding is of importance in relation to the data given below.

It was of further interest that the type of cells, Kupffer cells, lymph node reticular cells, vascular endothelial cells and others, which first began to take up the colored material within 3 to 5 minutes after the injection of E-AP-G into the blood, were the same ones that continued the process during the next 36 to 48 hours. After the longer periods there were simply more of them, more intensely colored. The differences seen were only quantitative not qualitative.

The experiments show with certainty that no free dye was present in the E-AP-G employed and that no unchanged dye was split off from it in the blood stream, during the 36 to 48 hours that the plasma remained blue. Had this taken place blue would have appeared in the bile and urine. Since the preceding experiments showed that the dye itself does not become segregated within R-E cells, it seemed probable that the coupled dye-protein had been engulfed and stored.

Before this supposition could be accepted certain possibilities remained to be considered. It is conceivable that, during the process of diazotization and coupling to form E-AP-G, the dye had become changed in such a manner that it had acquired a capacity for combining with proteins. Under these circumstances, should it be split off from the γ-globulin, while in the blood, it might enter the cells and be held there. As result the material seen in the cells would be a dye-protein altogether different from the injected E-AP-G. To test the point E-AP-G was mixed with egg albumin in the concentrations in which it was found in the blood of mice following the usual injections. After standing for a while, isoelectric precipitation effected complete separation of the components of the mixture. The albumin contained no trace of color. The test indicated that the dye of the E-AP-G had not acquired a capacity for combining with other proteins and consequently, should it become split off from the globulin, while in the blood stream, it would not be held by the proteins of the cells into which it entered. As result, one can regard the blue color seen within cells, after the injections of the AP in the preceding experiments, as E-AP-G either intact or changed only in its protein portion.

Since the appearance of some of the blue material in the Kupffer cells was extremely rapid, and since it was accomplished by a phagocytic type of cell, it seemed possible that the E-AP-G had been taken up intact. The intracellular distribution of the blue matter, whatever its nature, seemed to be too rapid to allow for an appreciable digestion of the protein portion of the AP in the blood stream, since it is well known that injected antigenic proteins can be found circulating in the blood for several weeks (27–29). Nevertheless the possibility remained that the protein portion of the E-AP-G had been changed, either while still in the blood stream and before it had entered the cells, or shortly afterwards. Spectrophotometric methods failed to identify the blue material after it had entered the cells. To determine whether or not the protein portion of the E-AP-G had been changed resort was had to serological methods.

Evidence that the Blue Material Seen in the Liver Is Stored Antigen

At first it seemed improbable that one could demonstrate the presence of antigen in liver tissue since antigenic proteins injected into the blood stream remain recognizable there for weeks. As result antigen would be present in the blood within the liver, and perfusion of the organ, before attempting to extract it, would neither remove all the blood antigen nor that which might be adsorbed on the tissues.

It has just been mentioned that the concentration of E-AP-G injected into the blood stream of mice, decreases rapidly in the first 48 hours, the blood becoming colorless in that time. Consequently the antigen content of the blood circulating in the liver must also decrease, and correspondingly the amount of antigen in contact with the tissues. While this takes place the blue material accumulates progressively for 48 hours in the Kupffer cells of the liver and in R-E cells of other organs, and no blue appears in the bile, urine, or feces. If one should find that the amount of extractable antigenic material in the blood and liver followed the same pattern, that is to say that it increased in the liver while it decreased in the blood, or, if there should be, 24 to 48 hours after the injection, more antigenic material in the liver than in the blood, then one could be sure that the blue material in the cells must be antigenically intact E-AP-G itself. This would be all the more certain since the finding of blue color in Kupffer cells, following an injection of E-AP-G, indicates that the highly diffusible dye is still coupled to protein.

Accordingly tests were made to study the antigen content of the blood and livers of mice injected with E-AP-G or with bovine γ-globulin.

Preliminary studies were begun with 30 gm. mice injected intravenously with 0.1 cc. of a 5 per cent solution of the bovine γ-globulin; the same amount of protein that was used in the tests with E-AP-G. After 5, 24, and 48 hours the animals were anesthetized and bled to death. The livers were removed at once, chilled in small glass dishes standing in chopped ice, ground with sand, extracted with various volumes of 0.85 per cent saline to obtain the desired dilu-

tion, and cleared by repeated centrifugation. All manipulations were carried out in the cold. Precipitin reactions with the sera and liver extracts showed much antigen in the blood at the end of the 5th hour, and progressively less in the next 2 days. No antigen was demonstrable in the cleared liver extracts.

Since all the visible blue material in the livers of mice injected with E-AP-G was found to be intracellular it seemed probable that the γ-globulin had remained with the cloudy cellular material that was discarded in the effort to obtain clear supernatant fluids for the precipitin tests.

Obviously, then, to detect the presence of antigen in the liver, it would be necessary to include the cloudy cellular material of the liver suspensions and to resort to complement fixation tests. For the latter, the technique described by Boyd (30) was employed, freely modified to standardize it for bovine γ-globulin, as antigen, and for antiglobulin as present in the sera of rabbits immunized to the bovine γ-globulin. The system was also tested and standardized for heterologous mouse tissue. In all instances, preliminary tests were made to rule out, or to determine, the anticomplementary action of the various cells or sera used, as also that of the γ-globulin and the E-AP-G. The anticomplementary effect of the latter, in the concentrations used, was negligible. All necessary precautions (30) were scrupulously observed. In the final tests the sera and liver suspensions were diluted 10, 20, 40 times and so on by multiples of 2 up to 20,480. As in the previous tests the livers were chilled at once upon removal, ground, and extracted in the cold, to prevent enzymatic attack upon the antigen, and the dilutions were made at once with chilled reagents. Next, the mixtures were inactivated in the water bath at 56°C. for 30 minutes. It seemed better first to dilute the chilled liver suspension rapidly in cold saline and then inactivate the dilutions, rather than first to inactivate the original, concentrated liver suspension and then make the dilutions. In this way, the possibility of a breakdown of antigen by proteolytic enzymes in high concentration should be greatly diminished.

In the first tests bovine γ-globulin was injected into the blood of several mice, employing either 3 times as much protein as had been used when the E-AP-G was injected, or an equivalent amount. As result, the antigen concentration in the blood was so great that, 48 hours after the injection, and even later, the antigen contained in the hepatic blood, which is estimated (31) to amount to 20 to 40 per cent of the organ's wet weight, masked any antigen that might be present in the liver tissue, and the titers of the sera and liver suspensions were approximately equal.

Less antigen was injected in a new series of tests; 50 μg. of bovine γ-globulin in 0.1 cc. of saline. Forty-eight hours later, when the livers had been given time to take up the maximum content of blue material, as shown in previous experiments with E-AP-G, the mice were killed, and the blood and livers were taken for study. In three experiments, in each of which different amboceptor and complement preparations were used, the findings were similar; there was always more antigen in the liver suspensions. Serum readings gave in one experiment only a trace of antigen, and in the other two, 1 plus at dilutions of 20 times and faint traces at dilutions of 80 and 160 respectively. By contrast, the liver suspensions read 1 plus at 80 dilutions in the first test and 1 plus at 160 dilutions in the other two, with slight traces at 1280 and 5120 times, respectively. The end point (1 plus) was 3 tubes farther out in the liver suspension in 2 of the tests and 2 tubes farther out in the third; this in spite of the fact that the liver suspensions were greatly diluted by the liver blood which contained less antigen than the tissue itself.

Finally the tests were repeated using the blue antigen E-AP-G. The findings were similar. Indeed this was true only 24 hours after injecting 0.1 cc. of E-AP-G, which had been diluted 100 times with saline. The liver suspension yielded 1 plus at 640 dilutions, with traces at 2560, whereas the serum showed 1 plus after only 20 dilutions—a difference of 5 tubes—and traces after only 40 dilutions, a difference of 6 tubes.

Precipitin tests were also carried out in these experiments. The sera gave positive reactions at 40 to 160 dilutions in them all. In all, the liver extracts were negative; indicating, by the absence of antigenic activity in the cleared liver extracts, that the antigen, like the blue material was present within the cells and had remained with the liver detritus which was discarded before performing the precipitin tests.

The finding of maximal amounts of blue matter in Kupffer cells of mouse livers, 48 hours after injecting E-AP-G into the blood indicated, as already pointed out, that the dye-to-protein bonds had not been disrupted. The finding, in the liver suspensions by complement fixation, of antigenic activity greater than that of the blood, indicated that the engulfed blue material, seen in the cells, was still sufficiently like the material originally injected to be antigenic. It can therefore be considered as taken-up antigen.

The Azoprotein Prepared from T-1824 as a Tracer Antigen

It is to be recalled that the first of the azoproteins to be described above was made from horse serum proteins and the dye, T-1824. Although this preparation, T-AP-H, was by far the most highly colored of the AP, it could not be used for the studies just described because of the behavior of the dye in the animal body. Injections of aqueous solutions of this dye into the blood stream of mice led to the appearance of blue granules and vacuoles in phagocytic reticuloendothelial cells all through the body, a typical *Speicherung*. It persisted for weeks. Because of this behavior the T-AP-H was open to one of the objections already discussed, namely that the dye, if split off from the protein to which it had been coupled, would appear in the R-E cells and remain there regardless of whether the split occurred while still in the blood or after the AP had entered the cells.

On the other hand, since the studies already described had shown that the dye of the E-AP-G was not split off from its protein, and further, that the AP remained antigenic in Kupffer cells for at least 2 days, it seemed worthwhile to find out whether the more highly colored T-AP-H would behave similarly. Should this be the case, the T-AP-H would serve as a much more brilliant tracer antigen than the E-AP-G and one could determine the initial sites of its distribution and early storage to great advantage. Of course, because of the ability of the dye, T-1824, to remain within cells for long periods, one could not hope to determine with it how long the T-AP-H remains as such within them, but only to trace the antigen to the sites at which it is first stored.

An experimental comparison of the behavior of the T-AP-H and E-AP-G in the bodies of mice, together with other experiments now to be described, showed that the highly colored T-AP-H serves as a better tracer than E-AP-G.

The Behavior of T-AP-H in the Tissues of Mice Compared with the Findings with E-AP-G

T-AP-H injected intravenously into mice in dosages like those employed for E-AP-G rendered the animals more deeply blue. However, the bluer plasma became colorless within 36

to 48 hours, as it did following the injections of E-AP-G. No precipitation or particle formation could be seen microscopically in the blood at any time, and although the T-AP-H colored the plasma for many hours, no blue appeared in either the bile, urine, or feces. Blue matter appeared in Kupffer cells and other R-E cells quite as fast as after injections of E-AP-G, the intracellular storage of color seeming to be too fast to allow for an appreciable proteolysis of the AP. Since it has already been shown that this does not occur with E-AP-G, it seemed likely that it did not happen to the T-AP-H.

Fresh and fixed tissue specimens obtained, as already described, showed that the distribution and storage of the two AP were exactly alike. The similarity of behavior warranted the supposition that the dye T-1824, like the dye, echt-säure-blau, was not split off from the coupled protein. Further tests showed this to be the case.

Tests with Solutions Containing Not Coupled Dye.—The dyes, T-1824 and echt-säure-blau, were added to horse serum in the same proportions as the coupled dyes of the corresponding AP. When the resulting mixtures were injected into some mice and the corresponding AP was injected into others, so that all received the same amounts of dye, the mice given the dye-serum mixtures showed much color in both bile and urine, while the mice receiving the AP solutions showed none. Further, when equivalent amounts of either of the dyes were injected, in aqueous solution into the blood of mice, color appeared in both bile and urine even more rapidly and in greater amounts than when they were introduced together with serum.

In the experiments in which the mixture of the dye T-1824 and serum was employed most of the dye must have been loosely bound to the serum proteins (15, 16), only very little remaining in the free state. Nevertheless the very small amount of free dye present in the mixture was detectable through its appearance in both the bile and urine. Since the injections of T-AP-H colored the liver and other viscera deeply blue it is obvious that the intense coloration could not have been produced by dye which had been split off from the protein of the T-AP-H before it entered the cells. Had this been the case the dye, free in the blood, would have appeared in the bile and urine. Clearly, the T-AP-H must have entered the cells as a dye-protein. Since the dye-protein E-AP-G retained its antigenicity for 2 days, at least, the T-AP-H chemically coupled in the same way to similar proteins, can be supposed to behave similarly as a tracer antigen.

The Distribution of "Tagged" Antigen in Certain Tissues

The azoproteins are soluble antigens. Nevertheless their distribution, following an intravenous injection, is like that of dyes of large molecule, fine particulate matter, or bacteria. This sort of take-up has been so fully described (23–26), that little needs to be said here. For the purposes of the present paper the distribution of the colored antigens will be discussed only as implicating certain organs known to be active either as sites of antibody formation or storage. More will be said in later papers about the distribution of antigenic AP in other organs and under different conditions.

The distribution of T-AP-H is exactly similar to that of E-AP-G. Both can be readily identified in fresh and fixed specimens. Because the color of the E-AP-G is much lighter and paler than that of T-AP-H, photographs of tissues containing the latter have been used, the better to demonstrate the findings.

However, the following descriptions apply equally well to the distribution of both AP preparations.

Findings in Lymph Nodes Following Intradermal Injection

Earlier work from this laboratory (32, 33), since confirmed in several others (34–40), has shown that those lymph nodes which are nearest to the sites of injection of pathogenic bacteria and viruses, form antibodies in high concentration against these agents before they appear in notable amounts elsewhere in the body. Since the phenomenon was first demonstrated in the cervical nodes of mice (32), after intradermal injections of various antigens into the lymphatics of the ears, it seemed a matter of interest to study the distribution of colored antigen when introduced into these nodes by the same route and in the same manner.

By techniques already described (41–44) about 0.01 cc. of the AP was introduced into the skin of the ears of mice, under the microscope, as superficially and with as little pressure as possible. Some of the blue fluid entered lymphatics torn purposely with the injecting needle, and it slowly drained through them to the nodes. There was no visible escape of the AP from the lymphatics at any time, as happens ordinarily when plain dye solutions are injected (41, 42).

Fig. 1 *a* (× 25) shows an unstained paraffin section of a cervical lymph node taken from a mouse 24 hours after introducing T-AP-H into the skin of the ear. The blue antigen appears black in the photograph. It lies in the cytoplasm of the cells situated in the subcapsular, cortical, and medullary sinuses, and at the hilus. The color was not found in the nuclei although blue granules often lay so close to the latter that they were clearly outlined. By contrast the lymph follicles in the cortical portion of the node and lymphocytes elsewhere, even those immediately next cells containing the blue antigen, took up none of it. The general picture is that of a *Speicherung* with vital dyes. Fig. 1 *b* is a photograph of a section from the same specimen, cut at some distance from the one shown in Fig. 1 *a*, and stained with hematoxylin and eosin to demonstrate the lymphoid tissue. In the process of staining, while bringing the section from high concentrations of alcohol to aqueous hematoxylin solutions, the AP was dissolved out. Although these preparations are not serial sections, one can identify in Fig. 1 *b* many of the structures in Fig. 1 *a*. Comparison shows that the lightly stained areas in Fig. 1 *b*, that is to say the cortical and medullary sinuses and the tissue at the hilus, containing endothelial, reticular, and other phagocytic cells, are the very ones that held the blue antigenic protein.

Phenomena Following Intravenous Injection of T-AP-H

Peripheral Lymph Nodes.—Figs. 2 *a* and 2 *b* show alcohol-fixed paraffin sections, unstained and stained respectively, of a cervical node of a mouse 24 hours after the animal received the usual intravenous injection of T-AP-H (0.1 cc. of the AP solution per 30 gm. of body weight). The distribution of the antigen, all the blue material being in the cytoplasm of sinus and reticular cells, is much like that seen in Figs. 1 *a* and 1 *b*, taken from an experiment in which

the antigenic material reached the nodes directly on the lymph stream. It is probable that the node pictured in Fig. 2 also received its colored matter from the lymph after the antigen had passed into that fluid from the blood.

Mesenteric Nodes.—Following intravenous injections of the AP the great mesenteric lymph nodes invariably became deeply colored. Fig. 3 shows an unstained paraffin section of a mesenteric node removed from a mouse 24 hours after the usual intravenous injection of T-AP-H. The blue material, black in the figure, is located in the cytoplasm of cells of the cortical and medullary sinuses and at the hilus, as in the peripheral nodes; the lymph follicles and lymphocytic tissue show none of it.

The distribution of the colored matter in mesenteric nodes is better shown in transverse sections. Figs. 4 *a* and *b*, 5, 6, and 7, show, at various magnifications, cross-sections of a node from a 31 gm. mouse, 48 hours after an intravenous injection of 0.15 cc. of the blue solution. The amount of intracellular blue material, probably maximal at this time, is greater than in the previous pictures, because of the larger dosage that the animal received. Fig. 5, unstained, shows the little round nodule at the top of Figs. 4 *a* and *b*, as it appears in a different section, magnified 100 times. Many colorless lymph follicles in the cortical regions are closely surrounded by antigen-containing cells. Fig. 6, magnified 90 times shows that part of the section pictured in Fig. 4 *a*, that has been boxed in with lines. Fig. 7 (× 400) is a photograph of another section, taken from the same node. The great quantity of the antigen taken up by the cells around the sinuses is worthy of special note, as too is its absence from the lymph follicles, although many of the latter are most intimately surrounded by antigen-containing cells. The endothelial cells too contain a great quantity of the blue material. The same distribution was found in fresh imprint preparations, in free-hand sections, and in frozen ones, consequently the results cannot be attributed to artifacts produced by fixation.

Liver.—The chief findings in the liver have already been mentioned. Little needs to be added here. It should be stressed again that from the 3rd or 5th minute after injecting the AP into the blood the Kupffer cells showed the presence of the tagged antigen. As the time interval between the injection and the examination was increased all specimens, both fresh and fixed, showed a rapid increase in the number of Kupffer cells storing the material and in the intensity of color in the individual cells. The color lay in the cytoplasm of the cells not in the nuclei, and the maximal storage occurred 36 to 48 hours after the injection when the plasma lost all visible color. Between the 3rd and 24th hours the endothelial linings of some of the portal and hepatic venules often contained much dark blue, and so too did the endothelia of the sinusoids. In tissues examined after 30 hours blue was no longer visible in the endothelia. The liver parenchyma cells took no blue. More will be said of the findings in the liver in a paper dealing with anaphylactic shock as induced with the colored antigen.

Skin and Connective Tissue.–By methods described in previous papers (41, 42), the skin and cutaneous vessels in the ears of mice were observed under the microscope during intravenous injections of the various AP solutions, and at various periods thereafter. There was no visible escape of color from the normal vessels except rarely where many small capillaries entered into venules; but escape did occur from injured vessels. However after a few hours blue granules could be seen accumulating extravascularly, and sections showed that they lay in the cytoplasm of histiocytes and other connective tissue cells. The blue deposits increased in number and intensity up to the 36th to 40th hour. In contrast, when plain dye solutions of equal dye concentration were injected into the blood, visible escape of color occurred in a few minutes and the tissues became a diffuse blue. After many hours color also accumulated in connective tissue cells, but it was a more brilliant blue than that of the T-AP-H and the granulations in the cells were finer.

Other Organs.—The blue antigen was found in all organs except the brain. It was especially rich in the kidney tubules and these organs became nearly as blue as the liver and mesenteric lymph nodes. It was present in large amounts too in the muscle cells of the uteri, in the spleen the omentum, in the lungs, and in smooth muscle all through the body.

DISCUSSION

Since the AP antigens can be directly seen in fresh and fixed tissues, at high or low powers of the microscope, or in the blood, lymph, or other body fluids, if in sufficient concentration, they can be more easily employed than radioactive tracers for the study of the distribution of foreign protein that has been introduced into the body.

The findings constitute a picture of the storage of a soluble "tagged" protein antigen within the tissues of mice, following its introduction into the blood or into the skin of the ears. The sites at which the antigen is found are presumably those from which the first stimuli to antibody formation arise. It is of interest that these sites are the very ones that have been shown by classical immunology to hold particulate foreign matter, whether antigenic or not. The seizure of the colored material by reticulo-endothelial cells almost everywhere in the body indicates that the antigenic stimulus to antibody formation can be brought to bear from practically all quarters of the body upon those tissues or cells that are capable of antibody formation. There seems to be no difference in the distribution and storage of foreign material in the body, whether it is antigenic in nature or not. It is of further interest that the distribution of the AP after intraperitoneal injection was eventually the same as after intravenous injection, a matter that will be taken up in later papers.

If, then, foreign antigenic material is held within cells of the R-E system, how long does it retain its antigenicity? Is it stored and protected within the cells, or is it destroyed there even more rapidly than it disappears from the blood? These questions cannot be answered as yet, but work is going forward on the subject. Blue color has been seen in R-E cells of mice as long as $3\frac{1}{2}$ months after injecting E-AP-G. Much of the material has disappeared by that time, but, as stressed above, the fact that any color remains at all shows that some sort of dye-to-protein-linked material is still present. Clearly the breakdown of protein in the R-E cells is slow.

Mice, intravenously injected with E-AP-G in the usual amounts, show, as will be described in later work, a very rapid fall in the antigen content of the blood. By the 12th day only traces remain, but these traces continue to be present for many more days. Is it possible that the traces represent a slow output of stored antigen from the R-E cells, as the latter slowly lose their color? If so, the prolonged formation of antibody may be consequent on sustained stimulation in this way. This important matter is now under investigation.

Other authors (11, 12), working with less intensely colored dye-azoproteins, have stated their belief that azoproteins may remain intact within kidney tubule cells and various reticulo-endothelial cells for as long as a year. However, no evidence except visual has been given to substantiate the supposition.

SUMMARY

Intensely blue dye–azoproteins have been prepared by diazotization and coupling of the highly indiffusible blue dye T-1824, Evans blue, with various serum proteins and egg albumin. The products, whether purified by precipitation with alcohol or by chromatography, have a constant dye-to-protein ratio and tests have shown them to be essentially free from unlinked dye. An extremely diffusible dye, echt-säure-blau, has also been coupled to bovine γ-globulin. These materials are adapted to physiological experimentation. They seem to behave in the bodies of mice like other proteins; they fail to appear in either the bile or urine of normal animals, and they are strongly antigenic.

When these soluble antigenic azoproteins are injected into the blood stream of mice for the first time they enter reticulo-endothelial cells in almost every organ of the body; the final distribution is like that of intravenously injected, finely divided particulate matter. The azoproteins appear in the cells which classical immunological studies have shown to be active in removing particulate antigenic materials or bacteria from the blood or body fluids. The Kupffer cells of the liver and sinus and reticular cells in lymph nodes, especially the great mesenteric node, are particularly active in the removal of the blue antigens from the blood, but many other R-E cells are active to a lesser degree. The storage of the antigenic material is in the cytoplasm only; it has not been seen within nuclei, nor has it been seen within cells of the brain.

Serological methods disclose that the blue material seen within Kupffer cells of the liver after as long a period as 2 days is still antigenic in its reactions. The blue azoproteins, therefore, serve excellently as tracer antigens, especially since they can be seen directly in fresh and fixed tissue preparations and in the body fluids.

BIBLIOGRAPHY

1. Landsteiner, K., *K. Akad. Wetensch. Amsterdam, Proc. Sect. Sc.*, 1922, **31**, 54.
2. Landsteiner, K., *J. Exp. Med.*, 1924, **39**, 631.
3. Landsteiner, K., and Levene, P., *J. Exp. Med.*, 1930, **52**, 347.
4. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1932, **56**, 399.
5. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1933, **57**, 633.
6. Heidelberger, M., and Kendall, F. E., *Science*, 1930, **72**, 252.
7. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1934, **59**, 519.

8. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62,** 467.
9. Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M., *J. Exp. Med.*, 1933, **58,** 137.
10. Sabin, F. R., *J. Exp. Med.*, 1939, **70,** 67.
11. Smetana, H., and Johnson, F. R., *Am. J. Path.*, 1942, **18,** 1029.
12. Smetana, H., *Am. J. Path.*, 1947, **23,** 255.
13. Hartwell, J. L., and Fieser, L. F., Organic Syntheses, New York, John Wiley and Sons, 1936, **16,** 12.
14. Howe, P. E., *J. Biol. Chem.*, 1921, **49,** 93.
15. Rawson, R. A., *Am. J. Physiol.*, 1942–43, **138,** 708.
16. Allen, T. H., and Orahovats, P. D., *Fed. Proc.*, 1949, **8,** 3.
17. Libby, R. L., *J. Immunol.*, 1947, **55,** 16.
18. Warren, S., and Dixon, F. S., *Am. J. Med. Sc.*, 1948, **216,** 136.
19. Pressman, D., Hill, R. F., and Foote, F. W., *Science*, 1949, **109,** 65.
20. Spateholz, W., Über das Durchsichtigmachen von menschlichen und tierschen Präparaten, und seine theoretischen Bedingungen, Leipzig, S. Herzel, 1911, 2nd edition, 1914.
21. Schulemann, W., *Arch. mikr. Anat.*, 1912, **79,** 223.
22. Kiyono, K., Die vitale Karmin Speicherung, Jena, Gustav Fischer, 1914.
23. Evans, H. McL., and Scott, K. S., *Carnegie Institution of Washington, Pub. No. 273, Contrib. Embryol.*, 1921, **10,** 3.
24. Cappell, D. F., *J. Path. and Bact.*, 1921, **32,** 595.
25. Cappell, D. F., *J. Path. and Bact.*, 1930, **33,** 175.
26. Kiyono, K., Sugiyama, S., and Amano, S., Die Lehre von der Vitalfärbung, Kyoto, Isseido, 1938.
27. Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, **85,** 571.
28. McMaster, P. D., unpublished data.
29. Longcope, W. T., and Rackemann, F. M., *J. Exp. Med.*, 1918, **27,** 341.
30. Boyd, W. C., Fundamentals of Immunology, New York, Interscience Publishers Inc., 2nd edition, 1947.
31. Ratner, B., Allergy, Anaphylaxis and Immunotherapy, Baltimore, The Williams & Wilkins Co., 1943.
32. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1935, **61,** 783.
33. McMaster, P. D., and Kidd, J. G., *J. Exp. Med.*, 1937, **66,** 73.
34. Ehrich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, **76,** 335.
35. Ehrich, W. E., *Ann. New York Acad. Sc.*, 1946, **46,** 823.
36. Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.*, 1944, **81,** 73.
37. Dougherty, T. F., Chase, J. H., and White, A., *Proc. Soc. Biol. and Med.*, 1944, **57,** 295.
38. White, A., and Dougherty, T. F., *Ann. New York Acad. Sc.*, 1946, **46,** 859.
39. Burnet, F. M., The Production of Antibodies, Melbourne and London, Macmillan Co., Ltd., 1941.

40. Gaidamovich, S. Ya., and Soloviev, V. D., *Am. Rev. Soviet. Med.*, 1947, **4**, 226.
41. Hudack, S. S., and McMaster, P. D., *J. Exp. Med.*, 1932, **56**, 223.
42. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1932, **56**, 239.
43. McMaster, P. D., *Harvey Lectures*, 1941–42, **37**, 227.
44. McMaster, P. D., *Ann. New York Acad. Sc.*, 1946, **46**, 743.
45. Fagraeus, A., *Nord. Med.*, 1946, **30**, 1381.
46. Fagraeus, A., *J. Immunol.*, 1948, **58**, 1.

EXPLANATION OF PLATES

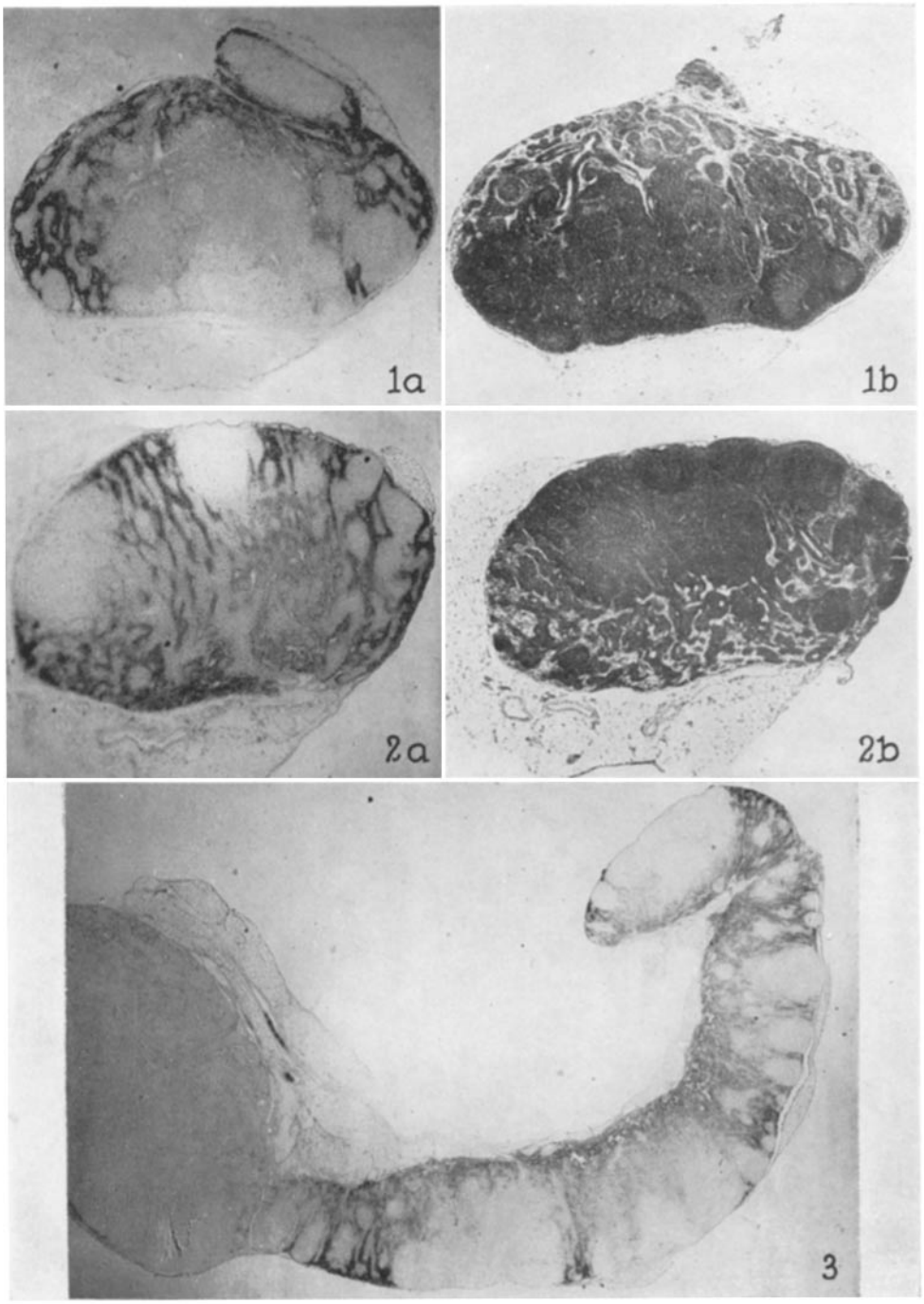
PLATE 32

FIG. 1 *a*. A photomicrograph of an unstained section of a cervical lymph node taken from a mouse 24 hours after introducing the blue azoprotein T-AP-H into the skin of the ear. The dark areas represent the distribution of the blue antigen lying in the cells of the sinuses; the lymphocytes have none of it (see text). × 25.

FIG. 1 *b* shows a section from the same node stained with hematoxylin and eosin to demonstrate the lymphoid elements. Although the photographs were not taken from serial sections many of the structures in Fig. 1 *a* a can be identified in Fig. 1 *b*. In the process of staining the latter section the azoprotein was dissolved out of the tissues; consequently the clear areas, the sinuses, are those which appear dark in the unstained section in Fig. 1 *a*.

FIGS. 2 *a* and 2 *b*. Photographs of sections from a cervical node removed 24 hours after an intravenous injection of T-AP-H. As in Figs. 1 *a* and 1 *b* an unstained section is compared with a stained one. × 25.

FIG. 3. An unstained longitudinal section of the mesenteric lymph node of a mouse 24 hours after an intravenous injection of the azoprotein. The dark areas represent the blue antigen taken up as described in the text. × 11.

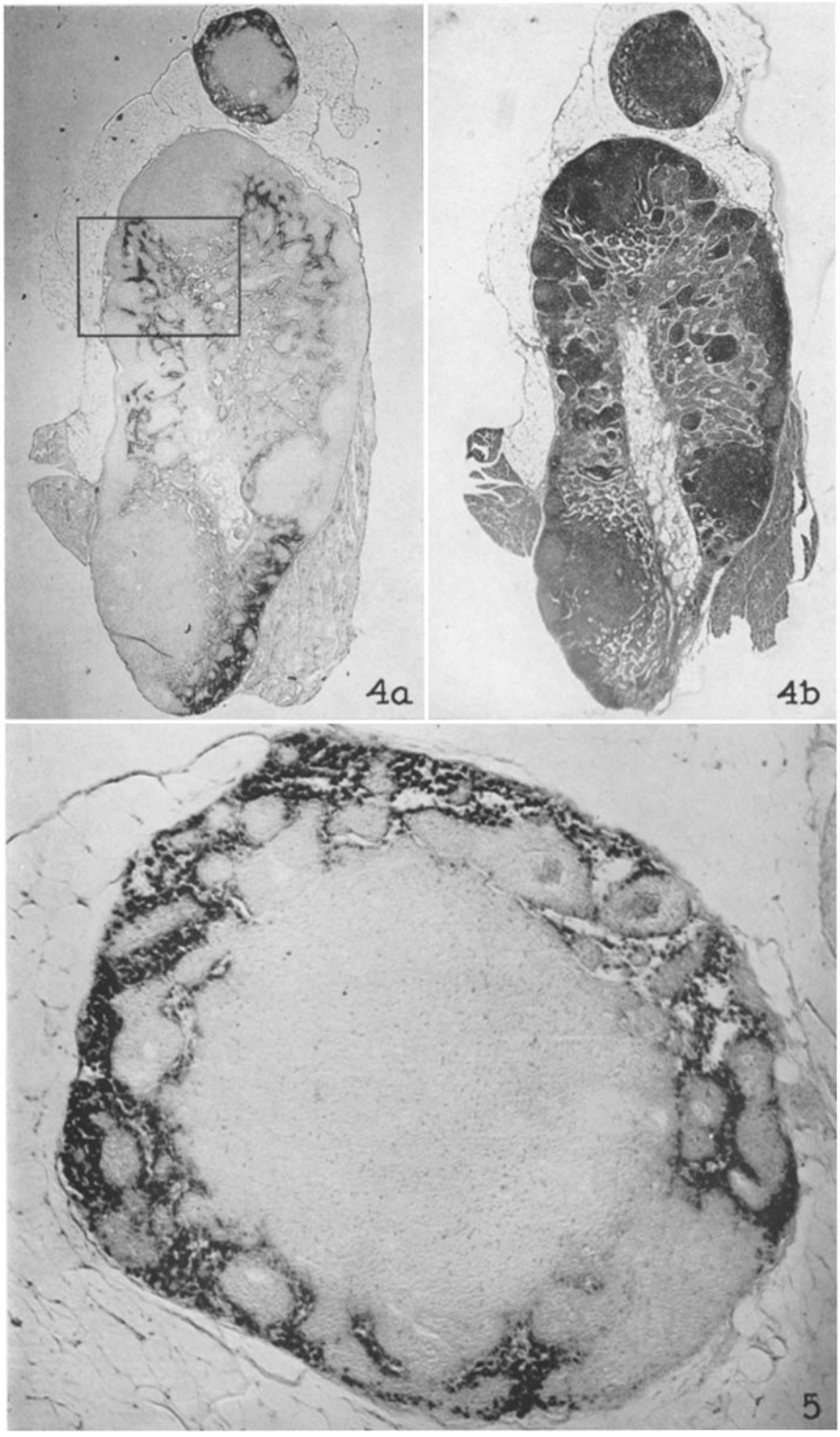


(Kruse and McMaster: Distribution and storage of blue antigenic azoproteins)

PLATE 33

FIGS. 4 *a* and 4 *b*. Unstained and stained transverse sections of the mesenteric lymph node of a mouse 48 hours after an intravenous injection of the azoprotein (see text). × 15.

FIG. 5. The round nodule at the top of Fig. 4 *a* as it appears at higher magnification and unstained in a different section. Again the black regions represent the blue azoprotein. The lymph follicles and lymphocytes in the medullary portion of the tissue contained no color, although they were intimately surrounded by cells containing the colored antigen. × 100.

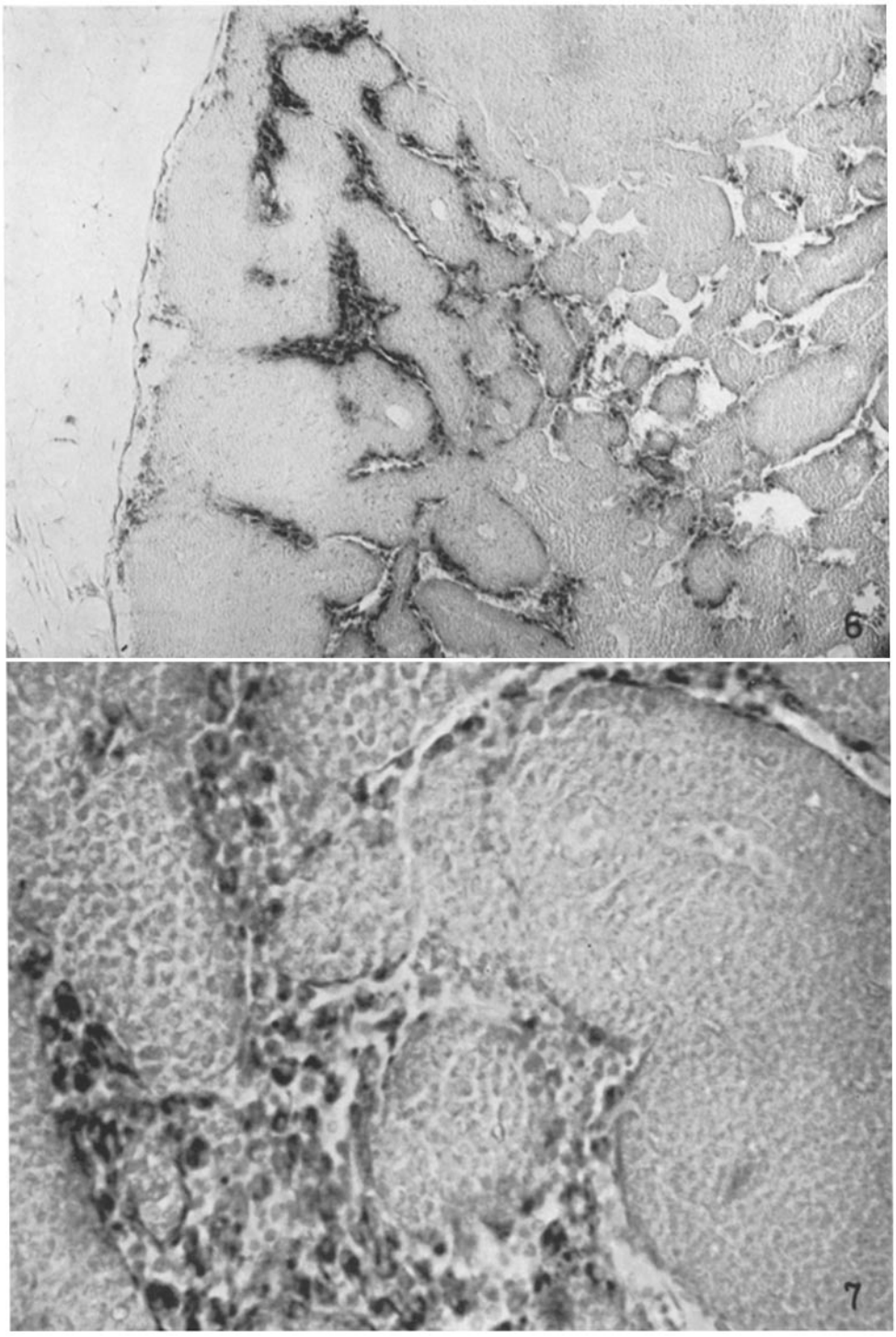


(Kruse and McMaster: Distribution and storage of blue antigenic azoproteins)

PLATE 34

FIG. 6. The photograph shows the region that is included in the small rectangle drawn in Fig. 4 *a*. The blue antigen, black or dark gray in the figure, stands out in the cells of the sinuses and endothelium against the light gray of the lymphocytes. × 90.

FIG. 7. A photograph of another section from the same lymph node. This section, too, was unstained. At this magnification, a black and white print does not show the sharp contrast seen in the actual section because the lymphocytes appear gray. Nevertheless the darker appearance of endothelia and cells in the sinuses indicates the presence of the blue antigen. It seems to lie within the cytoplasm of the cells. × 400.



(Kruse and McMaster: Distribution and storage of blue antigenic azoproteins)