THE PERSISTENCE IN MICE OF CERTAIN FOREIGN PROTEINS AND AZOPROTEIN TRACER-ANTIGENS DERIVED FROM THEM

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

(From the laboratories of the Rockefeller Institute for Medical Research)

PLATE 25

(Received for publication, March 12, 1951)

In previous work (1) the observation was made that some azoproteins injected intravenously into mice were still antigenic after storage in certain cells for several days. Experiments have since been undertaken to learn how long azoproteins or the native proteins from which they were derived can persist as antigens in the blood and tissues of mice. The matter is obviously of importance in relation to the larger problem of the mechanisms responsible for prolonged antibody formation.

Primary Phenomena

For the first experiments bovine γ-globulin[1] was utilized as antigen and conjugated, as already described (1), with a highly diffusible dark blue dye, echt-säure-blau,[2] to form an azoprotein designated as E-AP-G. As in the previous work this dye, injected as such intravenously into mice, was all excreted within a few hours, and during this period it failed to appear in granular form, or in vacuoles, within cells. The findings were very different with E-AP-G. After its injection blue material promptly appeared within various types of cells in many organs, chiefly in reticulo-endothelial cells, and remained within them in granular form for weeks.

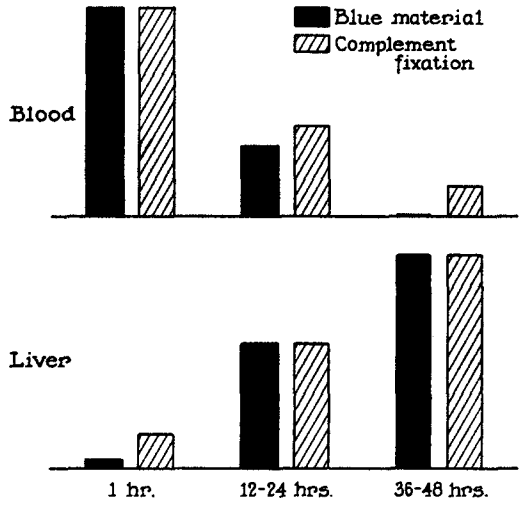
To extend these observations and to study the further fate of the antigens, mice of the Rockefeller Institute strain (1) were injected intravenously with 0.1 ml. of E-AP-G per 30 gm. of body weight, that is to say with about 3 mg. of protein in the conjugated form. Other mice received equivalent amounts of the unconjugated protein. Following these injections, at intervals of 1, 4, 12, 18, 24, 36, and 48 hours, 3, 4, 7, and 11 days, and at various longer intervals until the middle of the 4th month, pairs or groups of mice which had received the azoprotein were exsanguinated under ether or nembutal anesthesia. The color of their sera was compared with that of various dilutions of the azoprotein in tubes of similar size, and fresh tissues or fixed specimens, sectioned as already described (1) or cleared by the Splateholz (2) method, were compared by inspection, under the microscope, for their content of blue material. Complement fixation tests were made on the blood and on liver emulsions, obtained up to the end of the 3rd day, from some of the mice that had received either the con-

[1] Fraction II; Armour and Company, Chicago.

[2] Color Index No. 733.

jugated or the unconjugated protein, and precipitin tests were carried out with the sera by the usual methods (1).

The color changes taking place in the sera and livers during the first 3 days, when compared with the changes in the antigen content of the blood and tissues, showed that the blue matter stored in the cells was still the antigen, for that period at least, based on the findings of the paper already mentioned. Text-fig. 1 shows diagrammatically what was found in the blood and liver respectively at various times during the first 48 hours after injecting E-AP-G. After about 1 hour the blood had become intensely blue and its antigen content very high, as shown by the height of the black and the crosshatched columns respectively. The liver had in contrast little blue, and the antigen content of the liver emulsion was low,—no more than could be accounted

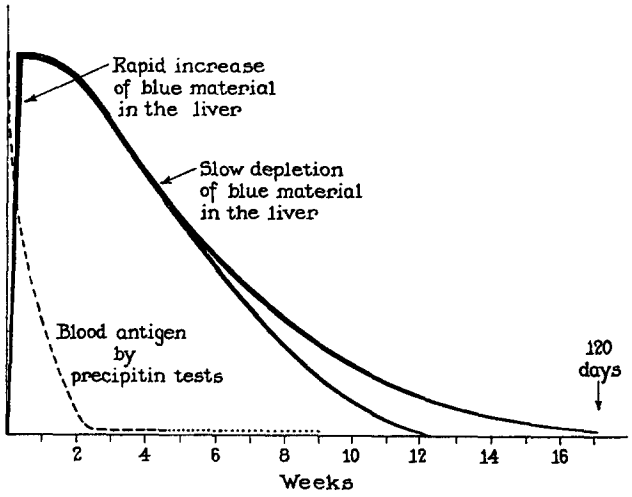


TEXT-FIG. 1. See text.

for by the amount in the blood within the vessels. During the next 12 to 24 hours the blood lost much color and its antigen content also fell rapidly, whereas the liver became intensely blue and its antigen content rose sharply. By the 36th to 48th hour all color had disappeared from the blood, to the naked eye, and its antigen content had fallen much further whereas the liver had attained to an intense blueness, which proved maximal, and its antigen content was far higher than that of the blood. It would appear from these facts and from those of the previous paper, already mentioned (1), that as the colored antigen disappeared from the blood it concurrently accumulated in the liver. Similar accumulations, but varying in intensity, occurred in other tissues. Despite these findings it could not be assumed that the blue matter seen in the cells after more than 2 or 3 days was still the antigen. Newer studies were now undertaken by other means—for reasons that will appear below—to find out how long the blue substance, whether antigen or not, can persist in the tissues of mice after injecting E-AP-G, and how long it is still antigen.

After a single injection of bovine γ-globulin or E-AP-G into the blood of mice, precipitin tests—carried out as in the previous work (1)—with the sera, days or weeks after the injections, disclosed a rapid fall in the blood antigen from its initial height. As Text-fig. 2 illustrates, schematically but not quantitatively, only minute traces of antigen were to be found in the blood after 2 weeks. The line of dashes in the chart depicting this fall has been made into a dotted one to indicate that, after several weeks, the tests showed only extremely faint traces of cloudiness such as might readily have been yielded by non-specific, interfering substances, not necessarily by antigen. On the 7th, 14th, and 21st days after injecting the mice once with antigen, precipitin tests for antibody failed to show its presence in detectable amounts. More will be said of this below.

By contrast, intravenous injections of E-AP-G led, as already mentioned, to a maximal storage of blue material in the liver and other tissues within the first 2 days. Thereafter this



TEXT-FIG. 2. See text.

blue material gradually disappeared. The heavy, descending, upper line in Text-fig. 2 depicts the slow depletion of color in the liver. The line has been forked to indicate that some color was found in every instance examined up to the beginning of the 13th week (85 days), and that in some instances it persisted for over 17 weeks (120 days). Color usually persisted a few days longer in the mesenteric lymph nodes.

Figs. 1 to 4 illustrate the retention of the blue material in the livers and mesenteric lymph nodes after 1 week and 2 months respectively. Fig. 1 is a photograph (× 350) of an unstained section, cut 20 μ thick, from a mouse's liver 1 week after injecting 5 mg. of E-AP-G. The dark specks in the picture are Kupffer cells and other endothelial structures in the organ stuffed with the blue material. Fig. 3 is a photograph (× 7) of the unstained, cleared, mesenteric node of the same animal, showing the blue material (gray to black in the picture) lying in the sinuses and trabeculae, as higher magnifications disclosed. Figs. 2 and 4, photographs of similar specimens taken from a mouse injected 2 months before with the same amount of E-AP-G, show less blue material in the liver and node. Tissues examined after longer periods showed progressively less and less blue until all disappeared between the 85th and 120th days.

Since the dye, echt-säure-blau, that was used to form E-AP-G does not, of itself, remain within cells, as already proven, the long persistence of the blue material indicated that dye, still coupled or combined with some protein or its products, had been retained within the tissues. It seemed possible that the colored material might still be the intact antigen, even after weeks, and the question arose: How could it be identified if it were present in the tissues in such minute amounts that its separation from other proteins by the ordinary procedures of chemical or serological identification was not practicable?

THE DEVELOPMENT OF A BIOLOGICAL TEST FOR THE DETECTION OF MINUTE TRACES OF ANTIGEN PERSISTING IN THE TISSUES OR BLOOD

It had been observed in the laboratory (3–5) that mice rendered anaphylactically sensitive by intraperitoneal injections of proteins displayed extraordinary vascular reactions in their ears microscopically, when challenged with a shocking dose of antigen. These reactions, which will hereafter be termed EVR (ear vascular reactions), occurred regularly in mice so weakly sensitized that they showed no other signs of anaphylaxis.

Plan of the Experiments

The extreme sensitivity of the EVR in actively anaphylactic mice made it seem likely that normal mice could be utilized in the following way to detect whether the persisting blue material was still specifically antigenic. The blood or ground up tissues of mice, to be called "donor" mice, which had previously received E-AP-G or plain bovine γ-globulin intravenously, were injected into the peritoneal cavities of other normal mice ("recipients") with the aim of sensitizing them. This could presumably be disclosed by eliciting the delicate ear vascular reaction (EVR) in either of two ways. If enough antigen had been absorbed to stimulate antibody formation, thereby rendering the recipients actively anaphylactic, the EVR should appear if shocking doses of antigen were given at the peak of sensitivity. Or one might attempt to elicit EVR in the recipient mice, 2 or 3 days after transferring the test materials, by injecting them with an appropriate antiserum. The appearance of the EVR, in the state of reversed passive anaphylaxis thus induced, would signify that antigen as such had persisted in the materials transferred from the donor mice, and that it had been absorbed by the recipients.

Experiments of the first sort, involving active anaphylaxis, failed, probably because the mouse forms little or no antibody when given only single injections of small amounts of certain antigens (6). Accordingly the second alternative was investigated further, the development, that is to say, of a detection test by the induction of reversed passive anaphylaxis.

The work divided itself into several steps. It was necessary first to learn whether mice, injected intraperitoneally with either E-AP-G or bovine γ-globulin, show the EVR in states of reversed passive anaphylaxis; and next whether the ear reactions are delicate enough to indicate the sensitization of recipient mice by the absorption of the small amounts of antigen that one might expect to transfer in the blood or organs from donor mice receiving antigen weeks or months before.

It seemed wise first to make the tests with the natural antigen from which the azoprotein had been derived (bovine γ-globulin) since, should the EVR not occur, there would be little purpose in carrying out further work with the colored tracer. Accordingly appropriate antisera were next prepared.

Preparation of Antisera.—Groups of rabbits were immunized against bovine γ-globulin by several courses, each of 3 daily intravenous injections, of 2.5 to 5 mg. of the protein, followed by rest periods of 4 days between each course (7). The animals were bled 5 or 6 days after the last injection. Other rabbits were given at least three intravenous injections of 2.5 to 5.0 ml. of 5 per cent aqueous bovine γ-globulin solution with intervals of 5 days between them. Nothing further was done to these animals for 3 months and then—to desensitize them—two or three subcutaneous injections of 0.3 ml. of the solution were made, within a few minutes, on the flanks. Five hours later each rabbit received an intravenous "booster" dose of 3.0 ml. of the antigen solution. Seven to 10 days later samples of blood were taken for precipitin tests, and the animals showing the highest titers were exsanguinated, with precautions for asepsis. The latter method yielded stronger antisera than the former. All the antisera were stored in 10 ml. lots in the refrigerator at 3°C., after the addition of merthiolate, 1 part in 5000.

Potency of the Antisera.—Practically all the tests described from here on were carried out with the two sera that yielded the strongest reactions. Since there was not enough of them for all the tests and for quantitative determinations of their antibody content as well by the usual methods (8–13), the potency of these sera was compared with that of a rabbit anti-bovine γ-globulin serum containing 0.3 mg. of antibody N per ml., kindly furnished by Dr. S. C. Bukantz of the Washington University Medical School. We are indebted to Dr. Walther F. Goebel of the Rockefeller Institute for the comparison which was carried out by a turbidimetric modification (14, 15) of the photronreflectrometer described by Libby (16–18). In this way the sera were found to possess approximately 0.72 and 0.57 mg. of antibody nitrogen per ml., respectively.

The Production of EVR in Reversed Passive Anaphylaxis.—Normal mice were injected intraperitoneally with solutions of bovine γ-globulin containing various amounts of the protein, ranging from 0.1 to 5.0 mg. One to 5 days later all received, under light nembutal anesthesia, intravenous injections of 0.1 ml. of one or the other of the antisera while an observer watched the smaller blood vessels in the ears of the mice under the microscope, by techniques to be described presently. In every instance vascular reactions occurred.

By another series of tests, which need not be detailed, the optimal conditions were determined for eliciting the sharpest EVR in mice that had received sensitizing doses of 0.1 to 5.0 mg. of protein intraperitoneally. These conditions, which were adhered to for the remainder of the work, were found to be met by an intravenous, shocking dose of 0.1 ml. of antiserum per 30 gm. of body weight, given 48 to 72 hours after the intraperitoneal, sensitizing injection.

The Sensitivity of the EVR.—Having established the fact that the EVR occur in reversed passive anaphylaxis, we attempted next to determine the sensitivity of the reactions in mice injected intraperitoneally with still smaller amounts of the antigen.

Bovine γ-globulin solutions were prepared in various concentrations ranging from 1000 to 0.5 μg. per ml. Upon several occasions each of these was injected intraperitoneally into groups of about five mice, in doses of 0.2 ml. per 30 gm. of body weight, so that the mice received amounts of the protein ranging from 200 μg. to as little as 0.1 μg. On the 2nd or 3rd days thereafter all were injected intravenously with 0.1 ml. of antiserum. With each series of tests normal mice were similarly injected. More will be said about them shortly.

It will suffice here merely to summarize the findings. The sensitized mice showed EVR roughly in proportion to the amount of antigen given. All those receiving more than 5 μg. of protein showed strong EVR. The majority of those given 5 μg. yielded postive reactions although about 15 per cent of them gave none. A similar state of affairs appeared in the mice given 2 μg. The detection of 0.5 to 1.0 μg. was obtained in more than half the trials, and in two mice out of five given only 0.1 μg. weak positive reactions appeared. Responses of similar delicacy were obtained in later work with mice injected with E-AP-G as the antigen to be detected.

It will be seen that the EVR served as an exceedingly delicate means for the detection of the foreign protein used in the experiments. It seemed wise next, before using the technique as a testing method, to study exhaustively the various non-specific vascular responses in the ear that might follow upon intravenous injection of foreign sera into normal mice and to compare them with the specific EVR, so that they might not be confused with one another.

Characteristics of the Non-Specific Vascular Reactions

Techniques for Observation.—The method for observing the minute blood vessels in the ears of mice, already fully described (3–5), was modified slightly. The animals, anesthetized by intraperitoneal injections of nembutal (0.4 ml. of a 1 per cent solution per 30 gm. body weight) lay as before in wooden molds with their ears spread out horizontally on white porcelain placques. The ears, lightly painted with paraffin oil, which greatly increased the visibility of the blood vessels, were brilliantly lighted by cooled, reflected light. In preceding work (3) the whole ear was observed under low power magnification (×15), but now a magnification of 100 times was used. Under these circumstances only a few vessels are under observation in a single field; and it was found best to survey the entire ear and pick out the most suitable field to be found in the zone lying about ¼ to ⅓ of the distance from the periphery of the organ to the base. In this regon of the ear the chief, central vessels have already branched at least three or four times, and with a fine ocular micrometer scale one can readily measure the caliber of at least one artery and vein. Besides the measured vessels many smaller ones and capillaries are included in the field.

For reasons which will appear below, it was found extremely important in all tests for the observer to familiarize himself with the rate of flow, in the field selected, of as many arteries, arterioles, veins, venules, and capillaries as possible, and especially to note the color of the tissues lying between the larger vessels. Micrometer measurements were taken at intervals of the vessels selected for that purpose, and the field as a whole was watched during the injection of antiserum and for 15 to 20 minutes afterwards. The precautions taken for injection of this latter will be outlined below.

Non-Specific "Injection Reactions" in the Ears of Normal Mice Receiving Serum Intravenously.—It is common physiological knowledge that intravenous injections of foreign, or even of homologous, serum can sometimes produce transient peripheral arterial constriction, especially if the serum is injected too rapidly, or if it is too cool or too hot. Intravenous injections of sera containing particles or clumps of bacteria, or which are cloudy for other reasons, nearly always produce vasomotor changes, and so too does the introduction of air. Since arterial constriction is one of the several specific blood vessel responses of sensitized mice (3), it was basically necessary to distinguish clearly between that of the non-specific "injection reactions" and that of the specific EVR. For this purpose, and also to learn how to reduce "injection reactions" to a minimum, normal mice were intravenously injected with rabbit and horse sera, either clear and sterile or cloudy and infected, in amounts of 0.1 ml.

per 30 gm. of body weight, at rates ranging from 15 seconds to 1½ minutes and at temperatures ranging from 20 to 50°C.

The cloudy sera produced arterial constriction more frequently than clear sera, but the rate of the injections and the temperature at which the sera were used were still more important. For example all sera, whether clear or cloudy, when thrown rapidly into the circulation, that is to say within 15 to 30 seconds, tended to provoke a brief arterial constriction in the ears. If given more slowly the incidence of reactions fell, or none appeared. All sera, clear or cloudy, whether they were employed at room temperature, or were cooler,—or too hot—gave rise to arterial constrictions frequently. To reduce them to a minimum it was found best to warm the syringe and injecting needle, loaded with clear sera only, in a water bath at 42°C., and to inject into the tail vein immediately, allowing as routine 40 seconds to 1 minute for the injection of 0.08 to 0.1 ml. Since the entire procedure usually required no more than 1 to 3 minutes the temperature of the injected serum must have remained close to body temperature. Under these circumstances some samples of sera gave no vascular reactions in the ears of normal mice, others produced arterial constriction with a frequency ranging from only 1 reaction in 10 animals to as many as 1 in 3. Cloudy or infected sera were not used for the experiments to be described here.

Under the optimal conditions outlined there appeared in the ears of all injected mice, whether normal or sensitized, an increase in the rate of blood flow during the injection and a paling of the color in the larger vessels, due no doubt to the increase in blood volume and the dilution of blood. By contrast, when "injection reactions" occurred, there appeared in addition brief arterial constrictions decreasing the diameters of the smaller arteries 20 to 50 per cent. These constrictions began, usually, about 20 to 30 seconds after beginning the injection, while it was still in progress. Prompt recovery was the rule after another 20 to 35 seconds. In some instances, however, the reactions began later, rarely as late as the 75th to 80th second, but even these "injection reactions" in normal mice never endured longer than the 2nd minute after the injection was begun. As will be seen below, the specific EVR were very different.

The Effects of Anesthesia: Pain Reactions.—If the animal is too lightly anesthetized, or if it is injected before the anesthetic has had ample time to act, movement of the ear upon the placque may cause arterial constriction, and slight contractions of the aural muscles may lead to changes in the contours of the ear vessels. Whenever either of these complications occurred the animals were discarded. It was found best, as routine, to make the test injection of antiserum 10 minutes after giving the standard intraperitoneal dose of nembutal. The free hand injection of the tail veins of the mouse is not always successful upon the first attempt. If it was necessary to make several attempts to enter the vein, or if the animal was too lightly under the anesthetic, the observer watching the ear occasionally saw partial contractions of some of the arteries or cyclic constrictions occurring rhythmically at intervals of 5 to 10 seconds. Whenever such reactions occurred the animals were discarded.

THE SPECIFIC EVR OF REVERSED PASSIVE ANAPHYLAXIS

Recognition of the specific EVR depends upon several phenomena, all of which may appear in differing intensity in strongly sensitized mice, while only a few or even only one, may be seen in the weakly sensitized animals. Mice sensitized with 5 to 10 μg. of protein or more usually showed all of the following phenomena.

Arterial Contractions.—There appeared first, during the injection of antiserum, the increase in the rate of blood flow within the arteries and the slightly lighter color of the blood, already mentioned as occurring during injections into normal mice. Then, in the tests on sensitized animals, a few or all of the arteries suddenly constricted, at times as early as 40 to 50 seconds after beginning the injection but usually after 1 or 2 minutes, sometimes after 3 or 4 minutes, or even after 6 to 12 minutes. The constrictions were general as a rule, but sometimes local ones occurred, either separately or superimposed upon the general spasm. Not infrequently the reactions were severe enough to completely obliterate some of the vessels. The ears blanched, and even when the spasms were relatively mild the blanching could be made out with the naked eye. Constriction endured for variable periods, depending on the severity of the reactions. Usually the maximum spasm, whether partial or complete, lasted about 1 minute, and then gradually during another 3 to 5 minutes the vessels returned to their previous calibers. More will be said of this below. Often when the constrictions began early and partial recovery had begun, a second series of spasms took place between the 6th to 12th minutes. This phenomenon never appeared in the "injection reactions" of normal mice.

Spasm of Veins.—In a majority of instances the veins also contracted, usually at the same time as the arteries, occasionally somewhat later, or rarely, prior to or even without the arterial spasm. Venous constrictions were not seen in the tests upon normal mice and they are believed to be pathognomonic of the specific EVR. Like the arterial spasms they were either general or local. The local constrictions were especially sharp, at times obstructing the veins. Obliterative spasm of whole veins, often seen in active anaphylaxis, appeared in the reversed passive form only when the reactions were extremely strong. Sometimes the spasms in the veins ceased when the arteries began to dilate, but in many tests they lasted 5 to 15 minutes longer and as a result the vascular bed became choked with blood.

In many of the tests, and during mild reactions especially, the walls of both arteries and veins appeared rugose, as though local fibrillary contractions had occurred, or as if constrictions of fibers in the outlying tissues or cutaneous muscles had squeezed the vessels from without. This too was not seen in the normal injected mice.

Early Changes in Blood Flow.—In most of the tests, shortly before the constrictions appeared, or during the spasm, or early in the period of recovery, the observer noticed a sudden slowing of flow. The blood cells became more clearly visible and they seemed to be more widely separated, an effect presumably due to plasma skimming at some constricted point in the artery lying between the heart and the microscopic field under observation. With the slowing of blood in the arteries, the venous flow slowed. During severe reactions stasis took place all through the ear. In milder reactions it occurred only near the ear's periphery, though within the field under study, while the arteries further toward the base showed a little flow and the larger veins became packed with cells. During still milder reactions stasis occurred in only one or two vessels while in the rest the flow of blood merely became slow.

In many experiments, especially those done upon animals that received very little antigen, the slowing of flow appeared without any constriction of arteries or veins in the microscopic field. It is important to record that sharp decreases in the rate of flow were never seen after serum injections into normal mice of the strain employed.[3] This phenomenon is a sign of a specific reaction.

Later Changes in the Rate of Blood Flow.—Usually in a few minutes the slow flow of blood began to quicken, and the blood cells in the arteries were no longer widely separated. The less severe the reaction, the sooner did the improvement take place, but often the flow remained slow for 15 to 30 minutes or more.

[3] Recent tests made with Swiss mice have disclosed the development of a reduced blood flow and a subsequent hyperemia in the ears of normal mice injected with serum. This strain is utterly useless for the tests here described, and doubtless many other strains of mice will also be found unsuitable.

Hyperemia in the Ears.—During EVR in active anaphylaxis hyperemia occurred (3), characterized by a slow and stately flow of blood in the widened vessels as recovery began, although the blood pressure remained at shock levels. The reversed passive anaphylaxis of the present work resulted in a similar hyperemia which seemed to develop in direct proportion to the degree of spasm in the veins, since it was especially severe in those instances in which the venous constriction endured after the arterial spasm had ceased. The capillary bed, venules, and larger veins became choked with densely packed blood cells and, as result, the ear appeared flushed to the naked eye. As a rule, the greater the amount of antigen given the mouse, the more intense the hyperemia. During severe or moderate reactions it made its appearance about 4 to 6 minutes after constriction began in the veins. During weak reactions it appeared later, but even in them it could still be seen at the end of the 15 to 20 minute period of observation. In some experiments in which venous reactions were not apparent the hyperemia was occasionally absent. Other mice, in contrast especially weakly sensitized ones, showed marked hyperemia although they evidenced very little spasm of either arteries or veins, or slowing of flow.

It is to be noted that the ears of normal mice, placed on porcelain placques as in these experiments and occasionally painted with oil, may show a mild degree of hyperemia. This phenomenon can be distinguished, however, from the one just described; it is far milder, and the rate of circulation is not decreased, nor do other vascular reactions occur.

Changes in the Blood Constituents within the Vessels.—Several authors (19–23) have reported cellular changes within the vessels of shocked animals. During anaphylactic shock in rabbits, Abel and Schenk (19) noted an increased adherence of leucocytes to the walls of the blood vesse.s and the formation of clumps of white cells in the blood stream. Knisely and his coworkers (22) have described in full the characteristic appearance of "sludged blood" with its clumping and packing of red cells within smaller vessels, both during shock and in certain infections.

These phenomena were prominent in the majority of our experiments, and they did not appear in normal control mice injected with the antisera.

It has been necessary to go into details in order to make plain the unmistakable character of the specific EVR. Fortunately most of the phenomena of specific EVR in sensitized mice are absent from the "injection reaction" occurring in normal animals; for example the constriction of veins, slowing of flow, stasis, packing of red cells, congestion of the capillary bed, sludged blood, clumping of white cells and marked hyperemia. As mentioned above, mice, sensitized with several μg. of protein as antigen, showed all or many of these signs, and no questions arose concerning the specificity of such strong or moderately strong reactions. Further, signs of shock, such as respiratory difficulty and ruffling of the fur, were often concurrently observed although they are not part of the EVR.

The Characteristics of Weak, Specific EVR.—In the course of the work it became necessary to deal with small amounts of antigen, as in the attempts already mentioned, to determine the least sensitizing dose of bovine γ-globulin. The tests of this sort were repeated later for verification of the previous findings, and finally in a few of the experiments yet to be described, the presence of notably small amounts of antigen in the blood and tissues of mice previously injected with it was sought by transferring these materials to normal recipient mice, which were then tested for the appearance of EVR.

In all such experiments as the sensitizing dose of antigen decreased the EVR became weaker. One after another the distinguishing phenomena disappeared until, during very weak reactions, only a single one remained,—which was not always the same one. In some instances it was spasm of the veins, in others stasis, or sludging of the blood, or some other of the characteristic signs of specific EVR. But frequently the last or only sign to remain was spasm of the arteries. Since the latter also appeared in "injection reactions," it seemed best either to rule out as negative all tests that showed only arterial constrictions or to find some way to distinguish specific from non-specific ones.

The choice was made as will now be discussed. At first the timing of occurrence of the arterial spasms during the specific EVR, as opposed to that of those appearing during the non-specific reactions, seemed to offer a satisfactory guide to a differentiation of the two. As already pointed out, the non-specific arterial spasms came on earlier as a rule than the specific ones, 20 to 35 seconds after the beginning of the injection. Recovery was usually complete within 90 seconds and not later than the second minute. In the sensitized mice the spasms began later, usually about 1 to 2 minutes after beginning the injection, or not for 3 to 4, or even 6 to 12 minutes. All these late occurring constrictions were manifestly specific in character. As also mentioned above, another phenomenon, not seen in injected normal mice, often appeared even in weakly sensitized animals—a second period of arterial constriction taking place after the first had subsided. Nevertheless, in some of the sensitized mice constrictions of the arteries began as early as 40 seconds after beginning the injection, that is to say, during the period in which non-specific "injection reactions" took place. Of course, such reactions could not be distinguished from "injection reactions" if they occurred in the absence of all other signs of specific EVR, or if they failed to persist for several minutes.

Criteria Adopted to Differentiate with Certainty Weakly Positive, Specific EVR from Non-Specific Responses. (End-Point for the Antigen Detection Test).—The finding that both non-specific and specific arterial constrictions can overlap in time rendered it advisable to rule out arterial constriction, unaccompanied by other signs of EVR, as conclusive evidence of a positive response, regardless of the time of its occurrence, although it seemed likely that the ruling might put some weakly positive reactions among those classed as negative. In practice, however, this necessity could usually be avoided. In all the experiments, six to nine test mice were injected with each dose of antigen employed or with the transferred blood or tissues from donor mice. Varying numbers of normal control mice were also injected. Under these circumstances, when the EVR were weak, some of the half-dozen presumably sensitized animals usually showed one or more of the pathognomonic signs of specific EVR (sludging of blood, stasis, etc.), often accompanied by constriction of the arteries too. Others of the same group showed at times only arterial spasm, and then were set down as negative, as of course were those showing no reactions. But it was the positive instances that counted. Whenever two or more of the test animals showed some of the vascular reactions, other than arterial constriction, the detection of antigen was considered established. If, on the other hand, only arterial constrictions—or no reactions—were observed the test was considered negative. The controls either yielded no reactions at all, or the occasional "injection reactions" typical for the particular batch of antiserum used in the test.

Enough has been said to indicate that among groups of weakly sensitized test mice

the constriction of arteries seems to be a slightly more delicate sign of sensitivity than the others. Nevertheless, in view of what has just been said, it was never accepted as a sign of specific EVR in the absence of the other distinctive phenomena. It has seemed better to err on the safe side rather than risk the classification of some non-specific reactions as positive.

In passing it should be noted that in hundreds of tests made upon sensitized mice we have observed from time to time non-specific arterial spasms preceding the specific ones in the same mouse. In some cases, only a few, the two occurred separately, and recovery from the first was followed after an interval by the onset of the second, but at times the reactions overlapped; an early constriction of the arteries took place with partial recovery for a few seconds, and then a sudden, more severe and enduring constriction before the vessel had returned to its original width. Phenomena of this sort never appeared in serum-injected, normal mice, while furthermore the double constriction was usually accompanied by some of the other signs of specific EVR.

TESTS FOR THE PERSISTENCE OF ANTIGEN

Having established the criteria to be used for the detection of antigen by means of specific EVR, we next attempted to determine how long several antigens persisted in the blood and certain tissues (the livers and mesenteric lymph nodes) of mice injected with them. It seemed wise at first to study the persistence of a natural protein, rather than that of the blue tracer, since should the natural antigen disappear from the tissues much sooner than had the blue material in our earlier experiments (85 to 120 days), then studies with the latter would be unprofitable. Accordingly we used the native protein from which the tracer was derived, namely bovine γ-globulin.

Mice of 22 to 26 gm. were injected intraperitoneally with 0.1 ml. of a 5 per cent solution of bovine γ-globulin (approximately 5 mg. of protein). At intervals of 2, 5, 8, 26, 32, 47, 56, 67, 76, 101, and 140 days, at least two and usually more such donors were anesthetized with nembutal and exsanguinated, under aseptic conditions, by snipping the large vessels in the opened chest cavity. After the blood had been pooled and citrated the livers were removed, stripped of their gall bladders and ligaments, pooled, and ground aseptically with 2 volumes of saline in a TenBroeck grinder. In some experiments the spleens or mesenteric nodes were also taken, pooled, and ground.

Normal recipient mice, lightly anesthetized, were then injected intraperitoneally with the pooled, whole, citrated blood procured as described, each receiving either 0.6 or 1.2 ml., which is one-sixth or one-third of the calculated total blood of a 30 gm. mouse. Depending upon the number of donors used, that is to say on the amount of pooled blood, six to nine recipient mice were injected. Next, the liver emulsion was injected intraperitoneally into other recipients in amounts equal to one-sixth or one-third of one whole liver. In this case too there were six to nine recipients, depending upon the number of donors. When ground spleen or mesenteric lymph nodes were transferred, each recipient got tissue amounting to an entire emulsified spleen or two or three lymph nodes. Similar transfers of blood, liver, and other tissues, from normal mice or animals injected with antigenic materials other than bovine γ-globulin, were also made to recipients as control tests upon several occasions. These recipients gave negative results. In some of the first tests half of the recipients were injected with 500 units of penicillin

G, together with the tissue extracts. This precaution was soon found unnecessary; practically all of the recipients stood the transfers without subsequent infection.

Forty-eight hours after all this was done some of the recipient mice were anesthetized and injected intravenously 10 minutes later with 0.1 ml. of the antibovine γ-globulin rabbit serum, to test for the appearance of EVR. In most of the experiments the number of recipients was too great to allow all of them to be injected and observed in a single day. Under these circumstances the tests were begun on the 2nd day after the transfer and continued throughout the 3rd day. No differences were noted between the animals studied on different days.

One other point deserves mention: in the tests done after many days, as will be seen, all or most of the recipients showed no EVR. To determine whether the lack of response in these instances was due to the absence of antigen in the transferred materials, or whether the mice had simply failed to exhibit EVR for some other reason, many of them—since they had already received their shocking dose of antiserum—were reinjected a few minutes later with antigen, using 0.1 ml. of a 0.2 per cent solution of bovine γ-globulin. All, without exception, reacted with very severe shock, sometimes fatal. Clearly the initial negative findings indicated no lack of responsiveness on the part of the mice, but rather an absence of antigen in the transferred materials or, perhaps, its presence in amounts too meager to be detected. More will be said presently about tests of this sort.

Findings

Presence of Antigen in the Blood.—The transfer of blood from the donors killed 2, 5, and 8 days after injection with antigen sensitized the recipients so strongly that the subsequent injection of antiserum produced severe shock. Even in an experiment made on the 26th day, strong EVR appeared in all six recipients; intense spasm of both arteries and veins, heavy sludging of blood, clumping of white cells, slow flow, and stasis in many vessels. Some of the mice also exhibited respiratory difficulty. When blood under test had been taken after a month, however, the responses became weaker. For example, in an experiment made on the 47th day only one recipient out of six showed moderately severe EVR, with both arterial and venous spasms, sludging of blood, a very slow blood flow and a delayed secondary constriction of many vessels after 6½ minutes, followed by severe hyperemia. Three of the others showed weaker reactions: arterial constrictions in all, slowing of flow in two, and moderate hyperemia in all. The remaining two recipients gave negative reactions. When the next test was made on the 56th day, two of the eight recipients were given twice the usual amount of blood, that is to say one-third instead of one-sixth of the calculated entire blood of a 30 gm. mouse. Nevertheless only very weak EVR appeared. Arterial constrictions in half of the recipients reduced the caliber of the arteries to 40 per cent of their original diameter, and in one animal the very small arteries (not arterioles) became obliterated. Although these spasms occurred at the proper time for specific reactions and endured in four of the mice for 4½ minutes after beginning the injection, the entire test would have been ruled negative had not the following phenomena also been seen. Two of the recipients showed secondary arterial spasms beginning after 6 and 8 minutes respectively, and followed by hyperemia which was strong in one of the mice and moderate in the other. In both instances the veins constricted and blood flow became slow. One of the two mice, an animal that had been given the larger

injection of blood above mentioned, also showed sludged blood and clumped white cells. The remaining recipients and four normal control mice injected with the same antiserum yielded no reactions. In four further experiments made on the 67th, 76th, 101st, and 140th days, none of the recipients exhibited any reactions whatever, not even "injection reactions," although in each test two were given twice the usual amount of blood.

From all this it can be said that, under the conditions of these experiments, minute traces of bovine γ-globulin seemed to persist as antigen in the blood of donor mice for as long as 56 days. Further, it is possible that minute traces might be demonstrable for a further unknown period of time if a sufficiently sensitive test could be devised to detect them.

The Persistence of Antigen in the Liver.—The tests with liver tissue gave markedly different results. Recipients of amounts equal to one-sixth of a liver, as procured from donors killed on the 2nd, 5th, and 8th days, showed strong EVR, though often not as severe as those of the mice that received pooled blood from the same donors. Later, however, the stronger EVR took place in the recipients that got liver tissue. For example, as just mentioned, the reactions obtained in recipients that got blood on the 47th day were weak and two of the animals yielded negative EVR, whereas all the mice receiving pooled liver tissue from the same donors showed moderately severe arterial and venous spasms. Sludging of blood and marked slowing of the circulation appeared in four of the six recipients and hyperemia in five. Again, in the test made on the 56th day, in which transferred blood yielded very weak responses, all of the mice receiving liver showed moderately severe reactions. In this test for the first time some of the donors were given twice the usual amount of liver, that is one-third of an entire organ. The reactions were no stronger than in those which got but one-sixth. Again, as mentioned above, the blood of donors transferred on the 67th day failed to produce any reactions. On the other hand, all but one of six liver recipients had positive EVR; four gave moderate reactions, that is to say not only arterial and venous constrictions but at least two of the other specific signs such as stasis, slow flow, sludging, or hyperemia. The fifth animal developed both arterial and venous constrictions, beginning 3 minutes after the injection of antiserum and enduring for four minutes more.

A test made on the 76th day gave similar results—negative EVR in recipients given blood, but positive EVR in all that got liver. The reactions were about as strong as those in the preceding test; three of the six liver recipients had spasms of arteries and veins, slowed blood flow, and sludging, while in the others spasms of both arteries and veins began 1¼ to 2 minutes after the beginning of the injection and lasted to the 4th to 5th minute. In this experiment antiserum was also injected into five normal mice. Four showed nothing, the fifth one displayed a non-specific "injection reaction" appearing at the 27th second and lasting only to the 58th after beginning the injection.

At the 101st day transfer of blood to nine recipients, and liver tissue to nine more, from four donors, led to no true EVR in the blood recipients, although one showed a 30 second, brief "injection reaction." Among the liver recipients the EVR were clearly positive but weak. Both arterial and venous spasms occurred in two with

slight sludging of blood, some slowing of flow, and hyperemia. A third mouse gave a similar reaction, with partial stasis besides, in about half the vessels in the ear. The arterial constrictions in these three mice endured 3¼ to 4 minutes, too long for "injection reactions," and in one of them a second constriction took place at the 6th minute. Four of the recipients showed only partial slight arterial constrictions, classified as negative, and two gave no reactions. In this experiment two of the mice had been injected with pooled liver tissue equivalent to two-thirds of an entire organ, and the others with half the amount. No differences were observed that could be attributed to the amount of transferred tissue. Normal control mice injected with antiserum yielded one "injection reaction" in five animals.

On the 140th day transfers of blood, liver, spleen, and mesenteric nodes were carried out from four donors to fourteen recipients. Large amounts of liver—two-thirds of a whole organ—and blood (half the estimated blood volume of one mouse) were given to some of these recipients to allow more opportunity for antigen to be absorbed. The others received the equivalent of one-third of one liver or one-third of the blood of one mouse. Two and 3 days later every test without exception was completely negative. There was not even a single "injection reaction." A finding like this, so completely negative, goes far to show that the mild or weak reactions found at the 101st day were truly positive.

From all the foregoing it is evident that, following intravenous injections of 5 mg. of bovine γ-globulin into mice, minute traces of antigen seemed to persist in the livers of the animals for 101 days and in the blood for 56 days. With the passage of time the detection tests became progressively weaker, indicating a slow loss of antigen from its storage site, a phenomenon that might account for the presence of the minute traces of antigen persisting so long in the blood.

As the experiments progressed and the tests for the detection of antigen remained positive longer than had been anticipated, it seemed important to ascertain whether false positive tests might be brought about by mechanisms other than the reaction of transferred antigen with the antibody of the injected antiserum.

Consideration of a Factor Possibly Accountable for the Production of False EVR.—As already mentioned, and as will be fully reported in a subsequent paper, precipitin tests on the blood of donor mice injected with bovine γ-globulin showed no detectable antibody. The donor mice, under the conditions of the experiments, were poor antibody formers. Nevertheless the question arose: Could the strong anti-bovine γ-globulin rabbit serum contain, coexistent with antibody, enough antigen to react with such antibody as might have been formed in the donor mice and brought over to the recipients when blood or tissues were transferred to the latter? If so the resulting reaction might augment if not cause EVR. Tests were made to determine the point.

Tests for the Presence of Antigen, Coexistent with Antibody in the Antisera.—It has long been known that rabbits immunized with a foreign serum may yield antisera which react, when mixed together, to produce a precipitate. At first it was assumed that a coexistence of antigen and antibody in each serum permitted the antigen of one to react with the antibody of

the other, or vice versa. Later others, denying this possibility, explained the phenomenon by the assumption that all sera contain a number of antigens (24–26). If, during the immunization of two rabbits, one of these antigens failed to produce antibody in one of the animals, then the resulting antiserum could contain traces of that antigen. If the other rabbit produced antibody to that antigen, then, on mixing the two antisera, the antigen in the first would react with the antibody in the second. The problem need not be further discussed since Naylor (27) has reviewed it fully and shown the latter supposition to be apparently correct.

The antisera used in our experiments were not produced by the injection of whole serum but instead with a relatively pure antigen, bovine γ-globulin, fraction II. Since, however, it may contain several antigenic substances, tests were made to ascertain whether a state of affairs like that just discussed might have influenced the findings.

In a first test for precipitation the two antisera used throughout this work, as also a third less potent one, were mixed in pairs in the proportions necessary to include the zones of antigen and of antibody excess. No clouding took place, showing that traces of antigen, if they had coexisted in these sera along with antibody, were not great enough to yield positive precipitin reactions.

It seemed best to test further by means of the sensitive EVR themselves. To accomplish this end a finding already mentioned above was utilized. It may be recalled that in several of the tests made upon mice sensitized with minute amounts of antigen the EVR failed to appear when the animals were injected with antisera, but these same animals, when reinjected with minute amounts of antigen to test whether or not they were reactive, developed strong EVR and severe shock. Indeed, in work to be reported later, it appeared that normal mice intravenously injected with small amounts of antigen could be thrown into severe shock by reinjecting them a few seconds later with antiserum, or *vice versa*. This phenomenon enabled us to test, in the following manner, whether or not traces of antigen in the antisera were present in sufficient amount to react with transferred antibody in the recipient animals and produce EVR although the serological tests for it had been negative.

Fifteen normal mice were injected with one of the antisera used throughout this work, then a few minutes later with the other, and finally with a third specimen of antiserum. No EVR appeared. Obviously, if traces of antigen were present in any of the antisera, they were not there in sufficient quantity to produce EVR.

In the course of the experiments another type of control test was used. Early in the work—when positive EVR had just been found after the transfer of material from donors on the 26th and 32nd days, and the possibility was first recognized that transferred antibody might be reacting with antigen traces in the antiserum to yield false EVR—evidence for the transfer of antibody from the donors to the recipients was sought directly by means of EVR tests. This was deemed wise in spite of the fact already mentioned that sera of donor mice, injected but once in the usual way with antigen, yielded negative precipitin tests for antibody at the 7th, 14th, and 21st days after injection. Accordingly, in the transfer tests done on the 47th day and in all those performed thereafter, several recipients were tested for the transfer of antibody by injecting them intravenously with antigen instead of with antiserum. For the purpose 0.1 ml. of 5 per cent bovine γ-globulin was used, either undiluted or at various dilutions ranging from 20 to 100 times. No EVR appeared. In every instance, however, the mice were reinjected a few minutes later with antiserum, and severe shock developed in every case, showing that the mice were very reactive. Clearly in these tests, made 47, 56, 67, 76, 101, and 140 days after injecting the donors, there was not enough transferred antibody to react with antigen in the various dilutions purposely offered to test for its presence.

These experiments demonstrated plainly that the EVR obtained in the routine tests by injecting antiserum could not have been produced by traces of antigen in the sera reacting with transferred antibody, although such traces might have been present. In work to be reported later the fate of antibody in the donor and recipient mice will be discussed further.

The Persistence of Azoprotein Antigens

The foregoing concerns itself with the persistence of bovine γ-globulin as antigen in mice. What can be said about the persistence of the colored azoproteins? Are they destroyed more or less readily than uncoupled proteins?

It will be recalled from experiments mentioned at the beginning of this paper, that mice injected with E-AP-G continued to harbor visible traces of blue material, whatever its nature, for 85 to 120 days in the liver and mesenteric lymph nodes. Since the tests for the protein, as antigen, from which E-AP-G was derived (bovine γ-globulin), were still positive after about the same length of time, 101 days, there was presumptive evidence, but by no means proof, that the blue material remained antigenic as long as it could be seen in tissues. Direct tests were obviously necessary to learn whether transfers of liver or other tissues from mice previously injected with azoprotein, would result in positive EVR reactions throughout the period in which the blue coloration remained visible.

Since the presumptive evidence, just mentioned, had been obtained with globulins, it seemed wise to make the comparative tests with a different protein, an azoalbumin. In so doing one could not only throw light on the problem as posed but could also learn whether different proteins persist for different periods as antigens. In addition by injecting some of the donor mice with the azoalbumin and others with the uncoupled protein from which it was derived, one could learn whether both yielded antigen detection tests for the same length of time.

For these purposes human serum albumin was coupled to the same dye, echt-säure-blau, that had been linked to globulin, resulting in a new azoprotein.

E-AP-HA.—A 25 per cent solution of human serum albumin, Red Cross Blood Bank, batch number 12618 (Merck and Co.), was diluted to 5 per cent with saline and coupled to diazotized echt-säure-blau by the methods already described (1), using of course the proper proportions to insure diazotization. The crude azoalbumin was then purified by six precipitations in alcohol, as also previously described, yielding a relatively pure echt-säure-blau-human albumin solution to be designated as E-AP-HA. This solution contained about 3 gm. of protein per 100 ml. and had the color equivalent of a 0.3 per cent solution of the dye as such.

Normal mice, 22 to 26 gm. in weight, were then injected intravenously, all on the same day, with 0.15 cc. of the E-AP-HA per 30 gm. of body weight. They were to serve as donors and hence they received approximately the same amount of dye-protein as the mice described earlier which got the azoglobulin, E-AP-G. At the same time an equal number of donor mice were injected with equivalent amounts of plain human serum albumin. At weekly intervals thereafter blood and ground liver and mesenteric lymph nodes were transferred to normal recipients as in the previous tests.

It soon appeared that the blue material was disappearing much more rapidly from the tissues than it had from the mice which had been injected with E-AP-G, although

the animals had received equivalent amounts of the azoproteins. Both the azoalbumin and the uncoupled albumin elicited EVR in the recipients but the response became weak sooner than in the earlier tests with bovine γ-globulin. The transferred blood elicited only weak reactions by the 14th day, and the last positive reactions from donors that received either the azoalbumin or the plain albumin were obtained on the 21st day. All the tests with transferred blood were negative a week later.

The amounts of colored matter in the livers of the azoalbumin-injected donors decreased so rapidly that but little blue material was left by the 22nd day, no more than that in the livers of mice 75 days after receiving the blue azoglobulin. The recipients getting ground liver tissue from these donors, injected with azoalbumin 22 days before, showed much weaker reactions than the mice of previous experiments that had received similar tissue fron donors injected with azoglobulin after the same time interval. By the 36th day the livers of the azoalbumin-injected donors contained almost no blue though the mesenteric lymph nodes held slightly more. One-third of the recipients that got liver tissue then showed every weak but positive EVR, but the mesenteric nodes elicited very slightly stronger reactions. Similar findings were obtained with tissues taken from the donors injected with the plain albumin.

Of far more interest were the findings from transfer experiments done after 44 days. The livers of the azoalbumin-injected donors, when examined in the gross or under low magnification (×20), were apparently free from blue color. The mesenteric lymph nodes of these animals on the other hand held traces of blue that were visible in the gross. The recipients getting the apparently colorless liver tissue yielded no EVR, whereas those injected with the tissue of the faintly colored mesenteric nodes showed positive EVR. Liver tissue from the donors that got albumin as such gave negative tests on the 42nd day.

In another experiment 14 donor mice were injected with the azoalbumin in the usual amount. Six weeks later some of these mice were examined, and again, the livers were apparently free from blue material while the mesenteric nodes held small amounts. The liver tissue, transferred to recipient mice, gave negative antigen detection tests whereas the transferred mesenteric nodes yielded faintly positive EVR.

Under the conditions of these experiments the tissues containing blue material yielded positive tests for antigen as long as the blue color remained visible in the gross in them. Moreover the azoalbumin and the albumin as such, from which it had been derived, yielded positive EVR during similar periods. It became evident that different proteins—human serum albumin and bovine γ-globulin—apparently persisted, as antigens, for different periods of time.

Finally, it was notable that following injections of azoalbumin the blue color disappeared in the gross from the liver in 42 days and from the mesenteric nodes after a few more days, whereas azoglobulin remained visible in these organs for 85 to 120 days, in spite of the fact that the same dye had been coupled in both series of experiments. Had the color in the liver been due to dye, split off from the protein to which it had been coupled initially and either unchanged within the cells or bound in some way to the cellular proteins of the mouse, then its persistence should have been the same in both cases. But not

so. The persistence of the colored material resembled that of the native proteins from which the azoproteins had been prepared, as shown by the tests for the persistence of globulin and albumin as such. This observation, taken with the other findings, seems to justify the conclusion that as long as the blue material could be seen in the tissues in the gross, or at low magnifications, it was still antigenic.

After these tests had been completed, sections of the livers, which were apparently color-free to the naked eye or to low-power magnifications and which yielded negative EVR in recipient mice, were seen under the microscope at magnifications of × 100 or more, to contain a very small proportion of Kupffer cells and endothelial cells still filled with blue material. It would appear that traces of antigen, too small to be detected by the EVR tests, were still persisting.

DISCUSSION

Many workers have studied the fate of antigens injected into various animals and into man, and most of them have reported that these persist for a much briefer period than we have found to be the case. Nevertheless a few workers have noted the persistence of antigens for periods sufficiently like those here reported to indicate that longer persistence might have been found if sufficiently delicate methods had been available to these experimenters.

In 1900, Ransom (28, 29) found diphtheria antitoxin still present in the blood of dogs 4 weeks after intravenous injection. Coca and Grove (30) reported that sera of patients sensitive to the protein of timothy grass were still present where injected into normal skin 4 weeks previously, but none of these authors sought for longer persistence. Later Weil (31) injected anti-horse rabbit serum into guinea pigs and succeeded in shocking them 70 days later with plain horse serum. Further tests indicated that the antigen (the anti-horse substance) was not in the blood but fixed on or in the tissues. In 1918 Longcope and Rackemann, studying the mechanisms of serum sickness, showed by precipitin reactions that horse serum persisted for 39 days (32) in the blood of a patient who had received 270 ml. of the serum, and shortly thereafter Longcope and Mackenzie (33) found evidence of an even longer persistence of the antigen. The next year Mackenzie and Leake, using similar methods (34), showed that horse serum persisted in the blood of two patients, who formed little or no antibody, for 52 and 67 days, whereas in those who formed antibody well it lasted not nearly so long. These observations bear upon the long persistence of antigen in our mice since, as already mentioned, these animals seemed to form little antibody when injected with antigen only once.

More recently Libby and Madison (35) have studied the fate of radioactive tobacco mosaic virus injected into the bodies of mice already immunized with the non-radioactive protein. They concluded that it persisted in the body for about a month. Perhaps a longer persistence would have been found if the mice had not been previously immunized. Pressman and his associates (36–42), injecting various radioactive

iodinated (I^{131}) antibody globulins into mice, as tracers, found that rabbit anti-mouse-kidney globulins, labelled in this way, were taken up by the kidneys in high concentration and by the livers and other tissues to a lesser extent. The radioactivity carrier, presumably the radioiodinated antibody, had a long half-life of 20 days in the kidney. It disappeared from the liver more rapidly at first, but later the loss took place more slowly. These findings indicate that traces of these proteins may endure in these tissues for months, probably, as Pressman suggests, because of a failure of the tissues to metabolize them. Still more recently, Pressman, Eisen, Siegel, Fitzgerald, Sherman, and Silverstein (41) have reported that proteins labelled with S^{35} are taken up non-specifically by the kidney and liver to a greater extent than radioiodinated proteins, and are retained longer.

In contrast to these findings others have reported relatively shorter periods of antigen persistence. In 1930 Topley (43), investigating the role of the spleen in the formation of antibodies, presented evidence for the take-up of antigen by that organ. After 24 hours much was present; by the 15th day little remained. Hawn and Janeway (44), and Schwab, Moll, Hall, Brean, Kirk, Hawn, and Janeway (45) working with serological methods found globulins and albumins persisting in the blood of rabbits for 2 and 3 weeks respectively. Dixon, Dammin, and Bukantz (46), using bovine γ-globulin labelled with I^{131}, and Knox and Endicott (47), employing bovine serum-albumin similarly labelled, found them persisting in blood of injected rabbits for only about 8 days or slightly longer. In recent months Dixon, Bukantz, and Dammin (48) with Talmage (49–51) have again obtained findings like those they had noted (46), and they report further that iodinated bovine γ-globulin was not retained by the tissues of rabbits, whereas radioactivated iodinated bovine γ-globulin, coupled to diazotized *p*-aminobenzoic acid (48) or R-salt-azo-biphenyl-azo radioactivated iodinated bovine γ-globulin (49), was retained for brief periods. The period of retention was far shorter than that of E-AP-G or E-AP-HA in the tissues of mice, as found in the present work. Very recently, too, Coons, Leduc, and Kaplan (52), using their fluorescein-isocyanate-antibody-antigen reaction, as an indicator, report that certain protein antigens can be found for only a few days at most, after their injection into mice. Gitlin, Latta, Batchelor, and Janeway (53) found the half-lives of bovine serum albumin and γ-globulin in the blood of rabbits, after intravenous injection, to be about 5 days, and the half-lives of their azoprotein derivatives only 1½ to 2 days. However, the curves would indicate that traces of these proteins might be found in the blood after much longer periods. In contrast to several of the findings just cited, it is to be recalled that Pressman, who worked with iodinated proteins found them persisting, as already mentioned (36–42), for several weeks in liver and kidney cells of mice.

Are species differences accountable for the contradictory evidence? In the present work and that of Pressman, mice were used, whereas most of the other experiments, except those of Coons, Leduc, and Kaplan (52), were carried out on rabbits. Talmadge, Dixon, and Bukantz have noted (51) that the speed of the disappearance of I^{131}-tagged γ-globulin from the blood of rabbits is an indication of the development of the immune response. In the present experiments, as already mentioned, mice injected only once with antigen formed no

antibody detectable in the serum by precipitin tests. Since the rabbit seems to form antibodies far better than the mouse (6), the difference may account for the longer persistence of antigen in the latter.[4]

Some, but not all, of the contradictions mentioned above can be accounted for by differences in technique. The methods used to extract tissue for tests of the persistence of antigen leave much to be desired. For example extracts of livers, removed only 1 to 3 days after injecting antigen into mice, in the doses used here, have failed to give positive precipitin tests (1) because the antigen remained with the tissue debris. It could not be demonstrated in the clear supernatant fluid, but positive complement fixation tests were obtained with the liver suspensions as such whenever much antigen was present. Ordinary serological methods (precipitin reactions) for the demonstration of antigens in the blood indicated, as shown in Text-fig. 2, that there seems to have been an almost complete disappearance of antigen from the circulation after 14 to 17 days, a period not very different from those reported by some of the authors just mentioned. Only by means of the EVR tests could a longer persistence of antigen be detected. Furthermore, the detection of antigen in the blood by serological means is generally carried out with serum. In the present work, by contrast, the recipient mice were given whole blood, with its red cells, polymorphonuclears, lymphocytes, monocytes, and platelets. Was the antigen persisting in, or adsorbed upon, any of these? Another possibility is that the mouse extracts traces of antigen from ground tissue or whole blood, injected into its peritoneal cavity, better than they can be extracted *in vitro*, and that in so doing, without any considerable loss of antigen, it renders itself hypersensitive. If this is true, it would seem reasonable to suppose that a method still more delicate than that depending on EVR might disclose an even longer persistence of antigen. In support of this supposition it may be recalled that, in testing for the sensitivity of EVR in mice injected with 5 μg. of protein, or less, some of the animals which got 1 μg. of antigen, or even as much as 5 μg., gave negative findings while others which got only 0.5 μg. or 0.1 μg. were positive. Findings of this sort would seem to indicate that minute amounts of antigen might persist in mice longer than we have been able to detect them, and yet be present in sufficient amounts to be immunologically active and to incite prolonged antibody formation.

The persistence of viruses has been looked upon as a special case because of their ability to maintain themselves within the body. However, the persistence of pneumococcus polysaccharides deserves mention. Felton, Prescott, Kaufmann, and Ottinger (54), and Felton (55) found polysaccharides in Kupffer cells and at other sites in mice 6 months after they had been injected with the substance. Coons and Kaplan (56) conjugated pneumoccus Type III polysaccharide antibody with fluorescein-

[4] Work is now under way on this theme.

isocyanate, and at various intervals after injecting rabbits with the polysaccharide as such introduced the fluorescein-antibody into the blood stream. The fixation of the antibody in various tissues was used as an indicator of the whereabouts of the remaining antigen. Fixation was found still to take place 6 months to a year after the injection of the polysaccharide, and Coons and Kaplan concluded that the polysaccharide had persisted that long in the body.

The distribution of our antigens in the tissues was much like that reported by those who have used azoprotein or different tracers (1, 36–42, 56–68). Haurowitz and Kraus (58), studying the take-up of proteins coupled to arsenic and iodine, also found them chiefly in the cells of the reticulo-endothelial system, but recently Crampton and Haurowitz (59) have shown that radioactivated ovalbumin, injected into rabbits, is apparently taken up in the liver, and that it appears mostly in the mitochondria of cells obtained from liver homogenates.

SUMMARY

Methods have been devised whereby the persistence of foreign antigens in mice can be detected. A highly diffusible, blue azo dye, echt-säure-blau was coupled to bovine γ-globulin and human serum albumin and injected into the animals. In this way deep blue tracer antigens were obtained. These were promptly stored in cells widely distributed throughout the body, especially in the reticulo-endothelial elements of the liver, spleen and mesenteric lymph nodes. The dye as such was not stored but rapidly excreted.

A blue coloration in the organs just mentioned was still perceptible after 85 to 120 days in the case of the azoglobulin and 36 to 44 days in that of the azoalbumin. To determine whether these substances had actually persisted, as well as to learn how long uncoupled globulin and albumin remained after injection, recourse was had to the phenomenon of reversed passive anaphylaxis, which was found to be characterized by extraordinary changes in the vessels of the ears (EVR) in the mouse, plainly visible under the microscope when called forth by an antiserum specific for the antigen to which the animal had been sensitized. So sensitive is the vascular response that as little as 0.5 to 0.1 μg. of protein as antigen, previously injected into the peritoneal cavity of a 30 gm. mouse, can be detected a few days later by an intravenous injection of antiserum.

By means of the EVR the globulin antigen has been detected in the blood and livers of injected mice for as long as 56 and 101 days, respectively; the albumin and azoalbumin for only 21 and 36 days. In the mesenteric lymph nodes of injected mice the albumin and azoalbumin antigens were found after 42 and 44 days, respectively.

The hepatic tissue and that of the mesenteric lymph nodes of mice injected with azoalbumin, containing in consequence stored blue material, when transferred to recipient mice yielded positive tests for antigen (EVR) as long as blue color could be perceived in these tissues with the unaided eye, or at low

magnification. After the color had disappeared from the tissues the transfer tests were found to be negative. This fact speaks for the antigenicity of the colored material.

In summary it is plain that certain antigenic proteins, after introduction into the blood stream of mice, are stored in certain tissues and that they may persist there for weeks or even months, far longer than has generally been supposed. This persistence of antigen within the body, especially after detectable amounts of antigen have apparently disappeared from the blood, provides a reason for prolonged antibody formation, a phenomenon for which no adequate explanation has hereto been offered.

BIBLIOGRAPHY

1. Kruse, H., and McMaster, P. D., *J. Exp. Med.*, 1949, **90,** 425.
2. Spalteholz, W., Über das Durchsichtigmachen von menschlichen und tierischen Präparaten, und seine theoretischen Bedingungen, Leipsic, S. Hirzel, 2nd edition, 1914.
3. McMaster, P. D., and Kruse, H., *J. Exp. Med.*, 1949, **89,** 583.
4. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1932, **55,** 417.
5. McMaster, P. D., *J. Exp. Med.*, 1941, **74,** 29.
6. McMaster, P. D., unpublished data.
7. Lee, K. H., Chow, B. F., and Wu, H. Chin, *J. Physiol.*, 1937, **11,** 193.
8. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1929, **50,** 809.
9. Heidelberger, M., Sia, R. H. P., and Kendall, F. E., *J. Exp. Med.*, 1930, **52,** 477.
10. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55,** 555.
11. Heidelberger, M., Kendall, F. E., and Soo Hoo, G., *J. Exp. Med.*, 1933, **58,** 137.
12. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61,** 559.
13. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1937, **65,** 647.
14. Goebel, W. F., Binckley, F., and Perlman, E., *J. Exp. Med.*, 1945, **81,** 315.
15. Perlman, E., and Goebel, W. F., *J. Exp. Med.*, 1946, **81,** 223.
16. Libby, R. L., *J. Immunol.*, 1938, **34,** 71.
17. Libby, R. L., *J. Immunol.*, 1938, **34,** 269.
18. Libby, R. L., *J. Immunol.*, 1938, **35,** 289.
19. Abel, R. G., and Schenk, H. P., *J. Immunol.*, 1938, **34,** 195.
20. Chambers, R., *Physiol. Rev.*, 1947, **27,** 436.
21. Zweifach, B. W., Chambers, R., Lee, R. E., and Hyman, C., *Ann. New York Acad. Sc.*, 1948, **49,** 553.
22. Knisely, M. H., Eliot, T. S., and Bloch, E. H., *Arch. Surg.*, 1945, **51,** 220.
23. Knisely, M. H., Bloch, E. H., and Warner, L., *K. Danske Vidensk. Selsk., Biol. Skrifter*, 1948, **4,** No. 7.
24. vonDungern, F., *Centr. Bakt., 1. Abt. Orig.*, 1903, **34,** 355.
25. Weil, R., *Proc. Soc. Exp. Biol. and Med.*, 1915, **12,** 37.
26. Denzer, B. S., *J. Infect. Dis.*, 1916, **18,** 631.
27. Naylor, G. R. E., *J. Hyg.*, 1950, **48,** 52.
28. Ransom, F., *Z. physiol. Chem.*, 1900, **29,** 349.
29. Ransom, F., *Z. physiol. Chem.*, 1900, **29,** 553.

30. Coca, A. F., and Grove, I. F., *J. Immunol.*, 1925, **10,** 445.
31. Weil, R., *J. Med. Research*, 1912–13, **27,** 497.
32. Longcope, W. T., and Rackemann, F. M., *J. Exp. Med.*, 1918, **27,** 341.
33. Longcope, W. T., and Mackenzie, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1920, **17,** 133.
34. Mackenzie, G. M., and Leake, W. H., *J. Exp. Med.*, 1921, **33,** 601.
35. Libby, R. L., and Madison, C. R., *J. Immunol.*, 1947, **55,** 15.
36. Pressman, D., and Keighley, G., *J. Immunol.*, 1948, **59,** 141.
37. Pressman, D., Hill, R. F., and Foote, F. W., *Science*, 1949, **109,** 65.
38. Pressman, D., *J. Immunol.*, 1949, **63,** 375.
39. Pressman, D., and Eisen, H. N., *J. Immunol.*, 1950, **64,** 273.
40. Eisen, H. N., and Pressman, D., *J. Immunol.*, 1950, **64,** 487.
41. Pressman, D., Eisen, H. N., Siegel, M., Fitzgerald, P. J., Sherman, B., and Silverstein, A., *J. Immunol.*, 1950, **65,** 559.
42. Pressman, D., *Cancer*, 1949, **2,** 697.
43. Topley, W. W. C., *J. Path. and Bact.*, 1930, **33,** 339.
44. Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, **85,** 571.
45. Schwab, L., Moll, F. C., Hall, T., Brean, H., Kirk, M., Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1950, **91,** 505.
*46. Dixon, F. J., Dammin, G. J., and Bukantz, S. C., *Fed. Proc.*, 1950, **9,** 380.
47. Knox, W. C., and Endicott, F. C., *J. Immunol.*, 1950, **65,** 523.
*48. Dixon, F. J., Bukantz, S. C., and Dammin, G. J., *Science*, 1951, **113,** 274.
*49. Bukantz, S. C., Dixon, F. J., Dammin, G. J., and Talmage, D. W., *Fed. Proc.*, 1951, **10,** 404.
*50. Dixon, F. J., Talmage, D. W., and Bukantz, S. C., *Fed. Proc.*, 1951, **10,** 409.
*51. Talmage, D. W., Dixon, F. J., and Bukantz, S. C., *Fed. Proc.*, 1951, **10,** 421.
52. Coons, A. H., Leduc, E. H., and Kaplan, M. H., *J. Exp. Med.*, 1951, **93,** 173.
*53. Gitlin, D., Latta, H., Batchelor, W. H., and Janeway, C. A., *J. Immunol.*, 1951, **66,** 451.
54. Felton, L. D., Prescott, B., Kaufmann, G., and Ottinger, B., *Fed. Proc.*, 1947, **6,** part 2, 427.
55. Felton, L. D., *J. Immunol.*, 1949, **61,** 107.
56. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, **91,** 1.
57. Breinl, F., and Haurowitz, F., *Z. physiol. Chem.*, 1930, **192,** 45.
58. Haurowitz, F., and Kraus, F., *Z. physiol. Chem.*, 1936, **239,** 76.
59. Crampton, C. F., and Haurowitz, F., *Science*, 1950, **112,** 300.
60. Sabin, F., *J. Exp. Med.*, 1939, **70,** 67.
61. Smetana, H., and Johnson, F. R., *Am. J. Path.*, 1942, **18,** 1029.
62. Smetana, H., *Am. J. Path.*, 1947, **23,** 255.
63. Seligman, A. M., and Fine, J., *J. Clin. Inv.*, 1943, **22,** 265.
64. Fine, J., and Seligman, A. M., *J. Clin. Inv.*, 1943, **22,** 285.
65. Warren, S., and Dixon, F. J., *Am. J. Med. Sc.*, 1948, **216,** 136.
66. Dixon, F. J., and Warren, S., *Am. J. Med. Sc.*, 1950, **219,** 414.
67. Gitlin, D., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74,** 138.
68. Latta, H., Gitlin, D., and Janeway, C. A., *Arch. Path.*, 1951, **51,** 260.

*See also subsequent reports (*Fed. Proc.*, 1951, **10,** 553–569) which appeared too late for mention in this paper.

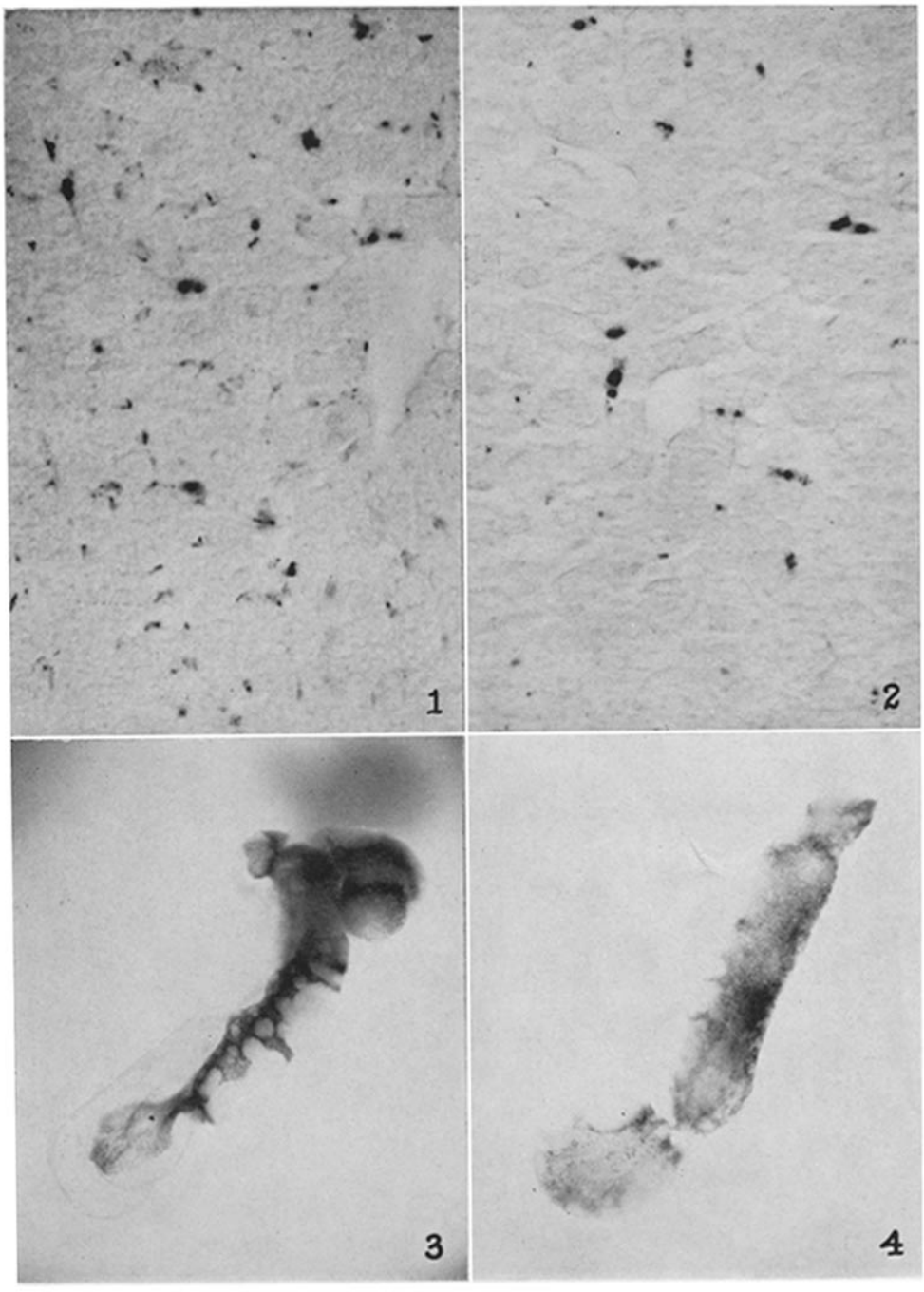
EXPLANATION OF PLATE 25

The photomicrographs of Figs. 1 and 2 were made by Mr. Julian A. Carlile. Figs. 3 and 4 were photographed by Mr. Richard C. Carter.

FIGS. 1–4. Retention of the blue material in the livers and mesenteric lymph nodes of mice after 1 week and 2 months respectively.

FIGS. 1 and 2 are photographs of unstained sections of liver 20 μ thick, after injection of the usual amount of E-AP-G. The dark specks in the tissue are the blue material. ×350.

FIGS. 3 and 4 are photographs of the mesenteric nodes of the same animals. Much blue material was still present in the node removed after 2 months. See text for further details. ×7.



(McMaster and Kruse: Persistence of antigens)