SUMMARY

1. Human diabetic and non-diabetic subjects excreted in the urine less than 3 % of an oral dose of 35 g. of sorbitol. No sorbitol could be detected in the faeces.

2. After 35 g. of oral sorbitol, there was no significant increase in blood sugar in normal subjects, but in diabetic subjects the blood sugar increased by a much smaller amount than after glucose. The concentrations of sorbitol in the blood were immeasurably small.

3. Small and insignificant amounts of sorbitol were present in the faeces after sorbitol was fed to patients on antibiotic therapy.

4. Experiments with uniformly labelled D-[¹⁴C]-sorbitol and D-[¹⁴C]glucose showed that in the normal and in the mildly diabetic subject at least 75 % of orally given sorbitol is rapidly metabolized to carbon dioxide. In a diabetic subject requiring insulin the recovery of labelled carbon in the respiratory carbon dioxide amounted to only 65 % and this lower figure was clearly due to the increased proportion of labelled material lost in the urine.

5. It was shown that a substantial proportion of oral sorbitol is rapidly converted into blood sugar and the pathway of this conversion is discussed.

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The Use of Radioactive Isotopes in Immunological Investigations

10. THE FATE OF SOME INTRAVENOUSLY INJECTED NATIVE PROTEINS IN NORMAL AND IMMUNE RABBITS*

By G. E. FRANCIS, J. D. HAWKINS AND A. WORMALL

Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C. 1

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Several workers have studied the elimination from the blood stream of antigenic proteins injected intravenously into normal or specifically immunized animals. The subsequent fate of these antigens after elimination from the blood has also been the subject of a number of investigations. However, none of these studies has yielded any detailed information about the rates of elimination of antigens from the blood of immune animals during

the first hour or so after injection, or of the immediate fate of these eliminated antigens, though it is during this period that any reaction with circulating antibody would be expected to occur. Such studies might therefore be expected to throw some light on the behaviour of antibodies *in vivo*, and to yield information on their role in the defence mechanisms of the body.

The proteins that have been used in most previous studies on their fate in animals have been labelled in different ways by the introduction of comparatively

* Part 9: Francis, Mulligan & Wormald (1955a).

large numbers of readily detectable groups not normally present in the protein molecule. These methods of labelling proteins, however, usually result in considerable modification of their properties, so that they behave differently from native proteins.

The artificial introduction of radioactive isotopes is a convenient labelling method for experiments of this type since the introduction of small amounts of isotopic material will result in the production of 'trace-labelled' proteins whose properties are practically identical with those of native proteins. In addition, radioactive isotopes can be used to label proteins which have undergone extensive chemical modification. The whole subject of labelled proteins has been comprehensively reviewed by Coons (1954).

Friedberg, Walter & Haurowitz (1955) have shown that measurements of radioactivity in tissues 9 days or more after the injection of antigenic proteins trace-labelled with ^{131}I are not reliable as estimates of the amounts of the injected proteins actually present, which may be 40–70 times that indicated by the ^{131}I measurement. This, however, does not necessarily invalidate results based on differences between normal and immune animals obtained in a relatively short period after the injection.

This paper describes experiments in which native proteins, trace-labelled with ^{131}I , were injected intravenously into normal and immune rabbits, and the persistence of these proteins in the blood stream was determined in detail during the first hour or two after injection. In addition, many of the rabbits were killed 1–2 hr. after injection, and the antigen content of certain tissues was determined. A preliminary account of part of this work has already been given (Almond, Francis, Hawkins & Wormall, 1954).

MATERIALS AND METHODS

Horse-serum γ -globulins. These were prepared from horse serum by Kekwick's (1940) method for the separation of human-serum γ -globulins.

Bovine-plasma albumin. A 30% solution of bovine albumin (Armour Laboratories) was suitably diluted with 0.9% (w/v) NaCl solution.

Isotopic labelling of antigens. In the earlier experiments (with γ -globulins) the proteins were labelled with ^{131}I by the introduction of about 4 atoms of iodine/molecule of protein, with the I_2 in KI method of Francis, Mulligan & Wormall (1951). In later experiments (with bovine albumin) the modified method of Gilmore, Robbins & Reid (1954), as described by Francis, Mulligan & Wormall (1955b), was used. In all cases the labelled proteins were freed from uncombined iodine by dialysis against 0.9% NaCl solution in Visking-brand transparent cellulose tubing. The amount of ^{131}I used in each preparation was calculated so that each rabbit received an injection of antigen containing about $20\text{ }\mu\text{C}$ of ^{131}I and giving an initial count rate of approximately

1000/min./ml. of whole blood in the apparatus used for radioactivity measurements.

Radioactivity measurements. Panax E.H.T. and scaler units (types 44 or 100C) were used throughout. For measurements of ^{131}I in the blood, measured samples of 1 ml. or less of blood were haemolysed with 10 ml. of 0.02N-NaOH and their radioactivity was measured in a Veall-type counter for liquid samples (Veall, 1948) (20th Century Electronics, type M6, M6H or M6M). Determinations of ^{131}I in tissues were made by a slight modification of the method of Perlman, Chaikoff & Morton (1941), the solution containing iodide being made up to a suitable volume and radioactivity measurements made directly on a part of this solution, in a Veall-type counter, instead of on a precipitate of silver iodide. In later experiments, tissues were homogenized in an MSE Ato-Mix Blender, and the radioactivity of samples of the homogenates was measured directly in a scintillation counter of the type described by Banks, Tupper, Watts & Wormall (1955).

Immunization. Most of the rabbits were immunized by the method of Proom (1943). Intramuscular injections of alum-precipitated antigen were given at about 14-day intervals, and good antisera were usually obtained 10–14 days after the second injection. In a few experiments with bovine albumin, rabbits were immunized by a course of intravenous injections of protein solution (3 mg. of N/kg. body wt.) at intervals of 7 days.

Qualitative and quantitative precipitin tests were carried out as described by Francis *et al.* (1955b).

Conduct of animal experiments. Adult rabbits (2.5–4 kg.) were injected with labelled antigen via the marginal vein of the right ear. Blood samples were obtained from the marginal vein of the left ear and collected in 1 in. diameter glass tubes in which one or two drops of saturated potassium oxalate solution had been evaporated. In many cases, after the injection of antigen into immune rabbits, varying degrees of anaphylactic shock were observed. Among other symptoms was a pronounced decrease in the peripheral circulation, rendering it difficult or impossible to collect any blood until the animal had recovered. This was largely overcome, in all the later experiments, by giving an intramuscular injection of an antihistamine (mepyramine maleate, 1.5 mg./kg. body wt.) about an hour before the antigen injection. Control experiments in normal and immune animals indicated that the antihistamine had no observable effect on the antigen elimination rate. The collection of blood samples was timed with a stop watch which had been started at the mid-point of the antigen injection; the times at the beginning and end of each bleeding were noted, and in calculating the results all the blood was assumed to have been taken at a time exactly half-way between these two times. Collection of the first blood sample was usually started not sooner than 2.5 min. after the injection, since preliminary experiments had shown that about 2 min. was necessary for the complete mixing of the injected antigen with the circulating blood.

Determination of blood volume. In some experiments the blood volumes of the rabbits were determined by the dye method (Courtice, 1943), modified by the use of a small 'loading' dose of 0.3 ml. of 0.2% solution of the dye (Evans Blue) given about 5 min. before the main dose. This was found necessary to saturate some extravascular receptors which rapidly remove a small amount of the dye from the circulation during the first few minutes after its injection.

Calculation of results. Throughout this work it has been assumed that the amount of radioactivity in the blood at any time is a direct measure of the amount of the injected antigen there (cf. Knox & Endicott, 1950). This may not be strictly true, for even within the first 2 hr. after injection, especially in immune rabbits, there may be a small amount of non-protein-bound iodine liberated into the blood stream as a result of de-iodination of the labelled antigen in the liver and other tissues (cf. Friedberg *et al.* 1955). However, the errors introduced by not making allowance for this breakdown are very small, and can reasonably be neglected in the experiments reported here.

The amounts of the injected antigen found in 1 ml. of each blood sample were plotted on a logarithmic scale against the times at which the samples were obtained. The points so obtained fell either on straight lines or on curves of the general shape shown by the continuous line in Fig. 1, which become straight after an initial period of up to 30 min. duration.

To analyse graphs of this second type, the equation for the line that fits best to the points on the straight section was calculated by the method of least squares, and this line was drawn on the graph and extrapolated to zero time. The ordinate values on this extrapolated line at the times

Fig. 1. Graphical analysis of typical curve of antigen persistence in the blood of a specifically immunized rabbit after the intravenous injection of antigen. (Antigen: ^{131}I -trace-labelled bovine albumin. Dose: 1 mg. of N/kg. body wt. Continuous line: experimental curve; broken lines: 'fast' and 'slow' components.)

corresponding to the points on the curved section of the graph were then subtracted from the experimentally determined ordinates of these points, and these differences were also plotted against the corresponding times. This second set of points was usually found to fall on a straight line, and the equation for the line best fitting these points was again calculated by the method of least squares, and the line drawn on the graph and extrapolated to zero time. The ordinate of a point on the experimental curve at any given time is thus the sum of the ordinates of the points on the two straight-line components at that time, indicating that the experimentally observed elimination rate is due to two independent concurrent processes each proceeding with an exponentially decreasing velocity. The half-lives of these two processes may be read off from the graphs.

The sum of the ordinates on the two component curves at zero time gives the total amount of injected antigen which would be present in each millilitre of blood immediately after the injection, assuming it to have mixed instantaneously with the whole of the circulating blood. Since the total amount of antigen injected is known, the animal's total circulating blood volume can therefore be calculated. The values calculated in this way were used in preference to those determined by the dye method when there was any discrepancy, e.g. when the turbidity of the serum rendered the dye method unreliable.

After the theoretical antigen concentration at zero time was calculated, either from the experimentally determined blood volume or by extrapolation of the component lines to zero time, all experimentally determined blood-antigen concentrations were expressed as a percentage of the initial concentration, and the curves redrawn. Fig. 1 shows the final curve in a typical experiment, the continuous line representing the experimentally determined elimination rate and the interrupted lines the two component processes. For convenience, the process which is virtually complete in 30 min. is referred to as the 'fast reaction', and the second process as the 'slow reaction'. The intercepts on the ordinate of the two lines at zero time also give the percentages of the total antigen present which are being subjected to elimination by the two processes separately.

Antigen in tissues. The amount of antigen found in any tissue was undoubtedly due in part to entrained blood, since no steps were taken to remove this blood in case this should also result in removal of any loosely bound antigen deposited in the tissue. Garvey & Campbell (1954) have shown that perfusion of the tissues washes out antigen deposited in liver or lungs, but the experiments recorded here were mostly carried out before this paper became available. In order to obtain an indication of whether antigen found in the tissues was entirely or only in part due to entrained blood, the results of radioactivity measurements on tissues are expressed as 'apparent blood volumes', i.e. antigen/g. of tissue divided by antigen/ml. of whole blood at the time of death. Evidently, if there is no deposition of antigen in the tissue, this ratio is the true blood volume (ml. of blood/g. of tissue) of the tissue. A comparison of the observed ratios in normal and immune rabbits should enable a decision to be made whether there has been any significant deposition of the antigen in the tissue of the immune rabbit.

In a few cases where animals received small doses of antigens which were almost completely eliminated from the blood by the time they were killed, the radioactivity of the tissues could be taken as a fairly accurate estimate of the

amount of antigen actually deposited, since the blood present in the tissues could have made only a negligible contribution to the total radioactivity.

RESULTS

Horse γ -globulins were chosen as the antigens to commence these studies since they are readily obtainable in a reasonably pure state by a simple method, and produce a good antibody response in rabbits. However, horse γ -globulins appear to behave as antigens in an abnormal manner in precipitin reactions *in vitro* (Francis, Mulligan & Wormald, 1954). They are not quantitatively precipitated by antibody, except possibly in regions of very large antibody excess, and in regions of antigen excess they are precipitated in amounts which are excessive relative to the amount of antibody in the precipitate. Since it appeared likely that they might also behave *in vivo* in a manner atypical of simple protein antigens, later experiments were carried out with bovine albumin.

Effect of dose level on persistence of protein antigens in circulating blood

The types of antigen-persistence curves obtained when normal and immune rabbits are injected intravenously with horse γ -globulin at various dose levels are shown in Figs. 2 and 3. The two immune animals injected at the higher rates (12.7 and 9.4 mg. of nitrogen/kg.) had approximately equal serum-antibody titres, but these were considerably higher than usual. In general, rapid elimination of approximately 50 % of the injected antigen occurred when injections were given at the rate of 1 mg. of antigen nitrogen/kg. body wt. The absolute amounts of antigen lost from the circulation in the first hour by these two rabbits were quite comparable (11.9 and 9.4 mg. of nitrogen), but the percentages were markedly different (35 and 58 % respectively). In the third rabbit, which received a much smaller dose, elimination was virtually complete. Normal rabbits in every case showed a much smaller percentage elimination in the first hour, and this percentage was dependent to a much smaller degree on the amount of antigen injected.

When bovine albumin was used as the antigen it was necessary to carry out a rough calibration of the antiserum before calculating the appropriate dose for injection. In a preliminary experiment 2 drops of the antiserum from each of four rabbits immunized with bovine-plasma albumin were treated with 5 drops each of serial dilutions of the antigen, and the amount of antigen required for maximal precipitation (which corresponds approximately to the equivalence zone) was judged by visual inspection. These rabbits were then injected with appropriate amounts of antigen to correspond to

(i) moderate antibody excess, (ii) slight antibody excess, (iii) slight antigen excess and (iv) moderate antigen excess. In all but the fourth rabbit nearly the whole of the antigen was eliminated from the circulation within 1 hr., but in the fourth only about 50 % was so eliminated.

In order to be able to analyse the curves satisfactorily it is essential that a significant percentage of the injected antigen should be removed, but sufficient should remain to render the measurements reasonably accurate in the later periods. Since the precipitation of horse γ -globulin by antibody never, in practice, approaches 100 %, this condition could nearly always be realized by injecting 1 mg. of antigen nitrogen/kg. body wt. into rabbits immunized against this protein, but a rough calibration of the serum was always carried out before

Fig. 2. Persistence of various doses of intravenously injected trace-labelled horse γ -globulin in the blood of normal rabbits. O, 5 mg. of N/kg. body wt.; ●, 1 mg. of N/kg. body wt.; ⊗, 0.33 mg. of N/kg. body wt.

Fig. 3. Persistence of various doses of intravenously injected trace-labelled horse γ -globulin in the blood of rabbits immunized against horse γ -globulin. ●, 12.7 mg. of N/kg. body wt.; O, 6.8 mg. of N/kg. body wt.; ⊗, 0.33 mg. of N/kg. body wt.

injecting rabbits immunized with bovine albumin, and the dose calculated to correspond to moderate antigen excess. Normal rabbits injected with bovine albumin gave, in nearly every case, straight-line persistence curves when plotted semilogarithmically.

Analysis of antigen-persistence curves

The antigen-persistence curves for a series of rabbits immunized by intramuscular injections of alum-precipitated bovine albumin, and injected with the trace-labelled antigen at varying intervals after the last immunizing injection, were analysed. Normal rabbits were also included in the group to act as controls.

The amounts of antigen injected necessarily varied with individual immune animals, and in the control rabbits injections of comparable size were given. The results (Table 1) show that the average half-lives of the slow reaction in the normal and immune groups were the same, but the reaction appears to have been slightly faster in immune rabbits tested a relatively short time after the last immunizing injection. The fast reaction, on the other hand, was observed in all immune animals, but in only two out of ten controls. In about one-

half of the immune rabbits of this series the velocity of the fast reaction did not correspond to an exponential function, i.e. the calculated curve was not a straight line but a smooth curve. It was not possible, however, to analyse this curve further to establish whether it consisted of two or more exponential processes. The percentage of injected antigen removed by the fast reaction in these cases is therefore determined by difference, and no half-life can be given. The significance of this finding will be discussed more fully elsewhere (Francis & Hawkins, in preparation). In a series of experiments carried out with horse γ -globulin as the antigen essentially similar results were obtained, but a relatively small percentage of the antigen was also eliminated by a fast reaction in normal rabbits (9-25% as compared with 26-67% in the immune rabbits). In both normal and immune rabbits the graphs of the fast reaction corresponded with simple exponential functions having similar half-life values to those observed in the first series.

In neither series did there appear to be any relationship between the time an animal had been in the immune state and either the half-life of the fast reaction or the percentage of antigen eliminated by this reaction.

Table 1. *Analysis of persistence curves of ^{131}I in the blood of normal and immune rabbits after the injection of labelled bovine-plasma albumin*

Time after last immunizing injection (days)	Antigen injected (mg. of N/kg. body wt.)	^{131}I removed by fast reaction* (%)	Half-life of fast reaction (min.)	Half-life of slow reaction (min.)
Normal rabbits				
—	0.09	11	3.7	265
—	0.09	0	—	275
—	0.16	0	—	269
—	0.16	0	—	226
—	0.29	0	—	309
—	0.30	0	—	310
—	0.37	0	—	209
—	0.91	10	6.5	328
—	1.19	0	—	199
—	2.20	0	—	245
Mean value				263
Immune rabbits				
8	3.04	43	4.6	182
8	2.90	33	3.4	129
14	0.64	(55)	—	144
15	1.97	39	7.9	268
15	2.39	38	5.0	203
15	0.60	22	11.3	147
15	1.20	(36)	—	282
15	1.17	(61)	—	285
22	2.58	(72)	—	277
22	1.35	(55)	—	300
22	1.62	(48)	—	450
133	0.11	76	8.2	350
133	0.10	67	10.1	420
Mean value				264

* Figures shown in parentheses were observed in rabbits in which the curve representing the fast reaction is complex; hence its half-life cannot be calculated. The figures are equal to that percentage of the ^{131}I which is not removed from the blood by the slow reaction.

Table 2. 'Apparent blood volumes' of tissues of normal and immune rabbits killed 1 hr. after the intravenous injection of labelled horse γ -globulin

For definition of 'apparent blood volume' see text (Materials and Methods).

Antigen injected (mg. of N/kg. body wt.)	Normal rabbits					Mean
	5.03	0.37	1.06	5.82	1.04	
Tissue						
Liver	0.16	0.16	0.18	0.18	0.068	0.15
Lungs	0.16	0.24	0.24	0.24	0.21	0.22
Spleen	0.19	0.19	0.23	0.21	0.14	0.19
Heart	0.11	0.023	—	—	—	0.07
Skeletal muscle	0.018	0.049	—	—	—	0.03

Antigen injected (mg. of N/kg. body wt.)	Immune rabbits					Mean
	12.6	6.79	5.84	1.07	0.57	
Tissue						
Liver	0.19	0.43	0.41	0.32	0.85	2.1
Lungs	0.71	—	0.36	0.32	0.28	0.36
Spleen	0.34	0.53	0.20	0.46	0.40	0.69
Heart	0.12	0.13	—	—	—	0.055
Skeletal muscle	0.014	0.013	—	—	—	0.038

Elimination of soluble antibody-antigen complexes

Bovine albumin trace-labelled with ^{131}I was added to a calibrated rabbit antiserum of known antibody titre to give a very small precipitate in the region of high antigen excess. After incubation at 37° for 1 hr. the mixture was kept at $0-4^\circ$ for 112 hr. to ensure complete precipitation of the insoluble antibody-antigen complexes, and centrifuged at 0° . The clear supernatant was injected into four normal rabbits, and the persistence of the labelled antigen determined. Each rabbit received 6.4 mg. of antibody nitrogen ($0.24 \mu\text{mole}$ of antibody) and 48 mg. of antigen nitrogen ($4.26 \mu\text{moles}$ of antigen). If it is assumed that antibodies are bivalent when they form soluble complexes in the antigen excess zone (Marrack, Hoch & Johns, 1951) then $0.48 \mu\text{mole}$ (5.44 mg. of nitrogen, 11.3%) of the antigen should have been in the form of these complexes. The remainder must have been present in the free state. The persistence curves were all simple exponential ones and had half-lives ranging from 167 to 242 min. (mean 197 min.).

Radioactivity measurements on cells and plasma

Blood samples from normal and immune rabbits injected with horse γ -globulin, taken at intervals from 5 to 60 min. after the injections, were centrifuged and the cells washed twice with 0.9% NaCl solution. Radioactivity measurements were then made simultaneously on the whole blood and on measured volumes of the washed and haemolysed cells. The results showed that a significant amount of radioactivity was centrifuged down with the cell fraction in the immune rabbits immediately after the injection (up to 25% during the first 10 min.) but that this diminished rapidly with time (1-2%

Table 3. 'Apparent blood volumes' of tissues of normal and immunized rabbits killed 60-90 min. after the intravenous injection of labelled bovine plasma albumin

For definition of 'apparent blood volume' see text (Materials and Methods).

Organ	No. of rabbits	Apparent blood volume		
		Range	Mean	
		Normal rabbits		
Liver	10	0.10-0.24	0.16	
Lungs	10	0.18-0.41	0.27	
Spleen	9	0.10-0.23	0.15	
Kidneys	6	0.12-0.34	0.21	
Immunized rabbits, tested 2-14 days after immunization, but showing no immune response				
Liver	12	0.12-0.26	0.18	
Lungs	13	0.17-0.40	0.28	
Spleen	11	0.12-0.19	0.14	
Kidneys	9	0.14-0.32	0.22	
<i>Individual values of 'apparent blood volumes' in immunized rabbits showing an immune response</i>				
Liver	0.95	0.72	2.0	6.7
Lungs	0.57	0.31	1.0	9.8
Spleen	0.83	—	—	4.7
Kidneys	0.45	0.21	0.69	—

after 1 hr.). By contrast, in normal rabbits only an insignificant amount of the radioactivity could be found in the cell fraction at any time. Similar results were obtained in rabbits injected with bovine albumin.

These results suggest that a precipitin reaction analogous to the familiar precipitin reaction *in vitro* may be occurring in the blood stream. If this is so, the precipitate must be rapidly removed from the blood, presumably by phagocytosis.

Deposition of antigens in tissues

A number of the animals used in the antigen persistence measurements were killed 1-2 hr. after the injection, and blood and tissue samples were removed for radioactivity determinations. The results are given in Tables 2 and 3. The apparent blood volumes of the various organs of different normal rabbits are reasonably constant for each organ irrespective both of the dose injected and the nature of the antigen. The figures for heart and skeletal muscle in the immune animals are not significantly different from those for the corresponding tissues in normal animals, but the apparent blood volumes of the livers, lungs and spleens of the immune rabbits show much greater individual variation, and are, with few exceptions, considerably higher than those of the corresponding organs of the normal rabbits. Thus there seems no doubt that the liver, lungs and spleen play some part in the removal of intravenously injected antigen from the circulation of immune rabbits.

In order to obtain a more quantitative picture of the part played by these organs, some immune animals were injected with very small doses of the antigens, so that only about 10% of the injected antigen was still circulating when the animals were killed 90 min. after the injection. The blood contained in the tissues could only have contributed very slightly to their total radioactivity, thus the actual amounts of antigen deposited in the liver, lungs and spleen could be determined fairly precisely. The results, expressed as the percentage of the injected dose present in the whole of each tissue, are shown in Table 4 and indicate that the liver is quantitatively the most important organ in removing antigen from the blood stream. This is mainly on account of its large weight compared with the other organs examined, for the concentration of antigen/g. of tissue is not invariably highest in the liver. The percentage of the injected antigen accounted for in the blood and the three organs examined is quite high, but several organs (e.g. lymph nodes, thymus and bone marrow) which may contribute to the removal of antigen have not been

examined in these experiments. The lymph and tissue fluid must also have contained significant amounts of the antigen.

Development of the immune response after injection of bovine albumin

The course of the development of the primary immune response in rabbits after an immunizing injection of antigen has been studied by Knox & Endicott (1950), and by Talmage, Dixon, Bukantz & Dammin (1951). They investigated the disappearance of bovine albumin and bovine γ -globulin from the blood of normal rabbits after a single intravenous injection, and showed that antibodies first appeared in the plasma about 9 days after the injection of albumin (12 mg. of nitrogen/kg. body wt.) and about 7 days after the injection of the globulin (6 mg. of nitrogen/kg. body wt.). Just before the antibodies were first detectable a marked acceleration of the rate of disappearance of the injected antigen was observed, which was interpreted as an early manifestation of the immune response. A different approach has been used in this work to study the same phenomenon.

Rabbits were given a single intravenous injection of non-radioactive bovine albumin (3 mg. of nitrogen/kg. body wt.) and then a test injection of ^{131}I -labelled antigen (0.3 mg. of nitrogen/kg. body wt.) 2, 5, 9 or 14 days later. The amount of the test injection was deliberately kept small, so that if the immune response had developed it would be readily detectable, and a considerable proportion of the injected antigen would be rapidly removed from the blood. None of these animals appeared to have any circulating precipitating antibody at the time of the test injection, as judged by qualitative precipitin tests, though it is possible that there may have been small traces which this test was not sensitive enough to detect. All the rabbits tested 2, 5 and 9 days after the immunizing injection failed to give the characteristic immune response of rapid elimination of part of the antigen from the blood stream during the first hour after injection, and the apparent blood volumes of their livers, lungs and spleens fell within the range usually encountered in

Table 4. ^{131}I Content of tissues of immune rabbits killed 90 min. after the injection of small doses of labelled antigen

Antigen injected ...	^{131}I content (% of injected dose)				
	Horse γ -globulin			Bovine-plasma albumin	
Tissue					
Liver	65	53	59	66	71
Lungs	5.9	4.1	17.5	8.4	2.3
Spleen	1.0	1.2	0.7	0.3	0.3
Blood	15*	10*	5*	4.4*	7.7*
Total recovery	86.9	68.3	82.2	79.1	81.3

* Calculated from the estimated blood volume.

normal animals (see Table 3). Three out of six animals tested 14 days after immunization showed a rapid elimination of about one-half of the injected test dose, and their organs had the high apparent blood volumes characteristic of immune animals. The other three animals in this group behaved in a manner indistinguishable from normal animals.

A later attempt to reproduce this phenomenon in six rabbits tested 14 days after the immunizing injection was inconclusive. Only one animal showed an immune response after the test injection. The serum of this animal did contain a very small amount of antibody, which was demonstrated by a more sensitive precipitin test than that used for testing the sera of the first set of animals.

These results suggest that the immune response first develops about 14 days after the intravenous injection of bovine albumin in amounts of 3 mg. of nitrogen/kg. body wt. Knox & Endicott (1950) showed that circulating antibodies to bovine albumin first appeared about 9 days after an intravenous injection of this protein. The reason for the difference between their results and ours is not apparent, but it might be due to the larger doses of antigen injected by these workers.

DISCUSSION

Talmage *et al.* (1951) have shown that 99% of an injected protein could be eliminated from the blood of immune rabbits in 24 hr., but the observations reported here demonstrate that, provided only small amounts of antigen are injected, over 90% may be removed from the circulation in less than 1 hr. by a 'fast reaction' having a half-life of approximately 10 min. It seems evident from the behaviour of the animals injected with different amounts of antigen (e.g., Fig. 2) that there is an upper limit to the amount of antigen which an individual rabbit is capable of removing by this fast reaction, and that this is determined by the amount of antibody available to react with it at the time of injection. The presence of small amounts of antigen in the cell fraction when the blood is centrifuged also supports the hypothesis that an *in vivo* precipitin reaction is associated with the mechanism of the fast reaction. The alternative explanation that the antigen may have been taken up by the blood cells does not seem very probable in view of the rate at which it leaves the circulation, though it is possible that some of the leucocytes may have acted in this way since they are believed to contain appreciable amounts of antibody. There was, however, no evidence of a fast reaction when soluble antibody-antigen complexes were injected into normal rabbits, which strongly suggests that the formation of a discrete precipitate is an essential part of this process.

The rapidity with which the precipitated antibody-antigen complex is completely removed from the vascular system remains, however, a most striking phenomenon.

When larger amounts of antigen are injected, the fast reaction is still evident, and although a smaller percentage of the injected protein is eliminated within the first hour, the amount removed by the fast reaction may still be determined by graphical analysis, either by reading off the intercept on the ordinate at time zero of the fast reaction component of the curve, or by subtracting from 100% the corresponding intercept of the slow component.

The fast reaction was also observed in all normal rabbits injected with horse γ -globulin, though only rarely with bovine albumin. We have frequently observed that γ -globulins are far more liable than albumins to undergo partial denaturation during chemical treatment, and it appears probable that some part of these 'more damaged' proteins, even though in a number of cases they were Seitz-filtered before injection, may be eliminated from the blood stream of normal animals by a phagocytic mechanism similar to that responsible for the removal of particulate foreign matter (cf. Francis & Hawkins, 1957). McFarlane (1956), in studies on the elimination of injected labelled plasma proteins over a period of several days, has also observed that proteins trace-labelled with ^{131}I may in some cases be eliminated from the circulation of normal rabbits in a manner different from that of biologically labelled [^{14}C]proteins, owing to a more rapid removal of part of the [^{131}I]protein, but this discrepancy disappeared if the [^{131}I]protein was first 'filtered' in the circulation of one animal before injection into another. This effect was also more marked with [^{131}I]serum globulin than with [^{131}I]serum albumin, and in our experiments also the occurrence of the fast reaction in normal rabbits injected with bovine albumin was exceptional.

The rapid rate of removal of injected antigens from the blood of immune rabbits is comparable with the rate of removal of injected particulate material such as colloidal chromic phosphate. Dobson & Jones (1952) have shown that this is removed from the blood of mice with a half-life of only 30 sec. This is certainly a faster reaction than is observed after antigen injection, but the mechanism for eliminating the two types of substance may be the same. The rate of elimination of particulate antibody-antigen complexes from the blood will be determined by at least three factors: the rate of mixing of antigen with the plasma so that it can react with all the antibody present, the rate of formation of the antibody-antigen precipitate and the rate of removal of that precipitate. Complete mixing of the antigen with the circulating plasma probably takes up to 2 min.: the rate of flocculation

of the antigen-antibody complexes in these particular systems cannot be assessed with any certainty, but it is likely to be of the order of a few minutes (cf. Boyd, 1947), so that one of these two factors is almost certainly the rate-limiting reaction. Thus it is quite likely that these insoluble antigen-antibody complexes are eliminated from the blood by the same mechanisms which remove other particulate material.

Native antigenic proteins are also eliminated from the blood of normal (non-immune) rabbits, but at a considerably slower rate. This loss of antigen from the blood is due to the diffusion of the antigen into the protein space of the animals, and it has been studied in more detail by Knox & Endicott (1950), Talmage *et al.* (1951), and Cohen, Holloway, Matthews & McFarlane (1956). The extravascular diffusion is usually complete in 24-48 hr. and is succeeded by an even slower loss of antigen from the blood, which is interpreted as being due to its metabolic breakdown. This last phase of antigen disappearance from the blood is not apparent in the short-term experiments reported here, but the loss of antigen by diffusion is constantly observed and in immune rabbits it is superimposed upon the more rapid elimination of the antigen from the blood by the fast reaction.

There is some suggestion from the experimental results (Table 1) that the half-life of the slow reaction in immune animals shortly after their last immunizing injection may be less than that observed in normal animals. It is not certain whether this difference is significant, but it might be expected that immune animals would have a slightly greater elimination rate during this second phase since (a) the concentration gradient of the antigen between plasma and lymph would be greater in the immune animals owing to removal of the antigen in the lymph by reaction with antibody there, and (b) any continued liberation of antibody into the blood stream would contribute to a more rapid elimination rate. In the series of animals injected with bovine albumin the half-life of the slow reaction does, in fact, show some tendency to increase when the animals have been in the immune state for a long time and are therefore unlikely to be still actively synthesizing specific antibody.

The possibility that soluble antigen-antibody complexes might be removed from the blood at a different rate from that of the uncombined antigen has also been tested, but there seems to be very little difference in the elimination rates of these two kinds of substances.

The tracing of injected antigens into the tissues has confirmed the observations of Libby & Madison (1947), Ingraham (1951), Laws & Wright (1952) and of Garvey & Campbell (1954), that the livers, lungs and spleens of immune animals take up antigen, but

has shown that this phenomenon occurs more rapidly than was demonstrated by these workers. When only small amounts of antigen are injected into immune rabbits, and almost all the antigen is removed from the blood in the first hour after injection, the total amount of antigen found in the liver, lungs and spleen in nearly all cases is only a little below that injected. It therefore seems probable that, though other organs undoubtedly play some part in the response of immune rabbits to antigen injection, these three organs are of major significance in this respect.

There are several ways in which antigens, injected intravenously into immune rabbits, could be deposited in the tissues. First, the antigen-antibody precipitates formed in the blood stream could either be filtered out directly by the capillaries or be transported to the tissues after phagocytosis by cellular elements in the blood. Secondly, they could leak out of the circulation into the tissues at places where hypersensitivity reactions have damaged the tissues (Garvey & Campbell, 1954). Thirdly, antigens could be taken up by antibodies which are bound to the tissues in some way. This third possibility will be discussed more fully elsewhere.

The first possibility was envisaged by Banks, Francis, Franklin & Wormall (1950) in their studies on the fate of lipovitellin injected into normal rabbits. This protein is precipitated by the low ionic strength of the plasma and is rapidly filtered out by the lungs. This factor might be expected to operate particularly in the lungs, for they contain the first capillary circulation that the intravenously injected antigen must traverse in its passage through the vascular system. Some form of filtration may also be occurring in the liver and spleen where the blood has to traverse sinuses containing large numbers of cells. Other particulate material is removed from the blood stream by filtration in the lungs, for Odeblad, Dobson, Odeblad & Jones (1955) have demonstrated that colloidal chromic phosphate forms small intra-capillary emboli in the lungs of mice after intravenous injection. However, in the elimination of antigens from the blood of immune animals it seems unlikely that this is the only mechanism operating, for there is no evidence that antigen is deposited in other tissues, such as skeletal muscle, which certainly have a capillary circulation.

The observations of Garvey & Campbell (1954) on the non-specific deposition of antigens in the lungs of immune animals are of importance in considering the possibility that antigens may be deposited in tissues because of hypersensitivity reactions. No attempt has been made to confirm these observations in the present work; it may be significant, however, that in the experiments described here,

antigens were still deposited in tissues when the animals had been protected from anaphylactic shock by injection of an antihistamine drug which would be expected to minimize, if not abolish, the hypersensitivity reactions which damage the tissues. This does suggest that even if antigens may be deposited in tissues non-specifically as a result of tissue damage, there are other means which are also operative.

SUMMARY

1. Horse-serum γ -globulins and bovine-plasma albumin, both trace-labelled with ^{131}I , have been injected intravenously into normal rabbits, and their persistence in the blood stream has been measured during the first 2 hr. after injection. A relatively small loss of the protein is observed during this period, and it has been shown that this loss is frequently brought about by two processes (the 'fast reaction' and the 'slow reaction'). A method for calculating the amounts of protein acted upon by each of these processes is described.

2. When the same proteins were injected into specifically immunized rabbits, a much larger proportion of the protein was removed from the blood by the fast reaction, probably owing to an *in vivo* precipitin reaction followed by a rapid phagocytosis of the precipitate.

3. The rapid removal of bovine-plasma albumin from the blood of immunized animals has occasionally been observed with rabbits which had received a single immunizing injection of the antigen 14 days previously, but this phenomenon was not observed 2, 5 and 9 days after the immunizing injection.

4. Soluble antibody-antigen complexes containing trace-labelled bovine-plasma albumin are removed from the blood of normal rabbits only slightly more rapidly than the antigen itself.

5. After the injection of antigens into immune rabbits, considerable deposition of antigen occurs in the liver, lungs and spleen. If such small amounts are injected that 80-90% of the injected antigen is removed from the blood in 1 hr., the recovery of ^{131}I in these organs is almost equal to that which has disappeared from the blood.

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