with ethanol (final concn. 80 %, v/v); the precipitate was washed with ethanol and with ether and dried *in vacuo*. For the isolation of the antibody the serum was roughly calibrated for antibody content and antigen was added in such amount that the precipitate was formed in or near the equivalence zone. The precipitate was washed with 0.9 % sodium chloride solution and then with water, and dried *in vacuo*. The radioactivity measurements were made in a windowless flow counter as described by Banks, Crawhall & Smyth (1956). The results are shown in Table 1.

If the total circulating plasma volume of the rabbit (4.13 kg.) is assumed to be 140 ml. and the plasma protein concentration 7 %, 9.8 g. of plasma protein will be circulating. Thus at 7.5 hr. the total

radioactivity incorporated into the plasma proteins was 49 μ C, or 1.6 % of the radioactivity administered. The actual count rate of the antibody fraction can be increased more than 100-fold by converting the hydrogen of the samples into methane and measuring the radioactivity of this gas (Banks *et al.* 1956).

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The Use of Radioactive Isotopes in Immunological Investigations

13. THE ROLE OF LEUCOCYTES AND NON-PLASMA ANTIBODY IN THE REMOVAL OF ANTIGENIC PROTEINS FROM THE BLOOD STREAM OF IMMUNIZED RABBITS*

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In a previous paper (Francis, Hawkins & Wormall, 1957) the behaviour of intravenously injected trace-labelled native proteins in normal and immune rabbits has been described. It was shown that, provided that suitable doses of antigen were injected, the immune rabbits rapidly eliminated some or all of the injected protein from the blood stream by a first-order reaction whose half-life was of the order of 5–10 min. This rapid elimination was believed to be dependent on the reaction of the injected antigen with circulating antibody. It was therefore decided to compare the amount of antigen that can be eliminated rapidly from the blood stream of an immune rabbit with the amount of antigen that could be precipitated *in vitro* by all its serum, under conditions as nearly comparable as possible with those prevailing *in vivo*.

It was found that more antigen could be rapidly eliminated from the blood after intravenous injection than could be precipitated *in vitro* when the same amounts of antigen/ml. of serum were used in the two experiments. This suggested that the injected antigen might be reacting not only with circulating antibody, but also with additional antibody not present in the circulation but capable of being made available with great rapidity. However, it is also possible that these two determina-

tions were made under such different conditions that a direct comparison between them was not valid. In order to decide between these two possibilities some similar experiments were carried out on rabbits 24 hr. or more after passive immunization, when all the antibody should be in the plasma or in fluids directly in equilibrium with it, and on rabbits at different stages during and after an immunization schedule.

A further possibility was that this non-plasma antibody might be released from lymphocytes circulating in the plasma and lymph, or from lymphoid tissue, since these cells are known to contain antibody.

Some immunized rabbits were therefore injected with cortisone, which has been reported to cause the liberation of considerable amounts of antibody from lymphocytes (Dougherty, Chase & White, 1945), to see if this treatment would have any effect on the relative amounts of antigen eliminated rapidly from the blood stream and precipitated *in vitro* by the animal's plasma. At the same time, both normal and immunized rabbits were injected with antigen, and total and differential leucocyte counts were made before and at intervals after antigen injection. The results of these experiments have confirmed and amplified those of Stavitsky, Stavitsky & Ecker (1949) and Stavitsky (1952).

* Part 12: Hawkins (1957).

A preliminary account of some of this work has already appeared (Francis, Hawkins & Wormall, 1955).

MATERIALS AND METHODS

Antigens. Bovine-plasma albumin and horse-serum γ -globulin were obtained and trace-labelled with ^{131}I as described previously (Francis *et al.* 1957).

Radioactivity measurements. These were made as described by Francis *et al.* (1957) except that in many experiments the radioactivity of blood samples was measured with a scintillation counter (Banks, Tupper, Watts & Wormall, 1955). The higher count rates obtained in this instrument (because of its greater sensitivity for the γ -rays emitted by ^{131}I) make it possible to inject smaller amounts of ^{131}I into the experimental animals. Thus as little as $2\text{ }\mu\text{C}$ of ^{131}I is needed to give an initial count rate of approx. 1000 counts/min./ml. of whole blood.

Immunization. Rabbits were actively immunized with alum-precipitated antigens, by the method of Proom (1943). For passive immunization sera from rabbits immunized against bovine albumin were pooled and treated with Na_2SO_4 (150 mg./ml. of serum) to precipitate the γ -globulins (some α - and β -globulins were probably also precipitated). The precipitated sludge was freed from SO_4^{2-} ions by dialysis against 0.154M-NaCl and then injected into the marginal ear veins of normal rabbits. Owing to the high protein concentration (approx. 5%) the amount injected (usually 10–15 ml.) frequently proved fatal to rabbits, death commonly occurring 8–24 hr. after injection. This was overcome by taking from the rabbits before injection a volume of blood about twice as great as the volume of protein solution injected.

Conduct of animal experiments. These were carried out as described by Francis *et al.* (1957). When horse-serum γ -globulin was used as the antigen injections of 1 mg. of N/kg. body wt. were given, but when bovine albumin was used in single test-injection experiments the amount injected was approximately double that required to give maximal precipitation with the serum *in vitro* (as determined by a rough calibration of a serum sample obtained from the animal immediately before commencing the experiment). These same relative injection levels were employed for both actively and passively immunized rabbits. Under these conditions, with bovine albumin, approx. 20–30% of the antigen and 50–60% of the antibody are precipitated *in vitro*.

Plasma-volume determinations. These were usually made as described by Francis *et al.* (1957). In the experiments in which leucocyte counts were made, it was considered undesirable to inject a dye for determination of plasma volume in case any of it might be taken up by the leucocytes and modify their staining properties. Plasma volumes were therefore determined by the use of ^{51}Cr -labelled red cells (Sterling & Gray, 1950). A measured volume (0.5–1.0 ml.) of the labelled cells was injected intravenously and samples of blood were collected from the opposite ear about 5, 10 and 15 min. later. The circulating blood volume was calculated from radioactivity measurements (in the scintillation counter) on measured portions of these blood samples and of a suitable dilution of the labelled cells, and the plasma volume was calculated by a simultaneous determination of the haematocrit value. For determination of antigen

persistence after injection of the ^{131}I -labelled antigen into these rabbits, the plasma from the blood samples was separated and its radioactivity measured. The radioactivities of the injected ^{51}Cr -labelled cells and ^{131}I -labelled antigen were such that even if as many as 10% of the cells in any one blood sample were haemolysed while the plasma was being obtained and separated, the extra radioactivity released into the plasma would not have been more than 2% of that due to the antigen present.

Theoretically, the determination of plasma volume by a method which actually determines red-cell volume is unsound, for there is no justification for assuming that the haematocrit value is uniform throughout the vascular system. However, the errors introduced by variations in the haematocrit value are probably small and can reasonably be neglected in the experiments described here.

Leucocyte counts. These were done by conventional methods (cf. Kolmer & Boerner, 1945). In each differential count about 300 leucocytes were counted unless the total count was less than 1000/mm.³, when only 100–150 were usually counted. Leucocytes were classified as lymphocytes, monocytes or granulocytes; the last class of cells was not further subdivided. Monocyte counts are not reported in this paper since the number of these cells counted in any one sample was so low that any differences between various blood samples were probably not significant.

Calculation of results. Antigen-persistence curves were analysed graphically as described by Francis *et al.* (1957). It was commonly found that the 'fast reaction' was a complex one and the curve representing it was not linear. In such cases extrapolation to zero time was done by visual inspection rather than by calculation.

The object of many experiments was a comparison between the amount of antigen that the whole of an animal's serum would precipitate *in vitro* and the amount of antigen actually removed from the blood stream by the 'fast reaction'. This has been achieved by expressing these two figures as a ratio which we have called the *vitro/vivo* ratio.

The amount of antigen that the whole of an animal's serum was capable of precipitating *in vitro* at the particular antibody/antigen ratio employed was calculated as the product of the plasma volume and the amount of antigen that a known volume of the serum would precipitate *in vitro*. Measured samples of antiserum obtained immediately before the injection of antigen were incubated with trace-labelled antigen in such amounts that the final antigen concentrations in the mixtures varied over a narrow range about the concentration which was expected to be found in the animal's plasma immediately after injection. After incubation for 1 hr. at 37° the mixtures were refrigerated overnight and the amount of antigen in each washed precipitate was determined by radioactivity measurements (Francis, Mulligan & Wormall, 1954).

The term 'apparent blood volume' used for expressing results of measurements of the uptake of antigen by tissues has been defined by Francis *et al.* (1957).

RESULTS

Four rabbits immunized with horse-serum γ -globulin and four immunized with bovine albumin were used in a preliminary experiment to deter-

Table 1. Vitro/vivo ratios observed in rabbits immunized against horse-serum γ -globulins or bovine albumin

Intramuscular immunizing injections (1-6) were of the alum-precipitated protein; intravenous immunizing injections (7 and 8) were of the native protein.

Method of immunization	Antigen eliminated by fast reaction <i>in vivo</i> (% of injected dose)	Antigen precipitated <i>in vitro</i> (% of injected dose)	Vitro/vivo ratio
Rabbits immunized with horse-serum γ -globulin			
1	27	15	0.56
2	44	28	0.64
3	39	28	0.72
4	31	28	0.90
Rabbits immunized with bovine albumin			
5	47	24	0.51
6	50	31	0.62
7	43	28	0.65
8	41	21	0.52

Table 2. Vitro/vivo ratios observed in immune rabbits at various times after their last immunizing injection of bovine albumin

Immunizing injections 1-6 were given intravenously; 7-10 were given intramuscularly.

Method of immunization	Time since last immunizing injection (days)	Antigen eliminated by the fast reaction <i>in vivo</i> (% of injected dose)	Antigen precipitated <i>in vitro</i> (% of injected dose)	Vitro/vivo ratio
1	13	55	24	0.44
2	14	76	32	0.42
3	14	61	25	0.41
4	20	72	36	0.50
5	20	52	13	0.25
6	20	55	31	0.56
7	82	65	25	0.38
8	132	67	67	1.00
9	133	67	61	0.91
10	133	75	59	0.79

mine the *vitro/vivo* ratios. Seven out of the eight rabbits tested removed considerably more antigen from their blood by the fast reaction than their sera precipitated *in vitro* (Table 1); in the eighth rabbit this difference was less marked and could have been due to experimental error.

This suggested that a considerable part of the antigen injected into immune rabbits might be removed from the blood stream by some mechanism other than by reaction with circulating plasma antibody. Further evidence was therefore sought to strengthen this conclusion and to investigate what this other mechanism might be. As a working hypothesis, it was assumed that there was some antibody not circulating in the free state in the blood, but still rapidly accessible to circulating antigen and capable of removing it from the blood. This we have termed 'non-plasma antibody', and a number of experiments were designed to confirm its existence and to assess its importance in antigen-antibody reactions *in vivo*.

Two sets of rabbits were tested at varying times after their last immunizing injection, in order to

discover whether the amount of 'non-plasma antibody' was dependent on the length of time the animal had been in the immune state. Six were immunized with bovine albumin by a course of four weekly intravenous injections of the native protein (3 mg. of N/kg. body wt.), and four received two immunizing injections of an alum-precipitated preparation. The results of determinations of the *vitro/vivo* ratio at various times after the last immunizing injection are shown in Table 2. There seems to be no increase in this ratio during the first 12 weeks after completion of the immunization schedule, but in the three animals tested 132-133 days after their last immunizing injection the ratio was much higher than in the other animals. It therefore seems probable that there was very little 'non-plasma antibody' in these rabbits.

Analysis of the persistence curves of the antigen in the blood of most of the rabbits immunized with bovine albumin showed that the fast reactions were not simple exponential ones: the curves representing them were themselves curved in a manner suggesting that they may have been made

Table 3. *Vitro/vivo ratios observed in rabbits after passive immunization with anti-bovine albumin*

Time between passive immunization and testing (hr.)	Antigen elimination by the fast reaction <i>in vivo</i> (% of injected dose)	Antigen precipitated <i>in vitro</i> (% of injected dose)	<i>Vitro/vivo</i> ratio
24	85	71	0.84
24	13	22	1.7
52	67	81	1.2
52	46	36	0.78

up of two (or even more) processes, designated the 'very fast' and 'fairly fast' components. Notable exceptions were the three rabbits tested 132-133 days after their last immunizing injection. The fast reaction in each of these animals was represented by a simple exponential curve. Maximum curvature of the curve representing the fast reaction is commonly observed when the *vitro/vivo* ratio is low. This is shown in Fig. 1, which shows the analysed persistence curves of the antigen in the blood of two rabbits, of which one had a *vitro/vivo* ratio of 0.25, and the other had one of 0.56.

*Antigen elimination from the blood
after passive immunization*

In order to simulate as closely as possible the conditions obtaining in actively immunized animals, the *vitro/vivo* ratios were not determined for at least 24 hr. after passive immunization. By this time the equilibration of the injected protein throughout the protein space of the body should be practically complete (Knox & Endicott, 1950), though Cohen, Holloway, Matthews & McFarlane (1956) have shown that complete equilibration of homologous γ -globulins in the rabbit may take as much as 140 hr.

Under these conditions it is unlikely that any great change in the antibody content of the serum will occur owing to extravascular diffusion between the time of withdrawing the sample of antiserum for calibration and the time of completion of the fast reaction. The results (Table 3) do not show any evidence of low *vitro/vivo* ratios comparable with those normally obtained in actively immunized rabbits. In two cases the ratio is actually greater than unity, though in one of these the analysis of the curve was less reliable than usual owing to the small percentage of antigen eliminated *in vivo*. It was also observed that all these animals, unlike most actively immunized ones, eliminated the antigen from their blood by a fast reaction that followed a simple exponential course.

A further observation was made on two passively immunized rabbits that were inadvertently injected with such a low dose of antigen that most of it was eliminated from the blood stream in the first 90 min. after injection. When the tissues of these animals were analysed for ^{131}I (Table 4) a

Fig. 1. Persistence curves of bovine albumin in the blood of two specifically immunized rabbits: Δ , *vitro/vivo* ratio of 0.25; $\circ$, *vitro/vivo* ratio of 0.56. Continuous lines are the experimental curves; broken lines show the analysis of these curves into the 'fast' and 'slow' components (see Francis *et al.* 1957).

Table 4. ^{131}I content of some tissues of actively and passively immunized rabbits

Animals were killed 90 min. after intravenous injection of ^{131}I -labelled bovine albumin.

Tissue	Percentage of injected ^{131}I in tissue			
	Actively immunized animals* (2)		Passively immunized animals† (2)	
Liver	66	71	36	29
Lungs	8.4	2.3	3.9	2.0
Spleen	0.3	0.3	1.1	1.8
Blood	4.4	7.7	6.3	13.3

* Taken from Francis *et al.* (1957).

† Injected with antigen 100 hr. after passive immunization.

very much smaller percentage of the injected dose was found in their livers than in the livers of actively immunized animals similarly injected.

Double injection of antigen into immune rabbits

If there are two mechanisms which remove intravenously injected antigens rapidly from the blood of immune rabbits, it should be possible to demonstrate the second 'fairly fast' mechanism in the following manner. First an amount of non-radioactive antigen equivalent to the amount of antibody circulating in the rabbit's plasma is injected (this amount is determined by a preliminary calibration of the antibody content of the serum and a determination of the plasma volume). About 30 min. later, when all of this antigen should have been removed from the circulating blood, a very small injection of trace-labelled antigen is given. Faster elimination from the blood of the antigen in this second injection than is observed in normal rabbits given a similar injection would indicate the presence of 'non-plasma antibody', especially if it could also be shown that there was no precipitating antibody in the blood immediately before the second injection.

Experiments along these lines were therefore performed, but gave rather inconclusive results, though there was a suggestion that the second injection of antigen was removed from the blood of the immunized rabbits at a rate intermediate between that observed after a single injection into immune rabbits (the so-called fast reaction) and that observed in normal rabbits. This suggested that such a phenomenon might be better observed over a longer period of time than the 60-90 min. during which observations were usually made after antigen injection. A further set of experiments was therefore carried out in which the antigen persistence in the blood of normal and immune rabbits was followed for 4-5 hr. after injection, the immune rabbits being first injected with an amount of non-radioactive antigen just sufficient to precipitate the whole of the circulating plasma antibody (Table 5). In all the normal animals a slow reaction with a half-life of 12-20 hr. was observed; some of the protein (never more than 25%) was eliminated by a faster reaction, which was sometimes complex. In the immune animals a slow reaction was again observed, but its half-life was considerably shorter than that of the slow reaction in normal animals. There was also a simple or complex fast reaction which removed more antigen from the blood than did the fast reaction in normal animals. The net result was that these immune animals eliminated considerably more antigen from their blood during the first 5 hr. after the injection than did the normal ones. A marked difference between these two groups was also

Table 5. Elimination of trace-labelled bovine albumin (0.3 mg. of N/kg. body wt.) from the blood of rabbits during the first 5 hr. after intravenous injection

Fast reaction		Slow reaction		Percentage eliminated from blood in 5 hr.
Half-life (min.)	Percentage reacting	Half-life (min.)	Percentage reacting	
Normal rabbits				
77	6	767	94	30
39	22	1155	78	35
Complex	22	710	78	42
Complex	22	873	78	38
35	23	833	77	40
Immune rabbits*				
32	42	580	58	60
27	32	218	68	74
Complex	33	238	67	72
Complex	23	203	77	73

* These were injected with an amount of non-radioactive bovine albumin equivalent to their total circulating antibody 30 min. before the injection of trace-labelled bovine albumin.

Table 6. Apparent blood volumes of tissues of normal and immune rabbits (with no circulating antibody) after injection of antigen

The apparent blood volume is the ratio

$$\frac{\text{antigen/g. of tissue}}{\text{antigen/ml. of blood}}$$

	Normal rabbits		Immune rabbits			
Liver	0.20	(0.16-0.32)	1.12	0.79	1.51	1.06
Lungs	0.41	(0.33-0.47)	7.30	1.78	0.77	0.75
Spleen	0.18	(0.14-0.23)	0.90	2.06	1.02	0.82

observed when measurements were made of the ¹³¹I content of their tissues (Table 6). The tissues from the immune rabbits showed the characteristic high apparent blood volumes of immune animals, whereas the tissues of the normal animals had apparent blood volumes that were scarcely higher than those observed in normal animals killed only 60-90 min. after injection (cf. Francis *et al.* 1957). It should be emphasized that when serum obtained from all these immune rabbits just before the second injection of bovine albumin was tested for the presence of antibody by the addition

of antigen, so that the latter was present at a concentration equal to that in the plasma immediately after the second injection, no significant amounts of antigen were precipitated. Therefore the elimination of the second dose of antigen must have been brought about by a mechanism distinct from any reaction with antibody in solution in the plasma.

Effect of antigen injections on leucocyte counts in normal and immune rabbits

In confirmation of the results of Stavitsky *et al.* (1949), Table 7 shows that the injection of bovine albumin into normal rabbits has no significant effect on the leucocyte count of the blood. By contrast, after injection of antigen into immunized rabbits there is a very rapid drop in the number of circulating leucocytes, both lymphocytes and granulocytes being affected. This leucopenia is most severe when antigen is injected in an amount which is in excess of that required to precipitate all the circulating antibody. Table 7 shows that in two rabbits which received smaller amounts of antigen

(so that at least 90% was eliminated from the blood in the 5 min. after injection) a much less marked leucopenia was observed.

Stavitsky *et al.* (1949) also showed that small doses of histamine (0.25–0.50 mg. of histamine phosphate, i.e. 0.08–0.17 mg. of free base/rabbit) had no effect on the leucocyte count. It is difficult to assess the amount of histamine liberated *in vivo* after an injection of antigen into an immune rabbit, but from their work *in vitro* Dragstedt, Ramirez de Arellano, Lawton & Youmans (1940) estimated that about 0.1–0.3 mg. of histamine (as free base) is liberated/kg. body wt. during an antigen-antibody reaction. This takes no account of any histamine that might be liberated from the tissues. Histamine has therefore been injected intravenously at levels of 0.7 and 2.0 mg./kg.; the larger of these two doses is likely to be at least as much as would be liberated after the injection of antigen into an immune rabbit. In order to simulate as closely as possible the conditions of the experiments in which antigen was injected the rabbits received the usual

Table 7. *Lymphocyte and granulocyte counts before and after the injection of bovine albumin into normal and immune rabbits*

All figures are number of cells/mm.³ of blood.

		Normal rabbits				
Time after injection (min.)		No. 1		No. 2		
		Lymphocytes	Granulocytes	Lymphocytes	Granulocytes	
- 30		3900	4100	3400	3600	
- 5		2800	3000	2500	3500	
5		3100	3300	2900	3100	
30		3500	3900	2200	2200	
60		3100	4100	1900	2900	
Immune rabbits injected with an amount of antigen of which 90 % was removed from the blood within 5 min. of injection						
		No. 1		No. 2		
		Lymphocytes	Granulocytes	Lymphocytes	Granulocytes	
- 30		5800	4700	4200	7000	
- 5		8200	4600	3200	7900	
5		—	—	1600	4500	
13		—	—	1600	2100	
30		3900	1300	800	900	
60		3400	2000	900	1500	
Immune rabbits injected with an excess of antigen not completely removed from the blood within 1 hr. of injection						
Time after injection (min.)	No. 1		No. 2		No. 3	
	Lymphocytes	Granulocytes	Lymphocytes	Granulocytes	Lymphocytes	Granulocytes
- 30	1500	3400	3300	3300	3600	4000
- 5	1400	3000	2000	2300	3100	4400
5	240	310	530	140	420	70
13	440	580	820	130	360	40
30	630	700	2500	400	420	65
60	750	1200	2700	380	670	95

Table 8. *Lymphocyte and granulocyte counts after the injection of histamine*All figures are number of cells/mm.³ of blood.

Dose of histamine injected (mg. of free base/kg.)	0.7		2.0	
Time after injection (min.)	Lymphocytes	Granulocytes	Lymphocytes	Granulocytes
- 60	3600	2300	3300	3900
- 10	2500	2600	2400	4300
2	1600	860	1200	520
15	1800	1700	1300	1200
30	2200	2600	1900	3500
60	2200	4000	2200	2500

Table 9. *Antibody concentration of sera of rabbits before and after injection with cortisone acetate*Dose of cortisone acetate was 2 mg./kg. Antibody concentrations are expressed as μ g. of antibody N/ml. of serum.

Time after last immunizing injection (days)	45 hr. before cortisone	21 hr. before cortisone	5 min. before cortisone	6 hr. after cortisone	<i>Vitro/vivo</i> ratio
17	420	380	340	280	0.73
21	—	—	280	250	—
23	—	—	200	160	—
29	440	440	390	380	0.29
52	—	700	670	640	0.63

dose (1.5 mg./kg. body wt.) of mepyramine maleate 1 hr. before the histamine injection. No signs of shock were observed. The leucocyte counts (Table 8) show that each dose produced an appreciable drop in both the lymphocyte and granulocyte counts. However, the effect was not as large or as prolonged as that seen after the injection of excess of antigen, and the leucocyte counts were nearly back to their pre-injection level 1 hr. after the histamine was administered.

Antigen elimination and leucocyte counts after administration of cortisone

In preliminary experiments it was shown that 2 mg. of cortisone acetate suspension/kg., injected intramuscularly, caused a marked reduction in the lymphocyte count of the blood 5–6 hr. later. This dosage was therefore adopted. Immune rabbits were bled one to three times at intervals before being injected with cortisone, and again 6 hr. after, and the amount of antibody nitrogen in the serum from each blood sample was determined. The results (Table 9) indicate that there was no increase in the antibody content of the serum after the injection of cortisone. In fact there was always a decrease, but as a decrease was also observed throughout the period during which observations were being made, it is considered unlikely that this was due to the action of cortisone. The rabbits were all used at least 17 days after their last immunizing injection, at which time their serum-antibody titres would be expected to be declining. A further

factor in this decline may have been the loss of blood, for each calibration involved the removal of 5–10 ml. of blood, corresponding to about 3–5 % of the circulating plasma volume.

Vitro/vivo ratios were also determined on three of these rabbits but the results (Table 9) were well within the normal range for immune rabbits not treated with cortisone (cf. Tables 1 and 2).

The leucocyte counts made during these experiments (Table 10) show a picture very similar to that observed after the injection of antigen into immune rabbits not treated with cortisone. Again there is a large drop in both lymphocytes and granulocytes, the fall in the latter being more pronounced, with some sign of recovery towards the pre-injection numbers 1 hr. later.

DISCUSSION

The experimental difficulties in obtaining an accurate measure of the amount of injected antigen rapidly removed from the circulation of an immune rabbit are very great, but the regularity with which low *vitro/vivo* ratios are obtained with these animals makes it reasonably certain that the phenomenon is a real one.

There seem to be two distinct types of explanation possible: (a) that the plasma antibody is in some way acting more efficiently *in vivo* than *in vitro*, or (b) that there is some source of rapidly available antibody (here termed 'non-plasma antibody') other than that in free circulation in the

Table 10. *Lymphocyte and granulocyte counts before and after the injection of bovine albumin into immune rabbits treated with cortisone acetate*

Dose of cortisone acetate was 2 mg./kg. All figures are number of cells/mm.³ of blood.

Time	No. 1		No. 2		No. 3		No. 4		No. 5	
	Lympho- cytes	Granulo- cytes	Lympho- cytes	Granulo- cytes	Lympho- cytes	Granulo- cytes	Lympho- cytes	Granulo- cytes	Lympho- cytes	Granulo- cytes
5 min. before cortisone	4500	5000	6000	5200	3900	4300	2800	3400	2600	2900
5 hr. after cortisone	1800	6800	1000	5600	1700	5300	1900	6300	1300	4500
6 hr. after cortisone, 5 min. before antigen	1600	4700	1300	3800	3300	10000	840	7200	700	3600
15 min. after antigen	340	140	500	180	1800	290	480	100	400	80
30 min. after antigen	980	350	1300	430	2500	390	670	100	730	140
60 min. after antigen	1300	940	1700	1100	3700	500	670	100	1600	830

plasma. That the actual plasma antibody is capable of precipitating more antigen *in vivo* than *in vitro* seems unlikely, since the precipitin reaction performed *in vitro* involves refrigeration as well as incubation, and it is well known that refrigeration causes a small but significant increase in the total amount of precipitate obtained (see, for example, Heidelberger & Kendal, 1935). Moreover, in passively immunized animals the *vitro/vivo* ratio does approximate to unity. There remains the possibility, however, that the amount of plasma antibody in actively immunized rabbits may be increased *in vivo* by drawing on reserves of antibody circulating in other tissue fluids. Gitlin & Janeway (1953) have shown that antibody is present in other body fluids in equilibrium with the plasma, and can readily pass from one fluid to another. However, this passage is normally a slow process compared with the rate of elimination of antibody from the blood stream by the 'fast reaction', so it is unlikely to be a significant factor in causing the *vitro/vivo* ratio to be less than unity. Although marked changes in the vascular endothelium occur when antigen combines with antibody *in vivo*, even in animals pretreated with mepyrmine (see, for example, Lecomte & Hugues, 1956), it is unlikely that these changes would be great enough to increase the rate of diffusion of antibody into the plasma sufficiently to account for the observed effect. Moreover, this reserve of antibody was presumably present to a considerable extent also in the passively immunized animals, and in those which had not received an immunizing injection for 19 weeks, which did give low *vitro/vivo* ratios. Although these animals had a lower serum-antibody titre than most of the other immune animals and might therefore be expected to have suffered an antibody-antigen reaction of less biological severity, resulting in smaller changes in the nature of the vascular endothelium, they yet had significant amounts of circulating antibody, comparable with that in some of the less highly immunized animals which gave low *vitro/vivo* ratios.

An alternative method by which the plasma antibody could act more efficiently *in vivo* than *in vitro* might be by the formation of soluble antigen-antibody complexes which are removed by the reticulo-endothelial system more rapidly than is the free antigen in normal rabbits. Francis *et al.* (1957) have shown that this is not the case with soluble complexes prepared in the far antigen-excess zone, but it does not necessarily follow that the biological behaviour of larger molecular aggregates left in solution after the precipitation of one-third to one-half of the antibody in the moderate antigen-excess zone will be the same as that of the simpler complexes found in the far antigen-excess zone, which are mainly of the type $Ab(Ag)_2$. In

order to settle this point these experiments have been repeated, with the supernatant fluid obtained by adding to the antiserum double the amount of (labelled) antigen required to give maximal precipitation, i.e. the same relative amount as used in the experiments reported here, and centrifuging off the precipitate. The results obtained were exactly comparable with those reported previously, i.e. there was no evidence at all of a 'fast reaction'.

In view of these facts it would seem that the alternative hypothesis, that the low *vitro/vivo* ratios regularly observed with actively immunized rabbits up to 3 months from cessation of the immunizing-injection schedule, must be due to the presence of 'non-plasma antibody', i.e. antibody not in free circulation but rapidly available for reaction with circulating antigen.

Graphical analysis of the persistence curves of bovine albumin in the blood of the immune rabbits also suggests that at least two processes are contributing to what has previously been called the fast reaction (Francis *et al.* 1957). It has not been possible to analyse accurately the curves representing this fast reaction, but they could be composed of two exponential curves, the 'very fast' component having a short half-life of 5-10 min. (which is probably the normal fast reaction due to reaction of the antigen with circulating antibody), and a 'fairly fast' component having a half-life of the order of 30-40 min., which might represent reaction with non-plasma antibody.

The results of the passive-immunization experiments, where the antigen is presumably reacting only with circulating antibody, suggest that the complex nature of the curve representing the fast reaction is an indication of the presence of 'non-plasma antibody'. Both from the nature of the curves, and from the *vitro/vivo* ratios observed, it appears that the rabbits tested 19 weeks after their last immunizing injection had no 'non-plasma antibody'. This could be explained by supposing that antibody when first formed is retained in the antibody-forming tissues and later released into the circulation, so that when antibody production has ceased all this initially tissue-bound antibody will be released into the blood stream.

The curves of the persistence of bovine albumin in the blood of normal rabbits during the first 5 hr. after its intravenous injection are different from those observed over shorter periods of time. The diffusion of an injected protein out of the blood stream into the lymph in various parts of the body is a complex process (Benson, Kim & Bollman, 1955), and though the persistence curves described in this paper have been analysed into slow and fast components (the latter sometimes a complex one) it is very difficult to give any definite physical meaning to these reactions. In fact, the analyses

have been performed only in order to give some quantitative expression to the differences observed between the persistence curves in normal rabbits and in immune rabbits given a double test injection of antigen. The persistence curves observed in these two groups differ in two main respects. The half-life of the slow reaction is considerably shorter in the immune animals (Table 5), and also the percentage of antigen that is acted upon by the fast reaction is greater than in the normal rabbits. Both these factors contribute to a considerably greater elimination of antigen from the blood of the immune rabbits during the 5 hr. experimental period. Thus the behaviour of a small second dose of antigen given 30 min. after a dose calculated to precipitate all the antibody in the animal's plasma seems to be intermediate between that observed after a single injection into immune and normal animals. In other words, there is still some mechanism for reacting with injected antigen and removing it from the blood stream, even when all available circulating antibody has been precipitated. The rate at which this mechanism can operate is a good deal slower than the rate of removal of antigen from the blood by reaction with circulating antibody.

Dougherty *et al.* (1945) showed by semi-quantitative methods that injections of adrenocortical steroids into anamnestic rabbits sensitized with sheep red cells produced a marked lymphocytopenia and a simultaneous sharp rise in the titre of plasma haemolysin, and Sanders, Florey & Barnes (1940) have also shown that the daily rate of passage of lymphocytes into the circulation, from the lymphatics, is considerably greater than the total number present at any one time. This secondary or anamnestic response to a non-specific stimulus has not been confirmed by accurate quantitative serological methods. For example, Heidelberger & Kendal (1935) were unable to obtain any increase in the titre of rabbit anti-ovalbumin serum by injecting large amounts of horse serum, and van der Scheer, Bohnel, Clarke & Wyckoff (1942) observed that rabbits hyper-immunized with type I pneumococci gave, after rest, an anamnestic response on injection of type I specific polysaccharide, but not on injection of horse globulin. Harris & Henle (1948) also observed no anamnestic response accompanying the lymphocytopenia produced by infection with influenza virus, and Fischel, LeMay & Kabat (1949) demonstrated that there was no increase in the rabbit-serum titre of anti-chick ovalbumin after X-irradiation or treatment with corticotropin, although a marked lymphocytopenia was produced, sometimes with and sometimes without a general leucopenia. In the experiments reported here also, it seems doubtful whether lymphocytopenia

can have had any significant effect in increasing the amount of available antibody, since the quantitative effect of 'non-plasma antibody' on antigen elimination is frequently considerably greater than that of plasma antibody, and it is unlikely that the amounts of additional antibody required could be made available by the lymphocytes in the short time involved. Moreover, the lymphocytopenia accompanying injection of cortisone had no effect on the *vitro/vivo* ratio and did not increase the circulating antibody titre of the serum.

Dragstedt *et al.* (1940) have previously shown that perfusion of lungs with blood to which has been added both an antigen and its specific antiserum leads to a fall in the leucocyte count, and Stavitsky *et al.* (1949) have demonstrated that a fall in polymorphonuclear leucocytes follows the injection of antigen into sensitized rabbits. The experiments reported here confirm these observations and, in addition, show that there is also a reduction in the number of circulating lymphocytes after injection of antigen.

If it is true, as suggested above, that the removal of injected antigen from the blood of immune rabbits is due partly to a reaction which proceeds more slowly than the reaction with circulating antibody, this rapid fall makes it seem improbable that circulating leucocytes are involved. The fall is more likely to be associated with the 'very fast reaction' of antigen with circulating antibody. A possible explanation is that the leucocytes might ingest the antigen-antibody precipitate, which we believe is formed in the blood stream (Francis *et al.* 1957), by phagocytosis. Another possibility is that the antigen might cause the clumping of the leucocytes, perhaps by combination with antibody on the surface of these cells. Such clumping of leucocytes in the blood of immune rabbits has been observed by Abell & Schenck (1938) after the injection of large doses of antigen. Any such clumps of leucocytes that might be formed would almost certainly be filtered out of the circulation very rapidly, and if there were any appreciable quantity of antigen immobilized in these clumps they could account for at least a part of the extra antigen which is removed *in vivo* above that which is precipitable by the antiserum.

It is difficult to decide what part, if any, liberation of histamine plays in this leucopenia. We have confirmed the observation of Bierman *et al.* (1951) that histamine itself causes a leucopenia, but even comparatively large doses (2 mg./kg. body wt.) have a rather smaller effect than the amounts of antigen we have injected. Thus it seems probable that this leucopenia is not entirely due to liberation of histamine.

The evidence so far discussed strongly suggests that there is a considerable amount of non-plasma

antibody which is still fairly rapidly accessible to the circulating antigen, and there is evidence that it may be bound in some tissues.

It is certain that the tissues of the doubly injected immune rabbits can take up some of the second injection of antigen, for they have the high apparent blood volumes characteristic of immune animals. If the non-plasma antibody is bound in the tissues it must be at sites where it would be accessible for reaction with the antigen in the blood. One possibility is that it might be bound in an organ in which the blood must traverse sinuses. Such an organ is the liver, and evidence that this organ may contain some tissue-bound antibody is provided by the finding that considerably more of the injected antigen is found in the livers of actively immunized than of passively immunized rabbits after the injection of small amounts of antigen, which are rapidly and nearly completely removed from the blood. This indicates that in actively immunized animals the liver possesses some mechanism, for removing antigen from the blood, which is lacking in passively immunized animals.

Dixon & Talmage (1951) have shown that there is no difference in the rate of catabolism of trace-labelled bovine-serum γ -globulin when administered to actively and passively immunized rabbits. However, the experiments reported in this paper do show differences in their response to injection of antigen. These differences are probably based on differences in the distribution of antibody in the animal, rather than on any enzymic adaptations accompanying production of antibody. It seems that the immune response of rabbits to injected antigens cannot be explained solely in terms of their reaction with circulating antibody.

SUMMARY

1. Actively, but not passively, immunized rabbits can eliminate rapidly from their blood more antigen than their sera can precipitate *in vitro*, except when they have been in the immune state for a long time.

2. When immune rabbits are given an injection of antigen sufficient to react with all the antibody in their plasma, a second small injection of antigen is eliminated from the blood more rapidly than a similar dose injected into normal rabbits. There is evidence for deposition of part of the second injected dose in the liver, lungs and spleen of the immune rabbits.

3. The significance of these observations is discussed and it is concluded that some non-plasma antibody is partly responsible for the immune responses observed when an antigen is injected into specifically immunized rabbits.

4. This non-plasma antibody is unlikely to be mainly in the lymphocytes or lymphoid tissue.

5. Injection of antigen into immune, but not into normal, rabbits causes a profound leucopenia, which is due to a fall in the numbers of both lymphocytes and granulocytes. This phenomenon is not modified by the prior injection of cortisone in amounts sufficient to cause a lymphocytopenia.

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The Effects of 8-Azaguanine on the Biosynthesis of Ribonucleic Acid in *Bacillus cereus*

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The incorporation of the guanine analogue 8-azaguanine into the micro-organism *Bacillus cereus* has already been described (Matthews & Smith, 1956; Mandel, 1957; Mandel, Sugarman & Apter, 1957*b*). Almost all the combined 8-azaguanine was found in the ribonucleic acid (RNA) fraction of the cells (Mandel *et al.* 1957*b*) and much of it was released from this fraction by pancreatic ribonuclease in the form of 8-azaguanosine 2':3'-phosphate (Matthews & Smith, 1956). When [¹⁴C]8-azaguanine was used, a small amount of the analogue was found in the deoxyribonucleic acid (DNA) fraction, and the 'acid-soluble' fraction of the bacteria was also found to be radioactive

(Mandel *et al.* 1957*b*). The addition of a purine or a purine compound to a bacterial culture in which growth had been inhibited by the presence of 8-azaguanine had the effect of restoring normal growth. Simultaneously, some of the 8-azaguanine was lost from the RNA fraction and was released into the medium (Mandel, 1957).

In the present investigation the effects of 8-azaguanine on synthesis of ribonucleic acid in *B. cereus* were examined. Since the analogue was incorporated into the RNA it seemed possible that a number of alternative effects might be expected. One possibility was that the total amount of RNA per cell might increase in order to compensate to some extent for the presence of the unnatural base. On the other hand, the normal amount of RNA could be present with some of its purines replaced

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