The Use of Radioactive Isotopes in Immunological Investigations

11. THE FATE OF SOME CHEMICALLY MODIFIED PROTEIN ANTIGENS IN NORMAL AND IMMUNE RABBITS

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Proteins, chemically modified by the introduction of atoms or groups that are not normally present, have been widely used by immunochemists, particularly for studies on the specificity of antibodies (see Landsteiner, 1945). Modified proteins containing radioactive halogen or coloured azo groupings have also been used in a few studies of the fate of proteins after injection into animals, since their rate of elimination from the plasma can be followed by radioactivity or colorimetric measurements, and histological techniques can be used to trace them in tissues.

Sabin (1939) injected a coloured azoprotein into rabbits and detected dye granules in cells throughout the reticulo-endothelial system. When repeated injections of this protein were made, specific antibodies were produced, and it was observed that the amounts of dye found in the liver and spleen were inversely proportional to the antibody titres of the sera. Kruse & McMaster (1949) injected azoprotein containing a very highly coloured and very diffusible blue dye (*Echt-Saure-Blau*) into mice and observed that the dye was still detectable in their livers after 15 weeks.

The rates of elimination of coloured azoproteins from the blood of rabbits have been studied by Pratt & Gregersen (1941), and by Gitlin, Latta, Batchelor & Janeway (1951). The latter workers observed that the more highly labelled the protein was, the more rapidly did it disappear from the blood stream. Qualitatively similar observations were made by Fine & Seligman (1943), when they injected proteins labelled with varying amounts of ^{82}Br into dogs, and observed that the rates of elimination of these proteins were proportional to the number of atoms of halogen introduced. These findings suggested that these chemically modified proteins behaved differently from native proteins when injected into rabbits and other animals. This was confirmed directly by Dixon, Bukantz & Dammin (1951), who showed that a native protein (bovine γ-globulin), trace-labelled with ^{131}I, was eliminated from the blood of normal rabbits much more slowly than an azoprotein derived from it by coupling with diazotized *p*-aminobenzoic acid. The azoprotein also appeared to be retained longer than the native protein in a number of tissues (particularly the liver and spleen), and its breakdown products were excreted much more slowly than those derived from the native protein.

Because they have been so widely investigated in other connexions, it was decided to study the behaviour of these chemically modified proteins after intravenous injection into normal and specifically immunized rabbits, in continuation of the studies reported previously (Francis, Hawkins & Wormall, 1957). Since antibodies formed in response to the injection of one particular chemically modified protein (the homologous antigen) will react with other proteins into which the same determinant groups have been introduced (heterologous antigens), it was of interest to investigate the fate of both homologous and heterologous antigens in immune animals. Such a study is only possible if chemically modified proteins are used. Two types of proteins were selected: highly iodinated proteins, in which the principal determinant groups are the 3:5-diiodotyrosyl residues in the polypeptide chains (Wormall, 1930); and mustard-gas sulphone proteins (HO_2-proteins) produced by treating native proteins with an excess of 2-dichloroethyl sulphone (mustard gas sulphone, HO_2). The principal determinant groups of these proteins involve the 1:4-thiazan 1:1-dioxide structure (Boursnell, Francis & Wormall, 1946; Francis, Mulligan & Wormall, 1955*b*).

MATERIALS AND METHODS

Highly iodinated proteins (iodoproteins). Bovine-serum γ-globulins or rabbit-serum γ-globulins were iodinated as described by Banks, Francis, Mulligan & Wormall (1951), except that the concentration of added ammonia was reduced to 2N. For the preparation of radioactive iodoproteins a solution of 0·1 N-I_2 in 0·1 M-KI containing a suitable quantity of $Na^{131}I$ was used. The products were freed from unbound iodine, iodide, etc., by dialysis rather than by precipitation.

HO_2-Proteins. These were prepared from either ovalbumin or bovine-serum γ-globulins as described by Francis, Mulligan & Wormall (1955*a*). Again, purification was effected by dialysis rather than by precipitation.

Radioactivity measurements. In blood and tissues ^{131}I was measured as described by Francis *et al.* (1957).

In blood and tissues ^{35}S was determined after conversion into $BaSO_4$ by the method of Bailey (1937). The $BaSO_4$ was centrifuged and transferred quantitatively to nickel planchets 2·5 cm. in diameter with the aid of a few drops of 50% ethanol. The dried precipitates were counted under a bell counter (G.E.C. type EHM2S). Corrections for self-absorption were made as described by Francis, Mulligan & Wormall (1954).

Protein-bound ^{131}I. Blood samples were haemolysed with 5–10 vol. of 0·01 M-KI, and the proteins precipitated by the slow addition, with stirring, of 1 vol. of 100% (w/v) trichloroacetic acid. The precipitated proteins were centrifuged, washed twice with 5 vol. of 10% trichloroacetic acid, dissolved in N-NaOH and made up to a convenient volume for radioactivity measurement.

Weighed samples of about 5 g. of liver homogenate were mixed directly with 5 ml. of 20% trichloroacetic acid, centrifuged and washed twice with 10 ml. of 10% trichloroacetic acid. Because of the difficulty of dissolving the precipitate, radioactivity measurements were made on the combined supernatants; the protein-bound ^{131}I was then determined by the difference between the ^{131}I in the whole homogenate and that in the trichloroacetic acid extract.

Immunization of rabbits and injection of radioactive antigens. These were performed as described by Francis *et al.* (1957).

Calculation of results. The percentages of radioactivity in the blood at various times after the injection of radioactive antigens were calculated as described by Francis *et al.* (1957), and graphs of the persistence of radioactivity in the blood were plotted. It is not possible to analyse these persistence curves mathematically in any simple way as can be done after the injection of native proteins into rabbits.

The amounts of radioactivity found in the tissues of the rabbits are expressed either directly, or as percentages of the amount injected, since the amount of radioactivity in the blood at the time of death was always so low that it did not contribute significantly to the amount in the tissue.

RESULTS

Iodinated proteins

All the immune animals used for these experiments were immunized with iodinated bovine γ-globulin, and at the time of the experiment had antisera which reacted strongly with the test antigen used. Three groups (three or four in each group) of immune animals were injected with iodinated rabbit γ-globulin (0·1, 0·3 and 0·95 mg. of nitrogen/kg. body wt. respectively), and one group with the homologous antigen (0·5 mg. of nitrogen/kg. body wt.). Four groups of normal rabbits were also injected similarly.

The curves (plotted on a linear scale) for the antigen persistence in the blood of individual rabbits in each group were in reasonable agreement, and there were no significant differences in the general shapes of the curves, or in the percentages of either antigen eliminated in the first 30 min. from the blood of control or immune rabbits. In all cases over 50% of the antigen was eliminated within 5 min., over 80% within 15 min. and 82–95% within 30 min. After reaching a minimum value by 30 min., in many cases the amount of ^{131}I in the blood commenced to rise again, as shown in the typical curve in Fig. 1. That this rise is probably due to the release of ^{131}I-containing fragments split off from the iodoproteins after they had been removed from the blood stream was shown by determinations (on two normal and two immune rabbits) of the amount of protein-bound ^{131}I 10–15 min. and 60 min. after the injection of radioactive protein. The non-protein-bound ^{131}I increased from less than 10% of the total radioactivity in the blood at 10 min. to over 30% at 60 min. with both normal and immune rabbits (Table 1), so that it seems unlikely that there is any marked difference in the rates of catabolism of iodoproteins in normal and immune rabbits, at least during the first hour after injection.

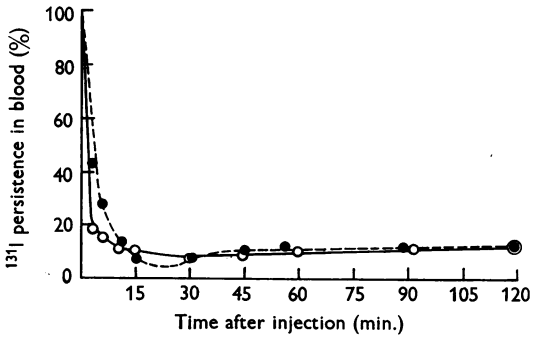

Fig. 1. Persistence of ^{131}I in the blood of a normal and an immune rabbit after the intravenous injection of highly iodinated bovine γ-globulin (0·5 mg. of N/kg. body wt.). ●– – –●, Normal rabbit; ○——○, immune rabbit.

Table 1. *Distribution of* ^{131}I *in blood of two normal and two immune rabbits after intravenous injection of highly iodinated rabbit γ-globulins* (0·3 *mg. of* N/*kg. body wt.*)

Time after injection (min.)		Normal (%)		Immune (%)	
10–14	Protein-bound ^{131}I	92*	91†	95*	94*
	Non-protein-bound ^{131}I	8*	9†	5*	6*
60	Protein-bound ^{131}I	62	58	69	66
	Non-protein-bound ^{131}I	38	42	31	34

* Samples of blood taken at 10 min.

† Samples of blood taken at 14 min.

The results of ^{131}I determinations on tissues of four rabbits killed 1 hr. after injection are shown in Table 2. The immune rabbits had considerably more ^{131}I in their livers and lungs than had the normal ones, even though the total elimination of the injected antigen from their blood was slightly less. In all cases, well over one-half of the ^{131}I in the liver was protein-bound, i.e. it was mainly due to the antigen and not to its breakdown products. Thus the deposition of antigen in these organs may, in some circumstances, be more characteristic of the immune response than the rate of elimination of the injected antigen from the blood stream.

Mustard gas sulphone-treated proteins (HO_2-proteins)

The rabbits used for these experiments were immunized with either HO_2-bovine γ-globulin, or with HO_2-ovalbumin, and the test injections were all made with the HO_2-γ-globulin (0·3 mg. of nitrogen/kg. body wt.), labelled with ^{35}S. Three of the four animals immunized with HO_2-γ-globulin had sera which reacted strongly with the test antigen, whereas the fourth showed only a weak reaction, but the two animals immunized with HO_2-ovalbumin had sera which reacted weakly with the homologous antigen and not at all with the test antigen.

The persistence of ^{35}S in the blood of some of these rabbits, for 60 min. after the injection, is shown in Fig. 2 (average curves for each group). The HO_2-protein is not removed from the blood of normal rabbits quite so rapidly as is a highly iodinated protein and there is no indication of the liberation of ^{35}S-containing fragments into the blood stream during the first hour after injection, since the blood radioactivity decreases progressively throughout this time. This label, therefore, seems a more

Table 2. ^{131}I *Content of tissues of two normal and two immune rabbits killed* 60 *min. after intravenous injection of highly iodinated rabbit* γ*-globulins* (0·3 *mg. of* N/*kg. body wt.*)

	Percentage of injected ^{131}I in tissue			
	Normal		Immune	
Tissue				
Liver	26·3 (70)*	27·3 (66)*	41·5 (80)*	43·5 (74)*
Lungs	0·5	0·3	11·4	8·9
Kidneys	3·2	3·3	3·1	2·8
Blood†	10·5	10·0	12·3	10·9

* Figures in parentheses give the percentage of liver ^{131}I which was protein-bound.

† Based on estimated blood volumes of 60 ml./kg. in each case.

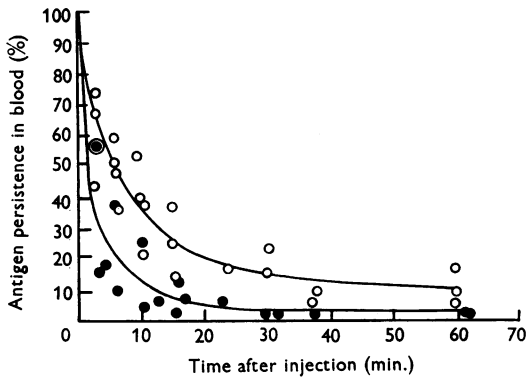

Fig. 2. Persistence of intravenously injected HO_2-bovine γ-globulin (0·3 mg. of N/kg. body wt.) in the blood of four normal rabbits and three immune rabbits. ○, Normal rabbits; ●, immune rabbits with sera reacting strongly with test antigen.

Table 3. *Antigen content of tissues of rabbits killed* 60 *min. after intravenous injection of 2-dichloroethyl sulphone-treated bovine* γ*-globulin*

Group *A*: two rabbits immunized with 'sulphone bovine γ-globulin'. Serum (1) gave a strong reaction with the test antigen, serum (2) a weak reaction. Group *B*: two rabbits immunized with 'sulphone ovalbumin'. Sera (1) and (2) reacted weakly with the homologous antigen, but gave no detectable reaction with the test antigen. Group *C*: two normal rabbits.

	Group *A*		Group *B*		Group *C*	
	(1)	(2)	(1)	(2)	(1)	(2)
Liver						
Concn. of antigen (μg. of N/g. of tissue)	6·6	11·0	6·8	6·1	3·9	5·4
Total antigen (%)	67	79	67	50	48	52
Lungs						
Concn. of antigen (μg. of N/g. of tissue)	0·69	1·8	0·94	0·27	0·23	0·21
Total antigen (%)	0·80	2·0	1·0	0·30	0·20	0·30
Blood						
Concn. of antigen (μg. of N/g. of tissue)	0·09	0·20	0·52	0·34	0·27	0·32*
Total antigen (%)†	1·7	4·0	10·2	6·8	5·4	6·3*

* Sample of blood at 30 min. The 60 min. sample, which would have had an antigen content only slightly lower, was lost.

† Based on estimated blood volumes of 60 ml./kg. in each case.

satisfactory one for chemically modified proteins, and HO_2-proteins are probably more suitable than highly iodinated proteins for studies *in vivo*.

The antigen was eliminated from the blood of the three highly immune rabbits considerably faster than from the blood of the normal rabbits, although its rate of elimination in the slightly immune rabbit injected with homologous antigen was not significantly different from that observed in the controls. The two rabbits immunized with HO_2-ovalbumin also behaved in a manner indistinguishable from the controls.

The results of analyses for ^{35}S in the lungs and livers of a small group of six rabbits are shown in Table 3.

As with the rabbits injected with iodoprotein, there was considerable deposition of the antigen in the liver and lungs of normal animals. The two animals immunized with HO_2-γ-globulin, however, showed a significantly greater deposition in these tissues, and this difference was actually more pronounced in the animal with the lower serum-antibody titre. Of the two animals immunized with HO_2-ovalbumin, whose sera gave no detectable reaction with the test antigen, only one showed any marked difference from the controls. In view of the differences in the amounts of antigen in the entrained blood in these tissues, calculations have also been made of the amounts of antigen actually deposited in the tissues, by making an allowance in each case for their blood content, based on the 'apparent blood volumes' of fifteen normal rabbits injected with ^{131}I-trace-labelled protein (Francis *et al.* 1957). These revised figures presented exactly the same overall picture as those given in Table 3, and they are therefore omitted from the table.

DISCUSSION

The fate of intravenously injected, chemically altered proteins differs considerably from that of native proteins (cf. Pratt & Gregersen, 1941; Dixon *et al.* 1951; Gitlin *et al.* 1951). In particular, they are eliminated very rapidly from the blood of normal animals (Haurowitz & Crampton, 1952), though the mechanism responsible for this behaviour is uncertain. It seems probable that it is connected with the extensive chemical alteration to the molecules which has occurred, accompanied by marked changes in their configuration. Crampton, Reller & Haurowitz (1953) have suggested that iodoproteins may form complexes with serum proteins which would rapidly be removed from the circulation.

The rate of elimination of these extensively modified proteins from the blood of specifically immunized animals during the first hour after injection is not necessarily greater than that shown by normal animals. In our experiments no significant differences could be observed in the elimination rates of iodoproteins by the two groups, but HO_2-proteins were eliminated significantly faster by immune animals having strong antisera at the time of injection.

The rate of deiodination of iodoproteins in the liver (and possibly other tissues) appears to be markedly greater than that of iodine trace-labelled proteins, but there is no evidence of any corresponding loss of the labelling group from HO_2-proteins in the short period studied. Iodoproteins therefore appear to be inferior to HO_2-proteins for investigations of this type *in vivo*.

Because of the large amounts of these antigens removed from the blood of normal rabbits, they are more extensively deposited in the livers than are native proteins. However, the recovery of radioactivity in the tissues examined is frequently rather low, for more than one-half of the injected antigen may not be accounted for. With the iodoproteins this may in part be due to catabolism of the antigen, followed by uptake of ^{131}I by the thyroid and excretion of non-protein-bound iodine through the kidneys. Certainly, the recovery of ^{131}I in the livers of normal animals injected with iodoprotein appears to be lower than the recovery of ^{35}S in the livers of normal animals injected with HO_2-protein, but in both groups it appears likely that there are considerable quantities of either the antigen or its breakdown products deposited in other tissues which were not examined.

More radioactivity is found in the livers and lungs of immune animals than in those of normal ones, even in circumstances where the antigen elimination rate from the blood is no faster than in normal animals. Thus the deposition of antigens in the liver and lungs seems to be a more constant feature of the immune response than their rapid elimination from the blood.

Proteins which have been extensively chemically modified may be regarded as more 'foreign' to the body than are native proteins from an immunologically distinct species, and it would appear that the bodily defence mechanisms against invasion of the blood stream by substances of this more 'foreign' nature are less dependent on a specific immune reaction than are the mechanisms concerned with substances of a less 'foreign' nature, even though the invading substances may be strongly antigenic.

SUMMARY

1. Highly iodinated γ-globulins and 2-dichloroethyl sulphone-treated γ-globulins are removed very rapidly from the blood of normal rabbits after intravenous injection. A considerable proportion of

the eliminated protein can be recovered in the liver but very little in the lungs.

2. Iodoproteins injected intravenously into specifically immunized rabbits are eliminated from the blood at the same rate as in normal animals, but immunized rabbits with good antisera to 2-dichloroethyl sulphone-treated proteins eliminate these proteins from their blood more rapidly than do normal animals.

3. With both antigens the deposition in the liver and lungs is greater in immune animals than in controls.

4. One hour after injection of iodoprotein at least one-third of the radioactivity in the blood is no longer protein-bound.

5. The deiodination of iodoproteins in the tissues renders them unreliable for studies *in vivo*, even for comparatively short-term experiments.

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The Formation of Hippuric Acid

THE INFLUENCE OF BENZOATE ADMINISTRATION ON TISSUE GLYCINE LEVELS

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The conjugation of benzoic acid with glycine has been extensively studied by many workers. One aspect of the problem that has received much attention is the origin of the glycine used for conjugation, but the exact source nevertheless remains uncertain. In the present investigation the problem has been approached by studying the concentration of certain tissue metabolites under various experimental conditions; this paper deals with the influence of the administration of benzoate upon the concentration of tissue glycine. The aim was to disturb the normal precursor → glycine reaction as little as possible, since the administration of relatively large amounts of suspected glycine precursors may cause unusual metabolic pathways to be followed. Few studies comparable with the present one appear to have been carried out. Christensen, Cooper, Johnson & Lynch (1947) and Vries & Alexander (1948) found that the administration of benzoate produced a fall in the concentration of free glycine in the blood of man, and Christensen, Streicher & Elbinger (1948) observed a similar decrease in the blood and liver of the guinea pig, although there was only a small change in the concentration of glycine in the kidney and no detectable change in muscle.

In the present investigation the influence of the administration of benzoate on the free glycine of various rat tissues and on the protein glycine of liver has been studied. Experiments in which blood glycine was investigated were also repeated on the rabbit since consecutive blood samples could be obtained from a single animal. In most of the experiments with the rabbit, urine samples were

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