The Use of Radioactive Isotopes in Immunological Investigations

12. THE BINDING OF A HAPTEN BY ANTIBODIES *IN VIVO**

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It is well known that haptens combine with antibodies *in vitro* (Marrack & Smith, 1932; Haurowitz & Breinl, 1933), although the forces which bind them together are comparatively weak. The *in vivo* neutralization of the physiological or pharmacological effects of haptens by antisera to antigens containing the corresponding determinant groups was demonstrated by Clutton, Harington & Yuill (1938) and by Butler, Harington & Yuill (1940). The former workers injected antisera to thyroxyl-proteins into rats and showed that in these rats thyroxine caused a smaller rise in the basal metabolic rate than in untreated rats. Butler *et al.* (1940) partially neutralized the antipyretic action of aspirin in rats by the prior injection of antisera to proteins that had been coupled with the azide of aspirin.

In the present study the binding of a hapten by antibodies *in vivo* has been inferred from experiments of a different type, in which the phenomenon has been investigated more directly. A radioactive hapten (3:5-di-iodotyrosine) has been injected into rabbits immunized against highly iodinated proteins [containing the 3:5-di-iodotyrosyl group as the main determinant group (Wormall, 1930)]. This injected hapten has been shown to persist longer in the plasma of these immunized rabbits than in the plasma of normal rabbits. Previous experiments along these lines by Mulligan (1950) were probably unsuccessful because large doses of di-iodotyrosine were injected, so that this compound was present in the plasma in considerable excess over the antibody.

MATERIALS AND METHODS

Isotopic labelling of di-iodotyrosine. The exchange reaction between di-iodotyrosine and I^- ion described by Miller, Anderson, Madison & Salley (1944) has been used to prepare small quantities of di-iodotyrosine of high specific activity. Di-iodotyrosine (1.2 mg., containing 5.06 μ equiv. of I) was suspended in 1.0 ml. of 0.2M-sodium acetate-acetic acid buffer, pH 4.6, and the required quantity of carrier-free $Na^{131}I$ and 0.1 ml. of 0.01M-KI (i.e. 1 μ equiv.) added. This mixture, in a flask fitted with an air condenser, was then heated for 1 hr. on a boiling-water bath. After the mixture

had been cooled, iodine was liberated from the inorganic iodide present by acidification with one drop of 10N- H_2SO_4 and addition of 0.1 ml. of 0.01N- KIO_3 . The iodine was extracted with several 1 ml. portions of CCl_4 , which were discarded, and the aqueous phase was then boiled briefly to free it of excess of CCl_4 , cooled and neutralized to pH 7.4. Under these conditions about 80% of the added radioactivity has been found to appear in the di-iodotyrosine on several occasions when this preparation has been carried out.

The $Na^{131}I$ supplied by the Radiochemical Centre, Amersham, is dispensed in 0.02M- $Na_2S_2O_3$ and it is essential that as little $S_2O_3^{2-}$ ions as possible should be introduced into the reaction mixture, as this substance inhibits the exchange reaction (cf. Gleason, 1955). In practice it has been found that as much as 0.2 ml. of 0.02M- $Na_2S_2O_3$ (containing the $Na^{131}I$) can be used without lowering the recovery of ^{131}I in the di-iodotyrosine.

Since di-iodotyrosine leaves the blood stream very quickly after intravenous injection (Tong, Taurog & Chaikoff, 1954), it was necessary to use large amounts of ^{131}I in order to trace the hapten satisfactorily. Thus some rabbits received as much as 500 μ C of ^{131}I , a dose which might be expected to have harmful biological effects if retained in the body for any length of time. However, Tong *et al.* (1954) have shown that 80% of an intravenously injected dose of di-iodotyrosine is excreted by rats in 24 hr., so that the injected rabbits were not likely to have suffered any harmful effect from the radiation.

Radioactivity measurements. These were made in scintillation counters of the type described by Banks, Tupper, Watts & Wormall (1955) coupled to a scaler and E.H.T. unit (Panax Ltd., Type 100C).

The radioactivity of all samples measured was such that they all gave net rates of counting which were above the background count rate. All counting was continued long enough to give a statistical accuracy of 2% or better.

Collection and fractionation of plasma. The solution of hapten was injected into the marginal vein of the right ear. Blood was collected from the marginal vein of the left ear into oxalated tubes and the plasma separated by centrifuging. Carrier di-iodotyrosine and KI were added to measured volumes of plasma and the proteins were precipitated by the addition of either 25 or 100% (w/v) trichloroacetic acid, so that the final trichloroacetic acid concentration was 10% (w/v). The precipitated proteins were centrifuged, washed thrice with 5 ml. of 10% trichloroacetic acid, dissolved in 1-2 ml. of N-NaOH and transferred to dishes for radioactivity measurements. The combined trichloroacetic acid washings were then treated with an amount of 0.02M- KIO_3 sufficient to react with all the KI present, the liberated iodine was extracted into CCl_4

* Part 11: Francis & Hawkins (1957).

(3 × 2 ml.), and the radioactivity of the aqueous phase (containing di-iodotyrosine and related compounds) was measured. The iodine was extracted from the CCl₄ into 1 ml. of an appropriate concentration of Na₂S₂O₃, the CCl₄ was washed twice with a little water and these aqueous solutions were combined in dishes for radioactivity determinations. The efficiency of this procedure was demonstrated by preliminary experiments in which known amounts of radioactive di-iodotyrosine and Na¹³¹I were added to serum and recovered in the appropriate fraction. Table 1 gives the details of these experiments.

Immunization of animals. Rabbits were immunized with alum-precipitated highly iodinated bovine plasma albumin according to the method of Proom (1943) and used about 14 days after their last immunizing injection. Their sera were calibrated as described by Francis, Mulligan & Wormald (1954), the homologous antigen being used.

Calculation of results. The radioactivity (counts/min.) in each fraction of plasma was expressed as a percentage of the total radioactivity injected, assuming immediate and uniform distribution throughout the circulating plasma. Plasma volumes were determined by the use of Evans Blue, as described by Francis, Hawkins & Wormald (1957). In most cases the recovery of radioactivity in the various

plasma fractions was checked by radioactivity measurements on the whole unfractionated plasma. The recoveries were usually satisfactory, the majority ranging from 90 to 105%. Any results outside these limits were discarded.

RESULTS

In some early experiments appreciable amounts of radioactivity were found in the protein fraction of the plasma from injected non-immunized rabbits. This must have arisen via the thyroid from the breakdown of the injected di-iodotyrosine. No comparable radioactivity was found associated with the plasma proteins of the immune animals, presumably because their thyroids were saturated with iodine derived from the breakdown of the antigen injected for immunization. In order to overcome this difference in behaviour between the normal and immune animals they were all given 0.05% of potassium iodide in their drinking water during the experiment and for the 5 days preceding it. This seemed to saturate the thyroids as judged by the fact that there was very little protein-bound ¹³¹I in the plasma either of normal or of immune rabbits after the hapten injection.

Only small amounts of di-iodotyrosine were injected, so that it should not be present in excessive amounts relative to the circulating antibody. Thus any hapten bound to the antibody would constitute a significant percentage of the total present. The molar ratio of di-iodotyrosine to antibody aimed at was about 0.5. Thus the actual amounts injected into different rabbits varied according to the antibody content of their sera, but

Table 1. Recovery of ¹³¹I-labelled di-iodotyrosine and ¹³¹I⁻ from serum added

	Radioactivity of compound added (counts/min.)	Radioactivity removed (counts/min.)	Recovery (%)
Di-iodotyrosine	1272	1239	97
Na I	2472	2351	95
Na I*	7476	7374	99

* Added to an antiserum to highly iodinated bovine albumin.

Table 2. Distribution of ¹³¹I in rabbit plasma after the intravenous injection of ¹³¹I-labelled di-iodotyrosine into three normal rabbits and three rabbits immunized against highly iodinated bovine plasma albumin

All figures are expressed as the amount of ¹³¹I present in the whole of the blood plasma, all the results being calculated as percentage of the amount of ¹³¹I injected. Mean figures are given, together with the ranges. The total radioactivity injected varied slightly from animal to animal, but averaged 1.7 × 10⁷ counts/min.

Time after injection (hr.)	Inorganic iodide		Protein-bound iodine		Residue*	
	Normal	Immune	Normal	Immune	Normal	Immune
3	4.4 (2.1-6.1)	7.8 (6.8-9.5)	0.39 (0.24-0.58)	0.54 (0.45-0.60)	1.6 (0.8-2.6)	1.8 (1.3-2.0)
6	4.0 (1.3-5.7)	7.0 (5.5-8.5)	0.15 (0.11-0.18)	0.27 (0.21-0.31)	0.43 (0.18-0.72)	0.79 (0.63-0.91)
9	2.6 (0.8-3.7)	5.9 (4.5-7.3)	0.17 (0.10-0.22)	0.27 (0.26-0.30)	0.23 (0.10-0.41)	0.42 (0.30-0.49)
11	2.1 (0.6-3.1)	4.7 (3.3-5.5)	0.13 (0.08-0.19)	0.22 (0.18-0.24)	0.27 (0.12-0.47)	0.44 (0.44-0.44)
24	1.2 (0.3-2.8)	3.3 (1.9-4.9)	0.07 (0.06-0.07)	0.09 (0.07-0.10)	0.10 (0.05-0.21)	0.14 (0.09-0.19)
32	1.1 (0.1-2.8)	2.5 (1.3-3.8)	0.05 (0.04-0.07)	0.09 (0.07-0.10)	0.06 (0.001-0.15)	0.11 (0.05-0.19)
48	1.0 (0.02-2.6)	0.9 (0.2-1.7)	0.03 (0.02-0.04)	0.04 (0.03-0.05)	0.04 (0.002-0.01)	0.09 (0.03-0.16)

* Assumed to be mainly di-iodotyrosine (see text).

the usual injection level was about 100 $\mu\text{g.}$ of di-iodotyrosine/kg. body weight. Control rabbits were also injected with slightly different amounts of the hapten so as to correspond as closely as possible with the different immune rabbits. However, no evidence was found of differences in behaviour due to the injection of different amounts of di-iodotyrosine within the range studied.

The results of a typical experiment with three immunized and three non-immunized rabbits are recorded in Table 2. The results show a wide scatter. However, very similar results were observed in two other experiments (in one of which the persistence of the hapten was observed in whole blood rather than in plasma), so it seems highly probable that the differences between the two groups of animals are real. Plasma samples from the immune animals in the first 24 hr. after injection of the di-iodotyrosine contained more di-iodotyrosine than the corresponding samples from the normal rabbits. Thereafter there was no significant difference between the two groups, but by that time the concentration of di-iodotyrosine in the plasma was extremely low. An unexpected observation was the presence of larger amounts of radioactive inorganic iodide in the plasma of the immune animals than in that of the normal ones. This must have arisen from the breakdown of the injected di-iodotyrosine and suggests that this substance is catabolized faster in the immune animals than in the normal ones.

DISCUSSION

Tong *et al.* (1954) have shown that ^{131}I -labelled di-iodotyrosine is very rapidly broken down after intravenous injection into rats, so that $1\frac{1}{2}$ hr. after injection nearly all the ^{131}I in the blood is in the form of inorganic iodide. Thus after injection of di-iodotyrosine it is necessary to fractionate the blood or plasma in order to determine how much di-iodotyrosine is actually present. The designations protein-bound iodine and inorganic iodide applied here to two of the fractions are clear-cut and reliable. The designation of the residue after removal of these two fractions as di-iodotyrosine is less certain, for Tong *et al.* (1954) have shown that compounds such as 4-hydroxy-3:5-di-iodophenyl-lactic acid and 4-hydroxy-3:5-di-iodophenylpyruvic acid may be found in rat's plasma after the injection of di-iodotyrosine. However, these compounds both contain the 4-hydroxy-3:5-di-iodophenyl group which is the determinant group of di-iodotyrosine and would therefore be expected to possess similar, if not identical, haptenic properties (cf. Snapper & Grunbaum, 1936). It therefore seems reasonable to conclude that the residual fraction, even if not an accurate measure of di-iodotyrosine, is a measure of substances that will function as haptens in the serological system under consideration.

The demonstration that more hapten is circulating in the plasma of the immune animals than of the normal animals during the first 24 hr. after injection provides definite, if somewhat indirect, evidence for the combination of hapten and antibody *in vivo*. The amount of hapten combined in this way seems to be extremely small under the conditions reported here. Thus in the experiment described in Table 2, the actual amounts of di-iodotyrosine in the plasma of the immune rabbits 6 hr. after the injection varied between 0.019 and 0.027 $\mu\text{m-mole/ml.}$, whereas in the normal animals the corresponding figures were 0.008 and 0.022 (mean 0.014) $\mu\text{m-mole/ml.}$ Thus the actual amount bound to antibody is likely to have been of the order of only 0.01 $\mu\text{m-mole/ml.}$ The antibody concentration in the rabbits' serum varied between 7 and 15 $\mu\text{m-moles/ml.}$ and was about a thousand times as great. When larger amounts of di-iodotyrosine (about 1 mg./kg. body weight; molar ratio of di-iodotyrosine to antibody, approx. 4) were injected into another group of rabbits, the absolute differences between the amounts in the plasmas of normal and immune rabbits were greater, and about 0.05 $\mu\text{m-mole}$ was bound to the antibody in 1 ml. of plasma. It seems probable that if still larger amounts of di-iodotyrosine were injected greater amounts would be bound to antibody, in correspondence with the known behaviour of *in vitro* systems. Owing to the comparatively low solubility of this compound at pH 7, this would not be possible without injecting very large volumes of solution.

The presence of more inorganic iodide in the plasma of the immune rabbits than in that of the normal ones is unexpected and its significance is not certain. It seems unlikely that there would be any difference between the two groups of animals in the rate of excretion of this substance, and if some of the di-iodotyrosine is bound to the antibody in the plasma it might be expected that it would be protected to a certain extent from catabolism in the immune rabbits. A possible explanation is that the immune rabbits might have become adapted in some way to catabolize di-iodotyrosine more rapidly than the normal rabbits. This would be a most interesting manifestation of the immune response, and this possibility is worthy of further investigation.

SUMMARY

1. The persistence in the blood plasma of various fractions containing ^{131}I has been determined after the intravenous injection of ^{131}I -labelled di-iodotyrosine into normal rabbits and rabbits immunized against highly iodinated bovine plasma albumin.

2. During the first 24 hr. after injection a substance whose properties resemble those of

di-iodotyrosine is present in higher concentration in the plasma of the immunized rabbits than in that of the normal ones. It is suggested that this is due to combination of the hapten with antibodies in the plasma of the immune animals.

3. During the same period inorganic iodide is also present in higher concentration in the plasma of the immunized rabbits than in that of the normal ones.

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The Mechanism of Carbohydrase Action

3. THE ACTION PATTERN OF β -AMYLASE*

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β -Amylase is a plant enzyme which hydrolyses α -1:4-linked polymers of D-glucopyranose by an endwise action. This action commences at the non-reducing end of the polymer, and the penultimate and subsequent alternate linkages are broken to yield maltose. These facts being established, there are two possible mechanisms for the enzyme action. These are a single-chain mechanism in which the enzyme completely hydrolyses one substrate molecule at a time or a multi-chain mechanism in which the enzyme attacks the ends of the polymer chain in a completely random fashion. *A priori* considerations suggest that single-chain action is unlikely, but the first investigations of the problem provided evidence in its favour (Swanson, 1948; Cleveland & Kerr, 1948). These authors' conclusions were criticized by Hopkins & Jelinek (1949) and by Bourne & Whelan (1950), both groups of authors

favouring multi-chain action. More recently, Kerr & Cleveland (1951) have provided further arguments in favour of single-chain action, while Hopkins & Jelinek (1954) and Bird & Hopkins (1954) take the contrary view.

There have been two methods of approach to the problem. The first is that used by Swanson (1948), Cleveland & Kerr (1948) and Kerr & Cleveland (1951). This employs native amylose as the enzyme substrate. The polysaccharide remaining at stages in the hydrolysis is isolated and its properties are compared with those of the original material. If the action is single-chain in character this residual polysaccharide should be unchanged amylose. In the second method, instituted by French, Levine, Pazur & Norberg (1950*b*), a much smaller substrate molecule is used in order to facilitate tests for the production of intermediate products. Using maltoheptaose, French *et al.* (1950*b*) observed that under optimum conditions for the enzyme action maltoheptaose was degraded to maltose and maltotriose without the intermediary formation of maltopentaose, i.e. the action was apparently single-chain. At

* Part 2: Whelan & Roberts (1953).

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