The Use of Radioactive Isotopes in Immunological Investigations

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

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(Received 11 February 1957)

It is well known that hapto ns combine with anti-
body s in vitro (Marrack & Smith, 1932; Hau ro witz
& Brei n, 1933), although the forces which bind
them together are comparatively weak. The in vivo
neutralization of the physiological or pharmaco-
logical effects of hapto ns by antisa to to antigens
containing the corresponding determinat groups
was demonstrated by Clutton, Harington & Yuill
(1938) and by Butler, Harington & Yuill (1940).
The former workers injected antisa to thyroxy-
pro teins into rats and showed that in these rats
thyroxine caused a smaller rise in the basal meta-
bo lic r at e than in untreated rats. Butler et al. (1940)
partially neutralized the antipro teic action of
aspirin in rats by the prior injection of antisa to
pro teins 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 experi-
ments of a different type, in which the phenomenon
has been investigated more directly. A radioac-
tive hapten (3:5-di-iodothyrosine) has been injected
into rabbits immunized against highly iodinated pro teins
[containing the 3:5-di-iodothyrosyl 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-iodothyrosine
were injected, so that this compound was present in
the plasma in considerable excess over the anti-
body.

MATERIALS AND METHODS

Isotopic labelling of di-iodothyrosine. The exchange reaction
between di-iodothyrosine and I⁻ ion described by Miller,
Anderson, Madison & Salley (1944) has been used to prepare
small quantities of di-iodothyrosyl of high specific activity.
Di-iodothyrosine (1-2 mg., containing 5-06 µequiv. 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
Na¹²³I and 0-1 ml. of 0-01M-KI (i.e. 1 µequiv.) 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-H₂SO₄
and addition of 0-1 ml. of 0-01N-KI. The iodine was
extracted with several 1 ml. portions of CCl₄, which were
discarded, and the aqueous phase was then boiled briefly
to free it of excess of CCl₄, cooled and neutralized to pH 7-4.
Under these conditions about 80% of the added radio-
activity has been found to appear in the di-iodothyrosine on
several occasions when this preparation has been carried out.
The Na¹²³I supplied by the Radiochemical Centre,
Amer sham, is dispensed in 0-02M-Na₂S₂O₃ and it is essential
that as little S₂O₃²⁻ ions as possible should be introduced into
the reaction mixture, as this substance inhibits the ex-
change reaction (cf. Gleason, 1955). In practice it has been
found that as much as 0-2 ml. of 0-02M-Na₂S₂O₃ (containing
the Na¹²³I) can be used without lowering the recovery of¹²³I
in the di-iodothyrosine.

Since di-iodothyrosine leaves the blood stream very
quickly after intravenous injection (Tong, Taurog &
Chaikoff, 1954), it was necessary to use large amounts of
¹²³I in order to trace the hapten satisfactorily. Thus some
rabbits received as much as 500 µC of ¹²³I, 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-iodothyrosine 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 scintilla-
tion 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 marginali vein of the left ear
into oxalated tubes and the plasma separated by centri-
fuging. Carrier di-iodothyrosine and KI were added to
measured volumes of plasma and the proteins were pre-
cipitated by the addition of either 25 or 100% (w/v) trichlo-
roacetic acid, so that the final trichloroacetic acid con-
centration was 10% (w/v). The precipitated proteins were
centrifuged, washed three with 5 ml. of 10% trichloroacetic
acid, dissolved in 1-2 ml. of x-NaOH and transferred to
dishes for radioactivity measurements. The combined
trichloroacetic acid washings were then treated with an
amount of 0-02M-KI0₂ sufficient to react with all the KI
present, the liberated iodine was extracted into CCl₄

(3 x 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. 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 & Wormall (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 & Wormall (1957). In most cases the recovery of radioactivity in the various plasma fractions was checked by radioactivity measurements on the whole unfractonated 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

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

<table>
<thead>
<tr>
<th>Radioactivity of compound added (counts/min.)</th>
<th>Radioactivity removed (counts/min.)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-iodotyrosine</td>
<td>1272</td>
<td>1239</td>
</tr>
<tr>
<td>Na ¹³¹I</td>
<td>2472</td>
<td>2351</td>
</tr>
<tr>
<td>Na ¹³¹I*</td>
<td>7476</td>
<td>7374</td>
</tr>
</tbody>
</table>

* Added to an antiserum to highly iodinated bovine albumin.

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### 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 x 10⁶ counts/min.

<table>
<thead>
<tr>
<th>Time after injection (hr.)</th>
<th>Inorganic iodide</th>
<th>Protein-bound iodine</th>
<th>Residue*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Immune</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>4·4</td>
<td>7·8</td>
<td>0·39</td>
</tr>
<tr>
<td></td>
<td>(2·1-6·1)</td>
<td>(6·8-9·5)</td>
<td>(0·24-0·58)</td>
</tr>
<tr>
<td>6</td>
<td>4·0</td>
<td>7·0</td>
<td>0·15</td>
</tr>
<tr>
<td></td>
<td>(3·3-5·7)</td>
<td>(5·5-8·5)</td>
<td>(0·11-0·18)</td>
</tr>
<tr>
<td>9</td>
<td>2·6</td>
<td>5·9</td>
<td>0·17</td>
</tr>
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<td></td>
<td>(0·8-3·7)</td>
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<td>(0·10-0·22)</td>
</tr>
<tr>
<td>11</td>
<td>2·1</td>
<td>4·7</td>
<td>0·13</td>
</tr>
<tr>
<td></td>
<td>(0·6-3·1)</td>
<td>(3·3-5·5)</td>
<td>(0·08-0·19)</td>
</tr>
<tr>
<td>24</td>
<td>1·2</td>
<td>3·3</td>
<td>0·07</td>
</tr>
<tr>
<td></td>
<td>(0·9-2·8)</td>
<td>(1·9-4·9)</td>
<td>(0·06-0·07)</td>
</tr>
<tr>
<td>32</td>
<td>1·1</td>
<td>2·5</td>
<td>0·05</td>
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<tr>
<td>48</td>
<td>1·0</td>
<td>0·9</td>
<td>0·03</td>
</tr>
<tr>
<td></td>
<td>(0·02-2·6)</td>
<td>(0·2-1·7)</td>
<td>(0·02-0·04)</td>
</tr>
</tbody>
</table>

* Assumed to be mainly di-iodotyrosine (see text).
the usual injection level was about 100 μg. of diiodotyrosine/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 131I-labelled diiodotyrosine is very rapidly broken down after intravenous injection into rats, so that 1½ hr. after injection nearly all the 131I in the blood is in the form of inorganic iodide. Thus after injection of diiodotyrosine it is necessary to fractionate the blood or plasma in order to determine how much diiodotyrosine 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 diiodotyrosine 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 diiodotyrosine. However, these compounds both contain the 4-hydroxy-3:5-di-iodophenyl group which is the determinant group of diiodotyrosine 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 diiodotyrosine, is a measure of substances that will function as haptons 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 diiodotyrosine in the plasma of the immune rabbits 6 hr. after the injection varied between 0·019 and 0·027 μmole/ml., whereas in the normal animals the corresponding figures were 0·008 and 0·022 (mean 0·014) μmole/ml. Thus the actual amount bound to antibody is likely to have been of the order of only 0·01 μmole/ml. The antibody concentration in the rabbits’ serum varied between 7 and 15 μmole/ml. and was about a thousand times as great. When larger amounts of diiodotyrosine (about 1 mg./kg. body weight; molar ratio of diiodotyrosine 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 μmole was bound to the antibody in 1 ml. of plasma. It seems probable that if still larger amounts of diiodotyrosine 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 diiodotyrosine 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 diiodotyrosine 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 131I has been determined after the intravenous injection of 131I-labelled diiodotyrosine 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.

It is a pleasure to thank Professor A. Wormall, F.R.S., and Dr G. E. Francis for constant advice and encouragement. The author is also grateful to Miss A. Routledge for her able and willing technical help during these investigations. The author is indebted to the Medical Research Council and the Central Research Fund of the University of London for grants (to Professor Wormall) which have partly covered the cost of these investigations.

REFERENCES


The Mechanism of Carbohydrase Action

3. THE ACTION PATTERN OF β-AMYLASE*

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(Received 25 February 1957)

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 (1950b), a much smaller substrate molecule is used in order to facilitate tests for the production of intermediate products. Using maltotriose, French et al. (1950b) observed that under optimum conditions for the enzyme action maltotriose was degraded to maltose and maltotriose without the intermediary formation of maltopentaose, i.e. the action was apparently single-chain. At