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THE IMMUNOLOGICAL REACTIVITY
AND BIOLOGICAL ACTIVITY OF IODINATED INSULIN

By
K. Brunfeldt, B. A. Hansen and K. R. Jørgensen

ABSTRACT

Pig insulin substituted with 1.0-10.8 I/mole was made in preparative quantities and analysed by elementary analysis, paper electrophoresis at pH 1.8, and UV spectroscopy. The preparations were tested for biological activity by intravenous injection into rabbits, by the mouse convulsion method, and by the effect on the 14C-D-glucose turnover in isolated rat fat cells. The reactivity of the preparations with pig insulin antibodies from guinea-pigs was tested by the double-antibody method in competition with 125I-insulin iodinated to about 0.7 I/mole. The reactivity with insulin antibodies was also tested with 125I-labelled insulin having a higher degree of iodination, by simultaneous iodination with 127I. Agreement between the calculated and the actual amount of iodine was demonstrated up to 8 I/mole. The UV absorption showed that at even higher degrees of iodination, iodine could be substituted into the tyrosine residues, showing that iodine also reacts with other structures in the insulin molecule. Weak non-covalent binding also seems to occur.

The iodination was found to have a reducing effect, as a function of the number of iodine atoms per mole insulin, on the reactivity with antibodies as well as on the biological activity. A reducing effect was also found, on the reactivity with antibodies as well as on the biological activity, when insulin was substituted with 0.9 mole fluorescein-isothiocyanate per mole insulin.

Blatherwick et al. (1927) demonstrated that iodine in a weak alkaline solution caused inactivation of insulin. Jensen et al. (1932) assumed that the cause of this inactivation was oxidation of disulphide bonds in the insulin. However, it was shown by Harington & Neuberger (1936) that insulin could be iodinated without being destroyed. On the basis of a determination of the number of tyrosine residues and the bound quantity of iodine, they concluded that the reaction with iodine involved a substitution in the tyrosine residues of insulin.
They demonstrated, moreover, that the iodination of the tyrosine residues caused a reduction of the biological activity. On intravenous injection into rabbits they found a reduction to 5–10%, and by the mouse convulsion method a reduction to 15% of the biological activity in the starting material. By partial de-iodination by catalytic reduction, part of the lost biological activity could be recovered.

Fraenkel-Conrat & Fraenkel-Conrat (1950) demonstrated that the loss of biological activity depended on the intramolecular distribution of the iodine which in turn depended on the method of iodination. In two preparations in which 50% and 56% of the tyrosine residues were iodinated, with disubstitution of 18% and 33% respectively, the biological activity of the two preparations, determined by the mouse convulsion method was 67% and 27% of that of the starting material. Lee (1957) concluded from the results of Fraenkel-Conrat & Fraenkel-Conrat (1950), that monosubstitution with 2 I/mole would not lead to any loss of biological activity.

de Zoeten & van Strijk (1961), determining the hypoglycaemic activity on rabbits of two preparations iodinated to 0.8 and 3.0 I/mole, found 80% and 30% respectively of the activity of the starting material.

Garratt (1964) measured the glucose uptake by isolated rat diaphragm following addition of insulin preparations obtained by ICl iodination. No reduction was found in biological activity with a degree of iodination of 3.2 I/mole, while 6.8 I/mole caused complete inactivation.

Izzo et al. (1964) studied the biological and immunological properties of insulin iodinated with a quantity of iodine corresponding to 0.6–11.1 I/mole. An iodine content of 1 I/mole or less did not cause any loss of biological activity as measured by the mouse convulsion, rat diaphragm, or rat fat pad method. Higher degrees of iodination caused gradual reduction of the biological activity. On the other hand, there was no effect of iodination on the reactivity of insulin with insulin antibodies until a degree of iodination of 10.4 I/mole was reached.

Rosa et al. (1966) studied the effect of iodination on the biological activity as determined by the rat fat pad method and the reaction of the S-S bonds with sulphite. They found that iodination resulted in an identical percental reduction of both properties. The most marked change occurred between 2 and 4 I/mole, when the reduction ranged from approximately 95% to approximately 25%.

Ooms & Arquilla (1966) demonstrated a reduction of the reactivity with insulin antibodies at 2 and 4 I/mole, but not at 1 I/mole. Identical results were obtained with and without pre-precipitation.

A brief survey dealing with iodine-labelling in the study of the biochemistry of insulin touching the topic of this paper has been published recently (Brunfeldt 1967).
The main object of the present study was to investigate the effect of iodination of insulin on the reactivity with insulin antibodies, as evaluated by the double-antibody method of Hales & Randle (1963). The biological activity of the preparations was tested on rabbits and mice as well as on isolated fat cells. The preparations used were characterized by elementary analysis, paper electrophoresis, and UV absorption.

MATERIALS AND METHODS

Insulin
Crystalline pig insulin containing approximately 7% H2O stored over saturated CaCl2, 6 H2O at room temperature was used for all preparations. The zinc content was 0.45% of dry weight. The biological activity determined by the mouse convolution test, was 25 units/mg dry weight.

Iodination with 125I
Derivatives having high specific radioactivity were prepared by iodination of 10 μg pig insulin by the method of Hunter & Greenwood (1962). The iodination was done as described by Brunfeldt & Jørgensen (1967). The degree of iodination with direct use of the carrier-free 125I- solution was about 0.7 I/mole. At higher degrees of iodination 125I- was added as 10 μl of a solution containing 11.495, 22.989, and 45.978 mg KI/100 ml, corresponding to a desired degree of iodination of 4, 8, and 16 I/mole, was pipetted into the ampoule before the addition of insulin.

Iodination with 127I
The iodination was carried out by slow addition of a 127I3- solution, which was about 0.1 N with regard to iodine. The 127I3- solution was made from 16.613 g KI + 6.347 g I2 + distilled water to make 500 ml. The normality of the solution was determined by adjustment with 0.1 N Na2S2O3 against KIO3. 400 mg (analytical values: 0.8-12.8 I/mole) or 250 mg (analytical values: 9.2-10.8 I/mole) pig insulin was dissolved to a concentration of 10 mg/ml in 0.5 N glycine buffer, adjusted to pH 9.5 with 60% KOH or concentrated NH3 water. By gel filtration K+, derived from the adjustment of the glycine buffer with KOH, and insulin, were only partially separated. By adjustment with concentrated NH3 water, on the other hand, an ash content in the ready product is avoided, and the NH4+ salt of insulin formed splits off NH3 on freeze drying. The iodination was carried out at 0°C during stirring. According to the degree of iodination the addition of the I3- solution was carried on for 1-4 hours. Stirring was then continued for another half-hour. Calculation of the necessary quantity of I3- solution was done as follows:

\[
\text{mg dry pig insulin} \times 2 \times \frac{1077.78}{\text{normality of I}_3^- \text{solution}} \times A = \text{ml;}
\]

A = desired number of gram-atoms iodine per gram-molecule of insulin: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, and 20.

Gel Filtration
Separation of protein and low-molecular compounds was carried out on Biogel P-4 or Sephadex G-25 fine. By ascending chromatography of the 127I-substituted prepara-
tions, a more reproducible separation was obtained than by descending chromatography. At the outlet of the column the absorption was recorded continuously at 2535 Å (LKB Uvicord) and for the $^{125}$I-substituted preparations, the radioactivity was also recorded. The radioactivity was measured with a scintillation detector connected to a Philips log-linear ratemeter. The effluent was carried from the outlet tube of the column through a piece of polyethylene tubing (I.D. 1 mm) passing below the scintillation detector. By gel filtration the greater part of Zn$^{++}$ was also removed from the insulin (Brunfeldt 1965). That part of the protein fraction which was eluted last was not collected, as it was contaminated with glycine. After the elution the protein fraction was freeze dried. The preparations were stored at 4° C.

**Biogel P-4**

The separation on Biogel was carried out by descending chromatography after iodination to 0–8.6 I/mole of 400 mg insulin. The reaction mixture was applied to a column, «Pharmacia» (100 cm $\times$ 2.5 cm I.D.) packed with Biogel P-4. The elution was done with 0.05 M NH$_3$ water (pH = 10.5), and the flow rate was 50 ml/hour.

**Sephadex G-25 fine**

The separation after iodination of 10 $\mu$g insulin with $^{125}$I, was carried out by descending chromatography. The reaction mixture was applied to a column (15 cm $\times$ 1.0 cm I.D.) packed with Sephadex G-25 fine. The elution was done with a barbiturate buffer, pH 8.6, containing 1% human albumin. The flow rate was 6 ml/hour. The solutions were stored in a 1/100 dilution with phosphate buffer, pH 7.4, containing 1% human albumin at approximately –20° C.

The separation on Sephadex of the $^{125}$I-substituted preparations was carried out by descending chromatography after iodination to 9.2–10.8 I/mole of 250 mg insulin. The reaction mixture was applied to a column (125 cm $\times$ 1.5 cm I.D.) packed with Sephadex G-25 fine. The elution was carried out with 0.05 M NH$_3$ water, pH 10.5, and the flow rate was 50 ml/hour. Later, it was found that ascending chromatography afforded better separation with up to 400 mg substance on a column LKB 4901 A.

**Fluorescein Labelling (F. T. C. insulin)**

Fluorescein-labelled insulin was prepared by reaction of insulin with fluorescein-isothiocyanate. The reaction is:

$$R\cdot N = C = S + R' - NH_2 \rightarrow S = C\left\langle \begin{array}{c} NHR \\ \end{array} \right\rangle$$

The preparation was made according to the method of Tietze et al. (1962). However, purification of the preparation on «active» coal was omitted, as the loss of protein in this process is considerable. Instead, purification was done by gel filtration on a column (100 cm $\times$ 3.2 cm I.D.) packed with Sephadex G-25 fine. As solvent and eluent, 0.05 M NH$_3$HCO$_3$, pH 9.0, was used. The flow rate was 96 ml/hour. After the elution the protein fraction was freeze dried. By the used procedure the fluorescein-group is coupled mainly to the N-terminal of the B-chain.

In addition, a preparation was made in which the addition of fluorescein-isothiocyanate was omitted.

**Analysis of the Preparations**

All the stated concentrations of insulin and insulin derivatives were calculated.
after drying to a constant weight in vacuum over P₂O₅ at room temperature, about 22° C, and corrected for ash content.

\[ ^{125}\text{I}-\text{insulin} \]

The degree of iodination of the \(^{125}\text{I}\)-insulin used, prepared by iodination with carrier-free \(^{125}\text{I}\) according to *Hunter & Greenwood* (1962) was about 0.7 I/mole, calculated on the basis of the specifications stated by The Radiochemical Centre, Amersham, England.

The assessment of the degree of iodination after iodination with carrier quantities of \(^{127}\text{I}\) was done by measuring the area below the curve, representing the recorded radioactivity at the outlet of the Sephadex column. The total radioactivity was taken to represent the total quantity of iodine, and the ratio of the protein-bound and the total radioactivity, to represent the degree of iodination. Calculated in this way the degree of iodination of the preparations was about 4, 6, and 7 I/mole insulin. When rinsing the ampoule, an attempt was made to rinse only the bottom of the ampoule.

\[ ^{127}\text{I}-\text{insulin} \]

The \(^{127}\text{I}\)-insulins prepared were analysed for iodine (Schöniger) and carbon. The degree of iodination was calculated as follows:

\[
\frac{\%\text{I}}{\%\text{C}} \times \frac{3074.82}{126.91} = \text{I/mole.}
\]

3074.82 is the total atomic weight of the 256 C atoms contained in one gram-molecule of pig insulin, and 126.91 is the atomic weight of \(^{127}\text{I}\). The result ± the standard deviation, S. D., was calculated as follows:

\[
K \times \frac{A \pm a}{B \pm b} = K \times \frac{A}{B} \pm K \times \frac{A}{B} \sqrt{\frac{a^2}{A^2} + \frac{b^2}{B^2}}
\]

\[
K = \frac{3074.82}{126.91} = 24.23, \ A = \%\text{I}, \ B = \%\text{C}, \ a = b = 0.2 \% \text{ absolute}.
\]

Thus the calculation of I/mole is correct only on the assumption that the number of C atoms remains unchanged after the iodination.

*F. T. C. insulin*

On the basis of the carbon content, the number of moles fluorescein-isothiocyanate per mole insulin was found to be 0.0 and 0.2 ± 0.3.

*UV Absorption*

UV absorption spectra of the various preparations, dissolved in TRIS-boric acid buffer, pH 9.1, in a concentration of 0.04 μM/ml, were recorded by a Beckman DK 2A spectrophotometer. The length of the cuvette was 1 cm. The measurements were performed at room temperature, about 22° C. At the stated pH, a maximum difference was obtained between the absorption ranges for unsubstituted and substituted insulin.

*Paper Electrophoresis*

Paper electrophoresis of the \(^{127}\text{I}\)-insulin derivatives prepared was carried out in 30 % acetic acid, pH 1.8, for 17 hours at room temperature, about 22° C, 100 V constant, amperage about 1.8 mA. Staining was with amido black. The preparations were dissolved to a concentration of 25 mg/ml in 30 % acetic acid, and 20 μl was added to each strip. However, it proved necessary to add urea to dissolve the preparation.
iodinated to 10.8 I/mole. Electrophoresis of the last-mentioned preparation was also
done in 20% acetic acid, 8 M urea, pH 3.27, for 20 hours at room temperature, about
22° C, amperage 1.0 mA constant, voltage 124–129 V.

**Biological Testing**

The biological activity of the ¹²³I-insulin derivatives was tested partly by intra-
venous injection into rabbits, partly by the mouse convulsion method, and partly by
their effect on the ¹⁴C-D-glucose turnover of isolated fat cells.

**Intravenous Injection Into Rabbits**

The preparations were dissolved up to a concentration corresponding to 0.2 mg un-
substituted insulin per ml in 0.9% NaCl by adding NaOH. The standard solutions
containing 0.2 mg per ml were prepared from crystalline pig insulin by the addition
of HCl. 0.1 ml per kg was injected intravenously into an ear vein of 8 rabbits.
Blood samples for blood sugar determination were removed 30 min before and im-
mediately after the injection of insulin and thereafter at the end of 10, 20, 30, 45,
60, 75, 90, 120, 150, 180, and 210 min. The blood sugar content was determined by
the method of *Hagedorn et al.* (1946). The area above the hypoglycaemic part of
the blood sugar curve was calculated by planimetry, and the results were compared
with those from standard preparations of crystalline insulin.

The fluorescein-labelled insulins were tested as described for the iodine-substituted
preparations.

**Mouse Convulsion Test**

The mouse convulsion test was carried out at 25° C and in other respects according
to the British Pharmacopoea 1963, using the screen described by *Thompson* (1946).

All preparations were dissolved to a concentration corresponding to 0.4 mg (10
units) unsubstituted insulin per ml. Dilutions of the more active preparations were
performed before injection of 0.25 ml per mouse. The preparations containing ≥ 8.6
I/mole were injected undiluted.

**Isolated Fat Cells**

The testing of the iodinated preparations on isolated rat fat cells was carried out
by the method of *Rodbell* (1964) as described by *Gliemann* (1965).

The determinations were carried out by reference to a standard curve showing the
insulin concentrations 0, 1.25, 2.5, 5.0, 10.0, 20.0, and 100 µ units/ml. Re-crystallized
ox insulin was used as standard. The preparations 0–2.0 and 3.0 I/mole were tested
in concentrations corresponding respectively to 10 and 30 µ units/ml unsubstituted
insulin. The preparations 4.1–10.8 I/mole were tested in a concentration corresponding
to 500 µ units/ml unsubstituted insulin.

**Immunological Technique**

For determining the reaction of the preparations with insulin antibodies the double-
antibody method with pre-precipitation (method B) of *Hales & Randle* (1963) was
used. This method has previously been described by *Brunfeldt & Jørgensen* (1967).
The accuracy (SEM) of the assay carried out with determinations in duplicate was on
an average ± 0.6% precipitated radioactivity (ordinate value) when counting 10⁴
pulses.

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RESULTS

Analytical Results

From Table 1 it is apparent that at iodination with up to 8 I/mole there is agreement between the calculated and the actual quantity of iodine per mole of insulin. At higher degrees of iodination there is an increasing difference between the calculated and actual values.

In testing for free iodine using thio-Michlers ketone (4,4'-bis-dimethyl-amino-thiobenzophenone) (Feigl 1958) on 25 µl glycine buffer, a positive reaction was obtained by adding 8 µl 0.05 N I$_3$ solution per ml buffer solution. During iodination it was only possible at the given addition rate of the I$_3$ solution and by adding a quantity corresponding to a degree of iodination of 10, 12, 15, and 20 I/mole, to demonstrate free iodine in the reaction mixture by an addition corresponding to iodination degrees higher than 7 I/mole. On the other hand, the reaction for iodine was negative after the addition of I$_3$ solution had been completed and the reaction mixture had been stirred for a few minutes. However, the more loosely bound iodine will be removed by gel filtration and drying, as the analytical values for the iodine content in preparations iodinated by addition of the same quantities of I$_3$ solutions were 9.2, 9.6, 10.3, and 10.8 I/mole.

UV Absorption

Absorption in UV of the individual $^{127}$I-insulin preparations showed increasing substitution in the 4 tyrosine residues of the insulin (A$_{14}$, A$_{19}$, B$_{16}$, B$_{28}$) with increasing degrees of iodination (Fig. 1). Thus, a shift of the absorption towards longer wavelengths and an increase in the molar extinction were demonstrable. At degrees of iodination higher than 8 I/mole there was an increased absorption around 311 mµ in relation to 8 I/mole. At an iodination

<table>
<thead>
<tr>
<th>Expected I/mole</th>
<th>Found I/mole</th>
<th>Expected I/mole</th>
<th>Found I/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>7</td>
<td>7.1</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>8</td>
<td>8.0</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>9</td>
<td>8.6</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>10</td>
<td>9.2</td>
</tr>
<tr>
<td>4</td>
<td>4.1</td>
<td>12</td>
<td>9.6</td>
</tr>
<tr>
<td>5</td>
<td>4.9</td>
<td>15</td>
<td>10.3</td>
</tr>
<tr>
<td>6</td>
<td>5.9</td>
<td>20</td>
<td>10.8</td>
</tr>
</tbody>
</table>

S. D. ± 0.1 (0.09–0.12) I/mole.
Fig. 1.
UV absorption of crystalline insulin and insulin substituted with 0–10.8 I/mole. TRIS-boric acid buffer, pH 9.1. Concentration 0.04 μm/ml.

degree of 10.8 I/mole, there was also an increased absorption at shorter as well as longer wavelengths.
Paper Electrophoresis

Using crystalline insulin there was a marked main fraction and traces of a slower and a faster migrating fraction as demonstrated by Sluyterman (1955) (Fig. 2).

With 0–4.9 I/mole there were no differences from crystalline insulin. With 5.9–10.3 I/mole there was an increasing quantity of a fraction anodal to the main fraction. At 9.6 and 10.3 I/mole there seemed to be yet another fraction anodal to the main fraction. At 10.8 I/mole there was merely a diffusely stained zone certainly due to precipitation of the preparation. In 20% acetic acid, 8 M urea, the preparation migrated as one broad band.

Biological Testing

As is apparent from Fig. 3 and Table 2 a decreasing activity was demonstrated with increasing degrees of iodination, by all three methods. While by the fat cell method, only traces of activity could be demonstrated by degrees of iodination exceeding 4 I/mole, intravenous injection into rabbits showed definite activity up to and including 9.2 I/mole, and the mouse convulsion test to 8.0 I/mole.

Fig. 2.

Paper electrophoretic fractionation at pH 1.8 of crystalline insulin and insulin substituted with 0–10.8 I/mole. Staining with amido black.
Effect of iodination on the biological activity of insulin measured by the isolated fat cell method, the mouse convulsion method, and by intravenous injection into rabbits.

*Fig. 3.*

Table 2.

Biological activity of $^{125}$I-insulin preparations.

<table>
<thead>
<tr>
<th>I/mole</th>
<th>fat cells % activity</th>
<th>mouse % activity</th>
<th>rabbit % activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>102 ± 6</td>
<td>98 ± 7</td>
<td>105 ± 16</td>
</tr>
<tr>
<td>1.0</td>
<td>69 ± 4</td>
<td>88 ± 8</td>
<td>91 ± 15</td>
</tr>
<tr>
<td>2.0</td>
<td>36 ± 2</td>
<td>63 ± 4</td>
<td>79 ± 14</td>
</tr>
<tr>
<td>3.0</td>
<td>19 ± 1</td>
<td>13 ± 1</td>
<td>48 ± 9</td>
</tr>
<tr>
<td>4.1</td>
<td>0.7</td>
<td>3.9 ± 0.5</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>4.9</td>
<td>0.5</td>
<td>4.5 ± 0.4</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>5.9</td>
<td>0.5</td>
<td>4.6 ± 0.6</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>7.1</td>
<td>0.5</td>
<td>2.2 ± 0.2</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>8.0</td>
<td>0.25</td>
<td>2.4 ± 0.2</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>8.6</td>
<td>0.25</td>
<td>trace</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>9.2</td>
<td>0.25</td>
<td>trace</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>9.6</td>
<td>0.25</td>
<td>trace</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>10.3</td>
<td>0.25</td>
<td>0</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>10.8</td>
<td>0.25</td>
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</table>

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Testing of fluorescein-labelled insulin showed, on intravenous injection into rabbits, hypoglycaemic activity of about 55% ± 12 of crystalline pig insulin, in the mouse convulsion method 57% ± 5 and in the fat cell method, 44% ± 3.

**Immunological Testing**

The immunological testing of the \(^{125}\text{I}-\text{substituted}\) preparations revealed a significant change in the course of the standard curve at degrees of iodination exceeding 1 I/mole (Fig. 4). On the other hand, identical standard curves were obtained with crystalline insulin and insulin substituted with 0 and 1.0 I/mole. The preparation called 0 I/mole was made by subjecting crystalline insulin to the same preparatory procedure as the iodinated preparations, apart from the addition of iodine. Between 3.0 and 4.1 I/mole there was a comparatively large difference, also demonstrable in another series of preparations from 0–8 I/mole. Between preparations containing 10.3 and 10.8 I/mole there was a very marked difference in reactivity.

To investigate whether the values depicted in Fig. 4 altered if the reaction time was longer, experiments were carried out on some of the iodinated preparations, prolonging the incubation period from the normal 24 hours to 48

![Fig. 4.](image)

The effect of iodination on the competition between \(^{125}\text{I}-\text{insulin}\) and \(^{127}\text{I}-\text{insulin}\) substituted with 0–10.8 I/mole with regard to binding to pig insulin antibodies.
and 72 hours (Table 3). There was a slight increase in the recovery of $^{125}$I-insulin when only this preparation was added. The recovery of $^{125}$I-insulin on addition of crystalline insulin and $^{127}$I-substituted insulin increased correspondingly. The increase demonstrated averaged 2.0 and 2.3% absolute after 48 and 72 hours' incubation respectively, in relation to the recovery after 24 hours' incubation. The values were calculated on the basis of the binding to antibodies when an excess of antibodies was present.

Experiments using $^{125}$I-labelled insulin in the same molar quantities, in which the degree of iodination was varied by adding $^{127}$I-, showed that the binding of I-insulin to the insulin antibodies in these experiments too, was appreciably reduced by an increase in the degree of iodination (Fig. 5).

$F. T. C. Insulin$

Immunological testing of fluorescein-labelled insulin revealed a definitely reduced reactivity with the insulin antibodies (Fig. 6).

**DISCUSSION**

As already mentioned, the calculation of the degree of iodination on the basis of the ratio %I and %C can be done only on the assumption that the number of carbon atoms per molecule of insulin remains unchanged. Conversion of the %C found for the various iodinated preparations to iodine-free insulin did not demonstrate a significant difference between the actual and the theoretical %C for 1.0–10.3 I/mole. For 10.8 I/mole, on the other hand, the %C was found to be too low. The %C of another preparation, also iodinated with a quantity of iodine corresponding to 20 I/mole and with an iodine content of 10.9 I/mole after gel filtration, was also too low, being 50.76, while the

<table>
<thead>
<tr>
<th></th>
<th>% Radioactivity in precipitate</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
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<tbody>
<tr>
<td>$^{125}$I-insulin</td>
<td></td>
<td>79.4</td>
<td>81.7</td>
<td>81.8</td>
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<tr>
<td>Standard</td>
<td></td>
<td>11.3</td>
<td>11.7</td>
<td>11.9</td>
</tr>
<tr>
<td>2.0 I/mole</td>
<td></td>
<td>13.6</td>
<td>14.7</td>
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<tr>
<td>4.1 &quot;</td>
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<td></td>
<td>67.7</td>
<td>68.9</td>
<td>69.7</td>
</tr>
</tbody>
</table>

Table 3.
The significance of the length of reaction time for the recovery of $^{125}$I-insulin in competition with $^{127}$I-insulin.
The effect of iodination on the binding of insulin to insulin antibodies estimated by the use of insulin substituted with $^{125}$I (0.7 I/mole) and $^{125}$I as well as $^{127}$I (4, 6 and 8 I/mole).

The effect of coupling with F. I. T. C. on the competition between $^{125}$I-insulin and insulin containing 0.9 mole F. I. T. C. per mole insulin.
The effect of iodination on the reaction between insulin and insulin antibodies. In A, calculated as per cent of the competitive effect of crystalline insulin at the same molar concentration. In B, expressed by the concentration of crystalline insulin which exerts the same competitive effect as the given concentration of the iodinated insulin, calculated as per cent of the molar concentration of the iodinated insulin.

The first two signatures in B appear to be filled up due to coincidence of all the three types of signatures.

Theoretical value was 53.22. Moreover, there was an increase in $\% \text{O}$ from the theoretical value 21.05 to 24.40. The $\% \text{H}$ was found to be 6.43, with a theoretical value of 6.65. Accordingly, the reduction of $\% \text{C}$ appears to be due, partly at least, to an uptake of oxygen, possibly caused by oxidative destruction of the imidazole ring in the histidine residues. Destruction of the imidazole ring in free histidine caused by iodine has been demonstrated by Roche et al. (1951) under conditions in which diiodo-tyrosine was stable. It is not known whether destruction of the imidazole ring of peptide-bound histidine can entail disruption of the peptide chain. By photooxidation of insulin at pH 7.0 and at 10° C it is possible to destroy the imidazole ring without disruption of the peptide chain (Weil et al. 1965).

N-terminal determination on 8.0 and 10.8 I/mole by the method of Fraenkel-Conrat et al. (1955) showed the normal amino acids: glycine and phenylalanine. An amino acid determination on a preparation substituted with 10.4 I/mole
after 24 hours’ hydrolysis in 6 N HCl at 105°C under nitrogen in an ampoule showed the theoretical ratio between mole lysine and mole arg, asp, glu, pro. ala, and phe. Thus, the present analytical results appear to show that iodination up to approximately 10 I/mole does not involve major changes in the composition of insulin. This is in keeping with Gruen et al. (1959) who could not on iodination with I₃⁻ solution to 8 I/mole, demonstrate any changes in the structure of the insulin molecule other than those which were to be expected because of the substitution with iodine. The changes demonstrated at 10.8–10.9 I/mole, however, call for further study of the conditions at higher degrees of iodination.

Disubstitution of the 4 tyrosine residues of the insulin molecule would correspond to a maximum degree of iodination of 8 I/mole. The analytical results show, therefore, that the iodine is able of reacting with structures other than the tyrosine residues in the insulin molecule. This is in agreement with Niemann (1966) who following enzymatic hydrolysis, demonstrated the occurrence of mono- and diiodo-histidine at iodination degrees of 6.1 and 8.1 I/mole. At 8.1 I/mole, for instance, 6% of the iodine was bound as monoiodo-histidine and 7% as diiodo-histidine. That the tyrosine residues are not fully disubstituted at 8 I/mole is also apparent from the finding that the UV absorption around 311 mµ at pH 9.1 increased further at degrees of iodination exceeding 8.0 I/mole (Fig. 1). At the given ratio (2:4) between the number of histidine residues and tyrosine residues the absorption at 311 mµ, i.e., at iodination degrees at which the histidine is mono- or di-substituted and at which uniodinated tyrosine residues do not occur, is determined by the iodinated tyrosine residues. This does most likely not occur, however, in tri-substitution of the histidine residues, since with substitution in 1-NH there is also absorption in the visible range (Brunings 1947). The preparation containing 10.8 I/mole is faintly brown-stained, and the increase in absorption in relation to 9.6–10.3 I/mole is, therefore, presumable due to substitution in the 1-position in the imidazole ring of the histidine residues. The ratio between iodinated tyrosine residues and histidine residues is conditioned by the marked difference in the rate at which the iodine reacts with the two amino acid residues. In experiments on the free amino acids, for instance, Li (1944) demonstrated that the iodine reacted 30–100 times more rapidly with tyrosine than with histidine.

Whether a slight degree of binding takes place to structures other than the tyrosine and histidine residues is as yet unknown. However, iodination of the phenylalanine residues under the given conditions must be considered less likely.

Formation of thyroxine may probably be excluded, at any rate at iodination degrees at which the iodine uptake is determined predominantly by the reaction with the tyrosine residues.
In the reaction mixture proper there must be a possibility of a non-covalent binding, e.g. by inclusion, as free iodine was not demonstrable during the reaction after brief stirring, even with an excess of iodine.

The paper electrophoretic separations (Fig. 2) carried out at pH 1.8 at which phenolic OH is not dissociated, show incipient separation at degrees of iodination at which the histidine residues begin to show demonstrable iodination. The appearance of a fraction anodal to the main fraction from 5.9–10.3 I/mole, and possibly yet another fraction anodal to the former from 9.6–10.3 I/mole, is therefore presumably due to the iodination of the histidine residues. Since only two new fractions are demonstrable, the reduction in positive net charge seems to be due to a reduction of the pK value for 3-NH⁺ in one or both of the histidine residues B₃ and B₁₀ in the event of disubstitution in the 2 and 5-position. Mono-substitution in the 2 or 5-position is stated to cause a reduction of the pK value from 6.00 to 4.18 in free histidine and at disubstitution in the 2 and 5-position leads to a further reduction to 2.72 (Brunings 1947). The corresponding values are stated by Schutte et al. (1966) to be 4.2 and 3.2. The explanation as to why the fractionation appears so clearly must be that the pK value for the 3-NH⁺ of the histidine residues is lower than for free histidine, presumably because of an effect on other positively charged groups. Destruction of the imidazole ring would also entail loss of a positive charge. With degrees of iodination at which a definite oxygen uptake could not be ascertained, however, this can only have taken place to a slight extent.

The significant reduction, to 69% of the biological activity found on testing on isolated fat cells at 1 I/mole, to 63% at 2 I/mole by the mouse convulsion method, and to 48% at 3 I/mole by intravenous injection into rabbits, is broadly speaking in agreement with the findings of other investigators, partly using other methods. de Zoeten & van Strik (1961), using rabbits, found the biological activity to be 80 ± 8% of that of crystalline insulin at a degree of iodination of 0.8 I/mole. By the rat fat pad method Rosa et al. (1966) demonstrated a significant reduction to 82% at a degree of iodination of 2.2 I/mole. At a range of iodination from 0.5 to 2.0 I/mole they found the values reduced, but not significantly, i.e. 94% and 90% respectively. Using the mouse convulsion method, Izzo et al. (1964) found 44% activity at 1.68 I/mole.

In assessing the effect of iodination it must be borne in mind that the iodination runs a heterogeneous course owing to the numerous substitution possibilities, 3⁴−1 = 80 due merely to the tyrosine residues.

Calculated on the basis of the reactivity of the individual tyrosine residues, the percental occurrence of the degrees of iodination 0, 1, 2, 3, 4, 5, 6, and 7, at an iodination degree of 0.8 I/mole, should be 41.8, 39.9, 15.1, 2.9, 0.3, 0, 0, at 3 I/mole 0.7, 7.4, 25.7, 34.5, 22.4, 7.7, 1.4, and 0.1 (de Zoeten & van Strik 1961). Indeed, by gradient electrophoretic fractionation Brunfeldt (1965) demonstrated a heterogeneous course of the iodination at iodination
degrees of 1–7 I/mole. The fractionation of 1 I/mole, however, appears to show a lower percental occurrence of unsubstituted insulin than 35% which may be calculated by interpolation on the basis of de Zoeten & van Strik’s (1961) values.

Part of the activity must then, at the lower degrees of iodination, be derived from unsubstituted insulin, and accordingly the biological activity of the iodinated part of the preparation must be lower than the biological activity obtained for the preparation as a whole.

The intramolecular distribution of the iodine is also, as mentioned in the introduction, of importance for the biological activity (Fraenkel-Conrat & Fraenkel-Conrat 1950). By iodination with $I_3^-$ under the given conditions, the tyrosine residues of the A chain is very easily iodinated. de Zoeten & de Bruin (1961) demonstrated that at an iodination with $I_3^-$ at pH 9.2 to 0.8 I/mole insulin, 86% of the iodine will be bound to the A chain and 14% to the B chain. At 3.0 I/mole the corresponding values were 79% and 21%. The amount of iodine bound as diiodo-tyrosine was by 0.8 and 3.0 I/mole 16% and 47% respectively in the A chain and 2% and 7% respectively in the B chain, corresponding to the presence of diiodo-tyrosine residues of 8% and 23.5% and 1% and 3.5% of the total number of substituted tyrosine residues. At an iodination degree of 3 I/mole, therefore, there should be a maximum of $54/2 \times 3 = 81$% insulin molecules containing a disubstituted tyrosine residue. The biological activity was reduced to 30% of that of crystalline insulin.

Lee (1957) performed a calculation on the basis of Fraenkel-Conrat & Fraenkel-Conrat’s (1950) experiments which appeared to show that mono-substitution by 2 I/mole is of no importance, while disubstitution entails loss of biological activity.

The reduction of the biological activity might also be due to an iodination of both or possibly one of the tyrosine residues of the B chain. Lee (1959), therefore, considered iodination of the B<sub>35</sub> tyrosine residue to be of decisive importance.

The substitution by iodine involves marked alterations in the chemical properties of the tyrosine residues, int. al. due to the electron attracting effect of the iodine, manifesting itself especially in a reduction of the pK value for the phenolic OH. For free amino acids the pK value for the phenolic OH at 25°C is stated to be 10.1 for tyrosine, 8.2 for 3-monoiodo-tyrosine, and 6.5 for 3,5-diiodo-tyrosine (Hughes 1957; Greenstein & Winitz 1961). However, the dissociation of the phenolic OH in the tyrosine residues is complicated by other negative charges in the insulin molecule. Inada (1961) has determined the pK values by spectrophotometric titration and found the following values: Free tyrosine 10.0, insulin – three of the tyrosine residues 10.4 the fourth, probably hydrogen bounded, 11.4. Gruen et al. (1959) also reported an average pK value of 7.9 for insulin substituted to 8 I/mole by iodination with $I_3^-$ in
glycine buffer, pH 9.5. However, this value is presumably rather too high, since as already mentioned, not all tyrosine residues will be disubstituted at this degree of iodination. Owing to the somewhat higher temperature in the organism, the pK value can be expected to be lower in in vivo experiments. It is reasonable to assume, therefore, that the disubstituted tyrosine residues are dissociated to a considerable extent in the organism.

It is not known whether a possible effect of an increased dissociation of the phenolic OH is due to the appearance of a negative charge or to disruption of hydrogen bonds in which the tyrosine residues take part, so that an alteration in conformation results. That one or more of the tyrosine residues take part in such external intramolecular hydrogen bonds is indicated by UV difference spectrophotometric measurements (Laskowski et al. 1960; Leach & Scheraga 1960). Intermolecular hydrogen bonds, in which the tyrosine residues play a part, must also be considered a theoretical possibility (Laskowski & Scheraga 1954). In the latter case the degree of polymerization would be influenced by the iodination. The degree of polymerization of the insulin, and hence its effective molecular weight in biological medium, is unknown (Krahl 1961).

Iodination, however, involves changes in the chemical properties of the tyrosine residues other than a reduction in the pK value of the phenolic OH. For instance, the binding of the 2 and 6 protons is apparently also influenced. Moreover, the steric effect exerted by the iodine atoms is important, as the iodine atom is considerable larger than the proton and of approximately the same size as the benzene nucleus (Brunfeldt 1965).

A common feature of all sequence-determined insulins from various species is the mutual position of the three S-S bonds. Rosa et al. (1966) demonstrated a strikingly similarity in the reduction of the biological effect and the reduction in the reactivity of the two inter-chain S-S bonds with sulphite. It is thus possible that the iodination of the tyrosine residues has an inhibitory effect on a reaction, in which one or more of the S-S bonds of insulin participate, and that this is in fact the process by which the hormonal effect is exerted and by which the insulin is at the same time inactivated.

It must be borne in mind, however, that the replacement of the S-S bond of oxytocin by the thioether bond S-CH₂ does not entail a reduction in the biological activity of oxytocin (Rudinger & Jošt 1964). Clauser et al. (1965) therefore, believe that the function of the internal S-S bond in the A chain is possibly to preserve the tertiary structure.

That the highly iodinated molecules do not react with the receptor, and thus block its reaction with endogenous insulin, seems to be indicated by the rabbit experiments in which none of the preparations caused hyperglycaemia. However, it is not possible to decide from any of the experiments whether the reduction in biological effect is due to an inhibition of the reaction with the
cellular receptor, or whether the iodination prevents the iodinated molecules from reaching the receptor.

As shown in Fig. 3 and Table 2 identical results were obtained with the non-iodinated preparations in the test systems used. Hence the discrepancies between the results obtained with the iodinated preparations, show that the non-iodinated and iodinated preparations are not dealt with in the same way in the different test systems. So the lower values obtained with the mouse convulsion method compared with the intravenous injections into rabbits may be due to a delayed resorption from the subcutaneous depot. That deiodination should cause the higher values obtained by these two methods compared with the values obtained by the isolated fat cells seems less likely, as by the intravenous injection into rabbits a decrease in activity is correlated to a shorter duration of the hyperglycaemia. However, a slight deiodination resulting in reactivation cannot be excluded by the lowest degrees of iodination. On the other hand the lower values obtained by the isolated fat cells, which also differ from those obtained by the rat fad pad method (Izzo et al. 1964; Rosa et al. 1966) and the rat diaphragm method (Izzo et al. 1964) may be due to an alteration of the surface properties of the isolated fat cells caused by the isolation procedure. Such an alteration may result in that a derivatization makes it impossible or more difficult for the insulin to pass the cell membrane or eventually react with it, than when the cells are intact. An inactivation by non-specific binding, however, seems less likely, since an increase in the concentration even up to 50 times that of the active preparations only brings about a barely demonstrable or no increase in the ¹⁴C-D-glucose turnover. In the mouse convulsion test a decreased resorption rate from the subcutaneous depot may tend to give too low values for the iodinated preparations. The results obtained with this method are in agreement with those of Izzo et al. (1964) who was able to demonstrate activity at 6 I/mole, but not at 9.6 I/mole.

Other derivatization also involves loss of biological activity as demonstrated by coupling with 0.9 mole fluorescein-isothiocyanate per mole insulin. Blocking of phenolic OH by acetylation also causes a loss of activity. This is presumably also caused by the blocking of phenolic OH by phenyl isocyanate and coupling in the ortho position with diazobenzene sulphonylic acid. However, phenylisocyanate also reacts with the free amino groups and with the guanidyl group, and diazobenzene sulphonylic acid also reacts with imidazole (Fraenkel-Conrat & Fraenkel-Conrat 1950).

Thus, while the reduction of the biological activity may be due to several factors, the effect of iodination on the reaction with antibodies must be due to a direct effect on the reaction between the two proteins.

The experiments using prolonged incubation demonstrate that the relation between the insulin-antibody-bound ¹²⁵I-insulin with a low degree of iodination and the other preparations tested does not alter on prolonged incubation.
This means that the course of the competitive reaction is normal and has been completed in 24 hours. The slight increase in the radioactivity bound to the insulin antibodies occurring on prolonged incubation, and which has also been demonstrated for \( ^{125} \text{I}-\text{insulin} \) only, is without doubt due to binding to less accessible positions in the insulin antibodies. The experiments using \( ^{125} \text{I}-\text{insulin} \) iodinated to higher degrees of iodination with carrier quantities of \( ^{127} \text{I} \) demonstrate that an effect of the iodination is also distinct in experiments using these preparations only. This means, as might be expected on the basis of the competition experiments, that the equilibrium between the insulin antibodies and the iodinated preparations is shifted to the left in the equation, as a function of the degree of iodination:

\[
\text{I-insulin} + \text{antibody} \rightleftharpoons \text{I-insulin-antibody}
\]

On the other hand, the reaction time until equilibrium has been attained, is the same. The somewhat higher degrees of binding of \( ^{125} \text{I}-\text{insulin} \) in Fig. 4 and Table 3 than in Fig. 5 are due partly to the values in the former cases being calculated as the per cent of the maximum binding to the antibodies, determined at an excess of these antibodies, while in the latter case they were calculated on the basis of the total activity. However, this cannot explain the entire difference which is due partly to the use of another batch of \( ^{125} \text{I}-\text{insulin} \) added in a somewhat lower concentration.

Derivatization with 0.9 mole fluorescein-isothiocyanate per mole insulin causes an effect similar to that of substitution with 3 I/mole (Fig. 6). Blocking of the free amino groups by acetylation is said to have no effect, while methyl-esterification of 92% of the carboxyl groups reduces the reactivity with the insulin antibodies (Grodsky et al. 1959).

Zinc ions do not appear to be of any importance for the reaction with the antibodies, as the preparations 0 I/mole and 1.0 I/mole, which contain only traces of zinc, react with the antibodies to the same extent as crystalline insulin with a zinc content of 0.45%. The presence of \( \text{Zn}^{++} \) in excess of the zinc content in the insulin preparations may be derived from guinea pig anti-pig insulin serum and rabbit anti-guinea pig serum, although they are used in dilutions of 1/8000 and 1/50 respectively. When testing the biological effect, \( \text{Zn}^{++} \) seems also to be of no importance, but the presence of \( \text{Zn}^{++} \) in the living organism or in the tissue preparations makes it impossible to assess its possible role in these experiments.

Although the reactivity with the insulin antibodies was tested in a considerably simpler system than the biological activity, it is also impossible to decide whether the tyrosine residues play a direct part in the reaction between the two components or whether the effect of the iodination is due to a change in conformation.

Comparison of the effect of iodination on the biological activity and on the reactivity with the insulin antibodies is difficult for several reasons. The
reaction of insulin in vitro with the antibodies was determined by measuring a state of equilibrium at known concentrations of the insulin preparations and constant concentrations of the antibodies (Fig. 4), while the determination of the biological activity is the result of an unknown dynamic process in which, owing to int. al. permeability factors, there may not be the same molar concentration of the different derivatives at the site of reaction, although this applies to the extracellular fluid. The results of the biological determinations are stated in all cases as the quantity of crystalline insulin which shows the same effect as the preparation concerned.

By expressing the reaction of the individual iodine derivatives with the insulin antibodies in per cent of the competitive effect of crystalline insulin at the same molar concentration, it is possible to obtain an expression of the reactivity of the preparation concerned, independent of the concentration, as the calculated values will be a direct function of the individual equilibrium constants (Fig. 7 A).

On the other hand, reading of the individual values for the iodinated preparations on the standard curve representing crystalline insulin will depend on the concentration and indicate how many μmole crystalline insulin exert the same competitive effect as the preparation concerned in the given concentration (Fig. 7 B).

When expressed in the last-mentioned manner, there seems to be a certain similarity between the effect of the iodination on the biological activity and the reactivity with the insulin antibodies. This might indicate that the insulin reacts with a high-molecular cellular component. However, other factors do not suggest this assumption. If it were true, a high degree of species specificity in biological activity should be expected. So it should be possible to demonstrate a difference in biological activity between pig and ox insulin owing to the difference in the reactivity with pig insulin antibodies. In clinical practice, however, it has not been possible to demonstrate a difference in hypoglycaemic activity between human, pig, and ox insulin, and the same applies to biological standardization. With the possible exception of guinea-pig insulin, insulin with an amino acid composition differing from that of other species, is said to produce the same biological effect (Smith 1966).

The present experiments have shown that substitution by iodine reduces the reactivity of insulin with antibodies and its biological activity, as a function of the degree of iodination. They have also shown that derivatization with 0.9 mole fluorescein-isothiocyanate per mole insulin reduces the reactivity with insulin antibodies and reduces the biological activity. However, it is not possible, on the basis of the present experiments, to elucidate the mechanism of the effect which derivatization with iodine or fluorescein-isothiocyanate exerts on the reaction with insulin antibodies or on the biological effect. So it has not been possible to show, whether the effect of the derivatization is a
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REFERENCES

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