THE ANTIGENIC PROPERTIES OF PIG INSULIN

By

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ABSTRACT

In the present study rabbits were immunized with insulin preparations of various degrees of purity. Antibody formation was determined by Boyden's haemagglutination method (Boyden 1951), by Berson et al.'s chromatoelectrophoresis (Berson et al. 1956) and by agar electrophoresis. In the two last mentioned methods highly purified ¹²¹I-pig insulin was used. The agglutination method gave a positive reaction with sera from rabbits immunized with the two least purified preparations, A-insulin and B-insulin, thus suggesting the formation of antibodies against impurities in these two preparations. On the other hand, agar electrophoresis, and to a lesser degree chromatoelectrophoresis, gave positive reactions with sera from all groups. Thus it was shown that antibodies were also formed in rabbits immunized with even the most purified insulin preparation. Antibody formation was, however, most pronounced in those rabbits which had been vaccinated with the most impure insulin preparation. By continuous paper electrophoresis the purest of the insulin preparations used for immunization could be separated into two fractions. The slowest migrating fraction tested by the haemagglutination test showed a weak but positive reaction indicating traces of impurities.

Thus, although the possibility of a cross reactivity between insulin and the impurities demonstrated cannot be rejected, the results obtained do not allow of any definite decision on this question. The negative inhibition with the purest insulin preparation, however, suggests that there is no common antigenicity.

Insulin antibodies are defined as proteins of a globulin character which are formed in the living organism in response to the injection of insulin and which react specifically with insulin. Both in man and animals it has been possible by various means to demonstrate the formation of antibodies following the injection of insulin preparations (Berson et al. 1956; Moloney & Goldsmith 1957; Lapresle & Grabar 1957). Even crystalline insulin preparations, how-
ever, must be regarded as mixtures of various modified forms of insulin and containing traces of impurities (Harfenist & Craig 1952; Fredericq 1956; Bangham & Mussett 1959). Consequently, it is difficult to evaluate the antigenicity of the insulin itself.

Lapresle & Grabar (1957) found that the antibodies demonstrable in insulin-treated diabetic patients by means of the passive haemagglutination-method were directed against an impurity in the insulin preparations and not against the insulin itself. On the other hand, Berson & Yalow (1959), using a chromato-electrophoretic technique, demonstrated that in almost all insulin-treated diabetics, proteins of an antibody nature were present which could bind 131I-labelled insulin. The reason for the negative reaction to insulin in Lapresle & Grabar's experiments (Lapresle & Grabar 1957), however, may have been due to the difficulty of binding insulin to antibody under the experimental conditions used. The positive reaction obtained in Berson et al.'s experiments (Berson et al. 1956), however, may have been due to a cross reactivity between insulin and impurities in the preparation injected.

Neither of the two investigations, therefore, allows of a decision as to whether insulin itself is antigenic.

The aim of the present investigation was to examine the significance of the impurities in the formation of insulin antibodies. For this purpose rabbits were immunized with pig insulin preparations of various degrees of purity.

**MATERIAL AND METHOD**

**Immunization**

White rabbits of both sexes, weighing 2–3 kilos, were vaccinated with pig insulin preparations made from the same raw material but of various degrees of purity.

5 ml of a solution containing 1 mg of insulin/ml sterile 0.9% NaCl acidified with HCl was pipetted into a Potter-Elvehjem homogenizer. 5 ml of Freund's incomplete adjuvant was added prepared from 7 ml Bayol F and 3 ml Arlacel A. When the complete adjuvant was used, 10 mg heat-killed tubercle bacilli was added. The incomplete adjuvant was sterilized at 140° C for three hours. The glassware was likewise sterilized. After the mixture was carefully homogenized, 1 ml was injected subcutaneously between the shoulder blades, *i.e.* an amount corresponding to 0.5 mg insulin. Vaccination was performed 7 times at weekly intervals. After an interval of 4 weeks, 1 ml was injected subcutaneously as booster. Complete adjuvant was used at first, but large subcutaneous nodules developed after 2–3 injections, so the procedure was continued with incomplete adjuvant. Rabbit No. 1 died from hypoglycaemia after the second injection and was replaced by a fresh animal which received only 5 injections. The remaining animals thrived. Blood samples were taken from an ear vein before starting the vaccination and one week after terminating the vaccination. After centrifugation at room temperature the serum was stored at -22° C.

The most impure of the pig insulin preparations, namely A-insulin, was injected into the group A rabbits. This was an amorphous material which had been taken out of preparation at an early stage. It had a biological activity of 18.0 ± 5% units/mg dry
weight, corresponding to 140 units/mg N. The biological activity was determined by the mouse convulsion test. After a single crystallisation of the A-insulin, an insulin preparation was obtained, namely B-insulin, which had a biological activity of 25.0 ± 6% units/mg dry weight, corresponding to 160 units/mg N. Group B rabbits were vaccinated with this preparation. A further purified and re-crystallized preparation, C-insulin, was used to vaccinate group C rabbits. The biological activity of this preparation was 27.3 ± 5% units/mg dry weight, corresponding to 173 units/mg N.

All sera were examined by Boyden's passive haemagglutination method (Boyden 1951) as well as by electrophoretic methods following incubation with ¹³¹I-insulin. The insulin sensitivity of the rabbits was also determined before and after vaccination.

**Passive haemagglutination**

Blood from selected sheep was stored in Alsever's solution at +4°C for a maximum of 14 days. The erythrocytes were washed three times in 0.9% NaCl before use, and a 2% suspension was then made in buffered saline, pH 7.2 (7 parts 1/15 M Na₂HPO₄ + 3 parts 1/15 M KH₂PO₄ + 30 parts 0.9% NaCl). The erythrocyte suspension was then incubated for 10 minutes at room temperature with equal parts of tannic acid solution (1:40 000, w/v in 0.9% NaCl); after careful washing twice with 0.9% NaCl and centrifugation for three minutes the erythrocytes were resuspended in 0.9% NaCl to give a 2% suspension.

Equal parts of antigen solution and erythrocyte suspension were incubated for 10 minutes at room temperature, then centrifuged at 500 × g for 3 minutes. After pipetting off the supernatant, the erythrocytes were carefully shaken up in the remaining fluid, and a 1:100 dilution of normal rabbit serum was added in an amount corresponding to the volume of fluid pipetted off. The washing procedure was repeated twice after centrifugation. After the last centrifugation, a 0.5% erythrocyte suspension was prepared in diluted normal rabbit serum, 1:100.

The amount of insulin adsorbed on to the erythrocytes was measured by using ¹³¹I-insulin as antigen. The amount constituted about 1 part in 1000 of the insulin used for sensitization (Table 1).

**Table 1.**

Amount of antigen in µg bound to sheep erythrocytes after incubation with ¹³¹I-insulin.

<table>
<thead>
<tr>
<th>Sheep erythrocytes</th>
<th>In 1 ml incubation mixture after sensitization</th>
<th>In supernatant after</th>
<th>In 10 µl packed erythrocytes corresponding to 1 ml incubation mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 washing</td>
<td>2 washings</td>
</tr>
<tr>
<td>Tannic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>0.4</td>
</tr>
<tr>
<td>No tannic acid</td>
<td>app. 310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td>5</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>0.3</td>
</tr>
</tbody>
</table>
The insulin preparations used as antigen were dissolved in buffered 0.9% NaCl, pH 7.2. «Optimum sensitizing amount» was 0.62-2.5 mg/ml. All sera were inactivated at 56°C for 30 minutes and were incubated with washed sheep erythrocytes at room temperature prior to use, so that any antibodies against sheep erythrocytes could be absorbed.

In test-tubes (diameter 2-3 mm, length 45 mm), a series of serum dilutions were made with 1% normal rabbit serum, pH approx. 6.5 (1:10, 1:20, 1:40, etc.). To each tube containing 0.1 ml of serum dilution, 0.1 ml of a suspension of sensitized red blood cells were added, and the suspensions were carefully mixed. One or two positive and negative control sera were included on each experimental day. The tubes were stored at room temperature overnight at a slope of 45°. An assistant who was familiar with the technique but unaware of what reactions to expect made the readings with the naked eye. In positive reactions, grainy agglutinates were found along the sides of the tube. The reciprocal value of the maximum serum dilution which still gave a clear positive reaction indicated the titre.

The inhibition test was made as follows: 0.1 ml inactivated antiserum, diluted 1:10 with 0.9% NaCl, was incubated for two hours at room temperature with 0.1 ml of a solution of A- or C-insulin. The concentrations of the insulin solutions used for the inhibition were 1000 – 500 – 250 – 125 – 62.5 – 31 – 15 mg/ml buffered 0.9% NaCl. pH 7.2. One tenth ml of the above mixtures was pipetted into a series of test-tubes, which thus contained one half of the above mentioned amounts of insulin, and to each tube 0.1 ml of the sensitized erythrocytes was then added. A positive agglutination reaction in the first tube, for example, indicated that 500 mg of insulin preparation was not sufficient to prevent a reaction between the sensitized erythrocytes and antibody in a serum dilution of 1:20. A negative test, on the other hand, showed that the reaction was prevented by using 500 mg of the insulin preparation.

**Iodination of insulin**

The insulin was iodinated in glycine buffer, pH 9.5, with iodine dissolved in CCl₄.

The iodination process was carried out in the glass apparatus shown in Fig. 1. Only highly purified pig insulin was used for iodination.

The procedure was as follows:

Two ml of CCl₄ + 1 ml H₂O was pipetted off into A. KI and KIO₃ were added by constriction pipette, usually in amounts of 75 and 15 µl of solutions containing 13.1 and 25.2 mg/100 ml, respectively. ¹³¹I was added as a clear, colourless carrier-free alkaline thiosulphate-free solution (pH 8-10) supplied from the Radiochemical Centre, Amersham, England. Then 0.2 ml of 1 N HCl was added, and after careful mixing and standing for 1 minute the I₂ was extracted with CCl₄ while mixing vigorously for 3 minutes. After the separation, the CCl₄ phase was added drop by drop to the insulin in B – usually 500 mg insulin in 0.5 ml glycine buffer. Vigorous stirring ensured uniform iodination. B was kept cooled with ice throughout the entire iodination process. The iodine extraction was done with a total of 3 × 2 ml CCl₄ which gave a 98% extraction of the radioactivity added.

After the iodination, which lasted about 40 minutes, the stirrer was rinsed with 200 µl of glycine buffer. The emulsion which had formed was broken down by centrifugation and the water phase transferred to a column (1 × 25 cm) packed with Sephadex G-25 medium. The protein was transferred practically quantitatively. The column was rinsed through with 1/15 m phosphate buffer, pH 7.2, 6 ml/h. In this way a rapid separation was obtained between protein and I⁻ so that the risk of radiation
Glass apparatus used for the iodination of insulin. Stirring was performed by the two small air turbines. (Quickfit).

damage to the insulin was reduced. After passage through the column, the absorption at 254 m\(\mu\) by means of an LKB Uvicord and the radioactivity was continuously recorded. This recording made it possible to control the distribution of the radioactivity between I-insulin and I\(^-\) (Fig. 2).

The I-insulin fraction was collected in a manually controlled fraction collector. The protein loss during gelfiltration was about 5 %. With the amount of insulin used the radioactivity in the iodide fraction was, for unknown reasons, greater than in the insulin fraction. This deviation from the theoretical value 1:1 could in some preparations be even more pronounced than shown in Fig. 2.

The amount of carrier iodine \(^{127}\)I corresponded to a degree of iodination of 1 atom iodine to 2.25 mol insulin, m. w. 5777.

One dimensional paper chromatography was performed (butanol, acetic acid, water,
Separation of I-insulin and \( I^- \) on Sephadex G-25, medium after iodination of 500 \( \mu g \) pig insulin with 10 \( \mu g I \).

- UV absorption at 254 m\( \mu \). Cell length 3 mm.
- Radioactivity.

The increase of the UV absorption after the protein fraction is due to a small amount of CCl\(_4\) transferred to the column together with the watery insulin solution.

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One-dimensional paper chromatography (butanol – acetic acid – water) of enzymatically hydrolyzed \(^{131}I\)-insulin.

- DI: 3,5-diiodotyrosine.
- MI: 3-monoiodotyrosine.
- ↑: Site of application.
- SF: Solvent front.
4:1:5) after enzymatic hydrolysis. 50 µl of the hydrolysate were applied. Carrier amounts of 3-monoiodotyrosine and 3,5-diiodotyrosine were added to the hydrolysate (Fig. 3).

The hydrolysis was performed with 100 µl of a 0.5 per cent solution of a pancreas enzyme preparation (Pancreatin NOVO) at 30°C for 20 hours on 2-2.5 µg of the iodinated insulin preparation dissolved in 2 ml 0.1 molar phosphate buffer, pH 7.5.

Scanning for radioactivity of the chromatogram demonstrated radioactivity arising from 3-monoiodotyrosine. Radioactivity arising from 3,5-diiodotyrosine could not be demonstrated with certainty. However, radioactivity was demonstrated arising from a not identified compound, most likely a not hydrolysed peptide, which may have contained disubstituted tyrosine. The radioactivity arising from 3-monoiodotyrosine was 3 times as great as the radioactivity from the unknown compound.

Chromatoelectrophoresis

Chromatoelectrophoresis was performed by the method of Berson et al. (1956). 131I-insulin was diluted in polysteryl containers with buffered saline, pH 7.2, to 1-5 µg/ml. 250 µl of serum was mixed with 25 µl of the 131I-insulin dilution to give a concentration of 0.1-0.8 µg insulin/ml serum. The mixture was incubated in a water bath for 15 minutes at 37°C and 20 µl applied to Schleicher-Schüll paper No. 2043 bmgl. The electrophoresis was made at room temperature in barbiturate buffer, pH 8.6, ionic strength 0.1 at constant current, 1 mA/cm, for 18 hours. The cathodic end of the electrophoresis chamber was elevated 2.5 cm so that the gamma-globulins could be removed from the site of application. After drying in a hot oven at 120°C for 30 minutes the paper strips were scanned for radioactivity by means of a Tracerlab chromatogram-scanner. A TGC-14 tube was used as detector, perfused with a mixture of helium and isobutane. Planimetry of the radiograms was carried out, and the percentage distribution between free and antibody-bound insulin was calculated. Antibody-bound insulin is defined as that part of the total activity which is found to correspond to the beta-gamma-globulins. Free insulin is situated a little anodic to the site of application. Amido black was used for staining of the serum proteins.

Agar electrophoresis

Agar electrophoresis and scanning for radioactivity were performed as described by Deichert (1964) using the above mentioned device for scanning the radioactivity. An amount of 5 µl was applied of a mixture consisting of serum and insulin solution in the proportion 10:1 incubated at 37°C for 15 minutes. The insulin concentration was 0.4 µg/ml serum. The electrophoresis was done in veronal buffer, pH 8.6, ionic strength 0.1. As the scanning in these experiments was done after fixation in 4% acetic acid and drying with filter paper, the ratio between free and bound insulin is too low due to a somewhat greater loss of free insulin than antibody bound insulin.

Testing of insulin sensitivity

All rabbits were tested for insulin sensitivity, both before immunization and 10 days after its termination. The purest of the insulin preparations, C-insulin, was used for testing. An amount of 0.02 mg/kg was injected intravenously into the ear vein of rabbits which had been fasting for 18 hours. The blood sugar was determined by the method of Hagedorn & Norman-Jensen.

Continuous paper electrophoresis

Continuous paper electrophoresis of C-insulin was performed as follows: 200 mg
Fig. 4.

Chromatoelectrophoretic separation of serum after incubation with approx. 0.4 μg ¹³¹I-insulin/ml.

A: K₄ serum before immunization with insulin.
B: K₄ serum after immunization with insulin.
↓: Site of application.

After the immunization binding of ¹³¹I-insulin to β-γ globulins occur to a marked degree.
Fig. 5.
Agar electrophoretic separation of serum after incubation with approx. 0.4 µg
131I-insulin/ml.

A: K₄ serum.
B: K₁₂ serum.
↓: Site of application.
The radioactivity maxima to the left of the application site represents the antibody-bound insulin.

C-insulin with a Zn⁺⁺ content of 0.53%/ was dissolved in 20 ml of barbituric acid buffer, pH 8.6, ionic strength 0.02, and fractionated in a Spinco model CP, 780–800 volts, 44 mA constant, in a refrigeration room at 4°C. Prior to fractionation, the paper was washed with 10 l buffer which was drained off. During the electrophoresis, the buffer was re-cycled. The rate of application of the insulin solution was 0.5–0.6 ml/h.

RESULTS

With the haemagglutination method, antibodies could be demonstrated in rabbits vaccinated with A- and B-insulin, but not in rabbits vaccinated with C-insulin (see Table 2). Furthermore, no reaction could be demonstrated between C-insulin and the antibodies formed. These results were confirmed by the inhibition technique (see Table 3).

The electrophoretic investigations showed that all three groups contained
### Table 2.
Demonstration of antibodies in rabbits immunized with pig insulin preparations of varying degrees of purity.

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-sera</th>
<th>Haemagglutination titer</th>
<th>Agar electrophoresis (app. 0.4 µg $^{131}$I-insulin/ml serum)</th>
<th>Chromatoelectrophoresis (app. 0.8 µg $^{131}$I-insulin/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A-insulin</td>
<td>B-insulin</td>
<td>G-insulin</td>
</tr>
<tr>
<td>A</td>
<td>K₁</td>
<td>160</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₂</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₃</td>
<td>320</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₄</td>
<td>320</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>K₅</td>
<td>320</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₆</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₇</td>
<td>80</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₈</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>K₉</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₁₀</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₁₁</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>K₁₂</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 3.
Inhibition test.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Test antigen</th>
<th>Titre without inhibition</th>
<th>Inhibition performed with</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₁ (1:20)</td>
<td>A-insulin</td>
<td>20</td>
<td>A-insulin</td>
<td>Total inhibition with 7.5 µg</td>
</tr>
<tr>
<td>K₁ (1:20)</td>
<td>-</td>
<td>20</td>
<td>B-insulin</td>
<td>Total inhibition with 7.5 µg</td>
</tr>
<tr>
<td>K₁ (1:20)</td>
<td>-</td>
<td>20</td>
<td>C-insulin</td>
<td>No inhibition with 500 µg</td>
</tr>
<tr>
<td>K₃ (1:20)</td>
<td>-</td>
<td>20</td>
<td>A-insulin</td>
<td>Total inhibition with 7.5 µg</td>
</tr>
<tr>
<td>K₃ (1:20)</td>
<td>-</td>
<td>20</td>
<td>B-insulin</td>
<td>Total inhibition with 7.5 µg</td>
</tr>
<tr>
<td>K₃ (1:20)</td>
<td>-</td>
<td>20</td>
<td>C-insulin</td>
<td>No inhibition with 500 µg</td>
</tr>
</tbody>
</table>
Fig. 6.
Continuous paper-electrophoretic separation of C-insulin.

sera which could bind $^{131}$I-insulin to beta-gamma-globulins which was not the case before immunization. The binding was greatest in serum from the two groups of rabbits which were vaccinated with the most impure insulin preparations and which gave a positive reaction in the haemagglutination method (see Table 2).

Immunization involved no reduction in the insulin sensitivity.

By continuous paper electrophoresis it was found possible to fractionate C-insulin into two clearly separated fractions (see Fig. 6).

The most rapidly migrating fraction contained 70–80 % of the amount of protein isolated and had a Zn$^{++}$ content of 0.24–0.35 %. The slower fraction contained 20–30 % of the amount of protein isolated and had a Zn$^{++}$ content of 0.01–0.09 %. When the fractions were re-run, no further fractionation was obtained. Comparison with amorphous precipitated insulin with a low Zn$^{++}$ content, showed that this had a migration rate corresponding to that of the slow fraction. These results are in agreement with those of Schlichtkrull (1958) who obtained a partial separation of insulin into two fractions by free electrophoresis, the larger and more rapid fraction having the greater Zn$^{++}$ content.

The fractions were dialysed against distilled water for 48–72 h at $+4^\circ$ C in rotating visking tubes and then freeze-dried. Both fractions formed sharp edged crystals with Zn$^{++}$. Tested on rabbits by intravenous injection no difference in hypoglycaemic activity could be demonstrated between the two fractions (see Fig. 7). Each preparation was injected in an amount of 0.02 mg/kg in the marginal ear vein of 4 rabbits.

The slower fractions were found to give a weak positive haemagglutination
Hypoglycaemic activity in rabbits of the two fractions obtained by continuous paper-electrophoretic separation of C-insulin. Each value represents the average of 4 animals.

- Slowly migrating fraction.
- Faster migrating fraction.

reaction in $K_4$ serum. The titre was 40–80. The most rapidly migrating fraction, on the other hand, gave a negative reaction.

The experiment indicates that the impurities present in A- and B-insulin are also present as trace in C-insulin and that these impurities are concentrated in the slower moving fraction.

**DISCUSSION**

The majority of rabbits in groups A and B formed antibodies demonstrable by the passive haemagglutination test. As these antibodies could not be demonstrated in rabbits of group C, the results indicate the presence of antigenic impurities in A- and B-insulin.

The findings of Lapresle & Grabar (1957) (see introduction) could thus be confirmed.

As Table 2 shows, however, the present investigation also reveals the disagreement mentioned in the introduction, between the results of the haemagglutination method and those of the electrophoretic methods. Thus, in almost all sera, the electrophoresis experiments showed binding of $^{131}\text{I}$-insulin to beta-gamma-globulins, while passive haemagglutination with C-insulin was negative. The reason for this may be that the haemagglutination method is less sensitive than the electrophoretic methods. Several factors may be responsible for this.

Feinberg & Flich (1957) showed, for example, that if the reaction mixture is allowed to stand, the antigen may be released from the sensitized erythrocytes and capture some of the antibodies in the solution. It is not known whether this abnormal effect occurs under the experimental conditions of the present experiments. The amount of antigen used in the haemagglutination experiments,
however, about 2 µg/ml undiluted serum, is double the amount used in the electrophoresis experiments, where 0.4–0.8 µg/ml of undiluted serum is used. A release of antigen could actually be of significance, as seen from the fact that 0.35 µg insulin would be sufficient to saturate the antibodies in the highest dilution of K4 serum (45% of 0.8 µg, see Table 2).

The difference in sensitivity could also be due to the use of diluted serum in the haemagglutination method, while practically undiluted serum was used for the electrophoresis. Dilution is, however, necessary if non-specific agglutinations are to be avoided.

Other conditions than the above might also reduce the sensitivity of the haemagglutination method. In the electrophoretic methods the demonstration of antibody occurs at pH 8.6 and with iodinated insulin, but no positive haemagglutination reaction could be obtained, even if the reaction proceeded at pH 8.6 in barbituric acid-buffered 0.9% NaCl with C-insulin as antigen. Similarly, A-insulin, which otherwise gives a strongly positive reaction, was also negative at this pH. Moreover, the use of 127I C-insulin as antigen instead of non-iodinated C-insulin did not lead to positive results under the usual conditions.

The inactivation of serum at 56° C for 30 minutes was shown to have no significance in the reaction between insulin and insulin antibody with the electrophoretic methods.

Finally, the possibility cannot be excluded that the absorption of antigen onto the erythrocytes may have an inhibitory effect on the reaction with the antibody.

In order to obtain reactions in the electrophoretic determinations due to a cross reactivity between insulin and impurities, these would have to be present in the purest of the insulin preparations used for the immunization, i.e. C-insulin.

The prerequisite for assuming that the positive reaction in the electrophoresis experiments with K9-12 sera originates from a cross reactivity between the insulin and impurities is thus satisfied.

Thus the investigation has not enabled us to decide whether it is the insulin itself which is antigenic, or if it is exclusively the impurities which cause the formation of antibodies with which the insulin can then cross-react. The fact that up to 45% of the iodinated preparation could be bound to the antibodies, excludes the possibility that the reaction takes place mainly between the impurities in the preparation and the antibodies. This argument naturally presupposes that the impurities are not iodinated to a considerably higher degree than the insulin, nor does this appear to be the case.

Subsequent studies using isolated impurities and highly purified insulin have shown, however, that the first explanation is the correct one (Deckert, to be published).
The demonstration of impurities, even in very highly refined insulin preparations, must call for caution in the interpretation of the results in immunological studies with insulin.

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