Insulin Receptors in Isolated Rat Hepatocytes

REASSESSMENT OF BINDING PROPERTIES AND OBSERVATIONS ON THE INACTIVATION OF INSULIN AT 37°C*

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Receptor binding of 125I-insulin was studied at 37°C and pH 7.4 in isolated metabolically active rat hepatocytes under conditions with degradation of only 5% of the extracellular insulin. It is concluded that within the physiological concentration range insulin binds to a homogenous class of noninteracting receptors as judged from kinetic and steady state experiments.

The net uptake of receptor-bound 125I-insulin showed a linear relationship between the rate constant of net uptake and the concentration of insulin in the range from 36 pm to 1 nm, as expected from a reversible, bimolecular reaction. The rate constants of association, $k_{a}$, and dissociation, $k_{d}$, were determined as 0.23 nm$^{-1}$ min$^{-1}$ and 0.12 min$^{-1}$, respectively. From the kinetic data the dissociation constant, $K_{d} = k_{d}/k_{a}$, was calculated as 520 pm and the number of receptors/cell as 16,100.

Following 1000-fold dilution of the concentration of extracellular 125I-insulin, the major part (88%) of the receptor-bound 125I-insulin dissociated from the cells with a half-time of $5.6 \pm 0.8$ min (S.E.) or $k_{d}$ of 0.12 min$^{-1}$ in accordance with the uptake experiments. The 125I activity was released as 50% immunoreactive insulin suggesting that insulin is inactivated in relation to receptor binding. The residual part (12%) was released as non-immunoreactive 125I activity with a half-time of about 3 h, suggesting internalization and degradation of part of the receptor-bound insulin. At 37°C the rate of dissociation was not enhanced by addition of 100 nm native insulin, whereas this was the case at 12°C. Thus, at 37°C the insulin receptors are not influenced by negative cooperative interactions.

At steady state the receptor binding of 125I-insulin in concentrations from 12 pm to 100 nm also conformed to a reversible reaction between insulin and one type of receptors. $K_{a}$ was estimated as $400 \pm 60$ pm (S.E.) and the number of receptors/cell was $15,200 \pm 1,400$ (S.E.). The observed $K_{d}$ is about five times lower than that previously described in adipocytes and the binding affinity of insulin from the Atlantic hagfish relative to pig insulin is $6.9 \pm 0.1$% (S.E.) in hepatocytes as compared to $23 \pm 2$% (S.E.) in adipocytes, suggesting structural differences between the insulin receptors in these two tissues. Other insulins tested did not show any difference between the relative binding affinities in the two cell types.

Finally, it was demonstrated that degradation of insulin in suspensions of hepatocytes occurs extracellularly as well as in relation to receptor binding. The soluble extracellular inactivating activity was reduced by washing and the extracellular inactivation of 125I-insulin was inhibited half-maximally by 120 nm native insulin while insulin from the Atlantic hagfish was an eight times more potent inhibitor.

In mammalian tissues, insulin and other polypeptide hormones exert their biological effects after binding to specific membrane receptors at 37°C (1, 2). Most studies on the characteristics of insulin receptor binding have been carried out at lower temperatures in order to reduce the inactivation of extracellular insulin concomitant to binding (1, 3–7). In purified liver plasma membranes (5) and isolated hepatocytes (6–8) from rat, insulin receptors have been studied at 20°C and 30°C, but not at a physiological temperature of 37°C. In these studies the steady state binding was consistent with a heterogeneous population of binding sites (5, 7, 8) or homotropic negative cooperative interactions (1, 9), whereas the kinetics of dissociation of the insulin-receptor complex suggested negative cooperativity (1, 9). In addition, in rat hepatocytes it has been found that half of the bound insulin is inactivated in relation to receptor binding at 30°C (8).

The present study was undertaken to characterize insulin receptor binding with respect to time course, affinity, and specificity in isolated, metabolically active hepatocytes at 37°C, where insulin exerts its biological effects. This was considered important to the understanding of the mechanism of action of insulin, since the binding characteristics depend on temperature (5, 6) and since the modification of insulin receptor binding by homotropic negative cooperativity is temperature-dependent and virtually not apparent above 30°C (9). In order to ascertain a constant insulin concentration throughout the experimental period, the inactivation of extracellular insulin was minimized at 37°C by use of cell concentrations 10 times lower (100 cells/ml) than those used by others (6–8). Such experimental conditions have previously been applied in receptor studies with adipocytes (10). A comparison between the binding characteristics of the two cell types is of interest, since in vivo they are exposed to different insulin concentrations (11), which might influence the number of receptors/cell (1, 12).

We present data from kinetic and steady state binding experiments at 37°C, which show that insulin in the physiological concentration range binds to a homogenous class of noninteracting receptors in rat hepatocytes. Furthermore, the
insulin receptors in hepatocytes are different from those previously described in adipocytes (10). Finally, we confirm the observation that about half of the receptor-bound insulin in hepatocytes is degraded in relation to binding and is released as inactive insulin (8). Preliminary results of this investigation have been presented (13).

**Methods**

**Chemicals**—125I-labeled pig insulin, prepared to a specific activity of 20 to 30 mCi/mg (14), was obtained from NOVO Research Institute, Copenhagen, Denmark. 125I-labeled hagfish insulin, with a specific activity of about 30 mCi/mg, was prepared by S. O. Emdin, Umeå, Sweden. (The properties of the two labeled insulin preparations have previously been described (10, 16).) Insulin from pig (27.6 units/mg), turkey, goosefish, pig proinsulin, and anti-insulin guinea pig serum were gifts from NOVO Research Institute. Insulin from hagfish, sea scorpion, shark, and the hagfish insulin analogue Desmethionine B31 hagfish insulin were gifts from S. O. Emdin and S. Falkmer, Umeå, Sweden. Insulin from chinchilla (18) and the modified insulins (19) Aₐ-acetyl, Aₐ-thiazolidine, and Aₐ BOC were gifts from S. Wood and T. L. Blundell, Sussex, England. Crude collagenase (CLS II) was from Worthington, Freehold, N. J. Silicone oil AR 200 was obtained from Serva, Heidelberg, Germany. Hepes and bovine serum albumin (Fraction V) were from Sigma, St. Louis, Mo. 4-Hydroxyethyl-1'H-inulin was from Amersham, England. All other chemicals were analytical grade from Merck, Darmstadt, Germany.

**Hepatocyte Isolation**—Hepatocytes were isolated from 48-h fasting female Wistar rats weighing 150 to 170 g. The method of Quistorff et al. (20) was used with the following modifications. Hyaluronidase was omitted from the perfusion medium and the liver was not incubated with collagenase after the perfusion because of reduced yield due to cell aggregation. Of the hepatocytes, 90% were found intact as judged was about 2.3 pmol/min/g wet weight of hepatocytes during 2 h as with collagenase after the perfusion because of reduced yield due to pyruvate and of ketone bodies from fatty acids were linear for at least 1 h. Binding of 125I-insulin at steady state was constant for 2 h after the isolation of the cells.

The hepatocytes were incubated at 37°C in a modified Krebs-Ringer/Hepes buffer, pH 7.4 (21), containing 25 mM bicarbonate and 10 mg/ml of bovine serum albumin. A cell suspension with a cell concentration of about 50 μl of packed cells/ml was preincubated for 30 min at 37°C and then diluted 5 or 50 times for use in inactivation or binding studies, respectively. The cell concentration was determined by centrifugation in a hematocrit tube and expressed as milliliters of packed cells/ml of suspension (volume fraction). A cell concentration of 1 μl of packed cells/ml (0.1%) of free insulin was 10 μl/ml of suspension (volume fraction). At appropriate times of incubation, 300-μl aliquots of the well mixed cell suspension were transferred to 550-μl propylene tubes containing 150 μl of silicone oil with a density of 1.04. After centrifugation at 3000 × g for 45 s in a Beckman microfuge, the hepatocytes formed a cell pellet in the oil separated from the buffer (22). The tubes were then cut at the middle of the silicone oil propylene mixture layer and the cell pellet was transferred to a Nahebauer counting chamber. The fraction of 125I-insulin at steady state was constant for 2 h after the isolation of the cells.

A cell concentration of 1 to 2.10⁶ cells/ml was considered constant during 30 min of incubation, where only 1% of the total insulin was bound to cells and a maximum of 5% was inactivated during 30 min at 37°C in the low cell concentration used. The receptor-bound 125I-insulin was defined as the difference between the bound 125I-insulin in the absence and the presence of excess native insulin in concentrations above 100 nM. The residual bound 125I-insulin in the presence of excess native insulin was defined as nonspecific binding.

Assuming a reversible reaction between one hormone and one type of noninteracting receptors, the insulin receptor binding reaction can be described by the law of mass action, and the following equations can be derived (10, 23).

**Expression of Results**—The results are expressed as the distribution coefficient of 125I activity between cells and buffer calculated as the ratio of bound 125I activity/liter of packed cells (D) and free 125I activity/liter of buffer (F). In the individual experiments (Figs. 3, 5, and 6) the fraction of bound in per cent of free 125I-insulin can be calculated as the product of the value of B/F and the cell concentration expressed in per cent, v/v. The concentration of free native insulin (I) was calculated from the amount of insulin added. This concentration (f) was considered constant during 30 min of incubation, since only 1% of the total insulin was bound to cells and a maximum of 5% was inactivated during 30 min at 37°C in the low cell concentration used. The receptor-bound 125I-insulin was defined as the difference between the bound 125I-insulin in the absence and the presence of excess native insulin in concentrations above 100 nM. The residual bound 125I-insulin in the presence of excess native insulin was defined as nonspecific binding.

We assume a reversible reaction between one hormone and one type of noninteracting receptors, the insulin receptor binding reaction can be described by the law of mass action, and the following equations can be derived (10, 23).

**Binding Studies**—The 1-ml aliquots of cell suspension containing hepatocytes in a concentration of 1 to 2.10⁶ cells/ml or 0.1 to 0.2%, v/v, were incubated in 10-ml propylene flasks containing 25 μl of buffer. Native insulin with or without 25 μl of native insulin in appropriate dilutions. The flasks were gassed with 95% O₂/5% CO₂, stoppered, and placed in a Gallenkamp incubator at 37°C. At appropriate times of incubation, 300-μl aliquots of the well mixed cell suspension were transferred to 550-μl propylene tubes containing 150 μl of silicone oil with a density of 1.04. After centrifugation at 3000 × g for 45 s in a Beckman microfuge, the hepatocytes formed a cell pellet in the oil separated from the buffer (22). The tubes were then cut at the middle of the silicone oil propylene mixture layer and the cell pellet was transferred to a Naehauer counting chamber. The fraction of 125I-insulin at steady state was constant for 2 h after the isolation of the cells.

At steady state the receptor-bound insulin equals

\[
(RI)_b = \frac{R_0 I}{K_c + I}
\]

where \(K_c\) is the dissociation constant (molar) and 300-μl aliquots of the well mixed cell suspension were transferred to 550-μl propylene tubes containing 150 μl of silicone oil with a density of 1.04. After centrifugation at 3000 × g for 45 s in a Beckman microfuge, the hepatocytes formed a cell pellet in the oil separated from the buffer (22). The tubes were then cut at the middle of the silicone oil propylene mixture layer and the cell pellet was transferred to a Naehauer counting chamber. The fraction of 125I-insulin at steady state was constant for 2 h after the isolation of the cells.

At steady state the receptor-bound insulin equals

\[
(RI)_b = \frac{R_0 I}{K_c + I}
\]

where \(K_c\) is the dissociation constant (molar) of free insulin.

The initial rate of formation of the receptor-insulin complex (v) is described by the equation:

\[
v = k_1 R_0
\]

where \(R_0\) is the concentration of receptors (mole/liter of packed cells).

The dissociation of the receptor-insulin complex follows a monoeponential time course:

\[
(RI)_b = e^{-k_1 t}
\]

where \(k_1\) is the rate constant of net uptake of insulin by the receptor and can be expressed as

\[
k = k_1 + k_1
\]

where \(k_1\) and \(k_1\) are the rate constants of association and dissociation, respectively, and \(I\) the concentration of free insulin.

The formation of the receptor-insulin complex (RI) with time (t) approaching equilibrium (eq) is described by the equation:

\[
(RI)_b = (RI)_0 (1 - e^{-kt})
\]

where \(k\) is the rate constant of net uptake of insulin by the receptor and can be expressed as

\[
k = k_1 + k_1
\]

where \(k_1\) and \(k_1\) are the rate constants of association and dissociation, respectively, and \(I\) the concentration of free insulin.

Inactivation of Extracellular 125I-Insulin—The inactivation of extracellular 125I-insulin in suspensions of hepatocytes at 37°C was studied in more detail in order to define incubation conditions in which the binding of 125I-insulin could be measured at constant insulin concentrations. As shown in Fig. 1 hepatocytes in a concentration of 10⁶ cells/ml inactivated...
Fig. 1. Time course of inactivation of $^{125}$I-insulin. Hepatocytes (1%, v/v) were preincubated in the absence of insulin. After 30 min of incubation (zero time), 100 pm $^{125}$I-insulin was added. The immunoreactive $^{125}$I-insulin was determined in the buffer, separated from the cells at the indicated times, and was expressed as percentage of total $^{125}$I activity (○). In parallel a suspension of preincubated hepatocytes was washed after 30 min of incubation by centrifugation and resuspension in fresh buffer. At zero time 100 pm $^{125}$I-insulin was added to the resuspended cells (△) and to the cell-free buffer from the preincubation (×). The immunoreactive $^{125}$I-insulin was determined as above.

55% of the added $^{125}$I-insulin in 20 min. Most of the inactivating activity was found in the extracellular medium and after washing the cell suspension showed a greatly reduced rate of inactivation. The extracellular inactivating activity was proportional to the cell concentration and only 5% of the extracellular $^{125}$I-insulin was inactivated by $10^6$ cells/ml during a 30-min incubation period. Therefore, 1 to $2 \times 10^5$ cells/ml were used in the binding experiments. The extracellular inactivation of $^{125}$I-insulin after 20-min incubation with an unwashed suspension of hepatocytes ($10^6$ cells/ml) showed half-maximum inhibition by 120 nM native insulin, while hagfish insulin was 8 times more potent as inhibitor (Fig. 2). Identical results were obtained with $^{125}$I-insulin from pig or hagfish as tracers. The inactivation of insulin was temperature-dependent and at 12°C the activity disappeared. Inactivating activity was not released from the rat liver when perfused during 1 h under near physiological conditions as described (25). This suggests that the extracellular inactivating activity originates from hepatocytes damaged during the isolation procedure. It seems unlikely that the inactivation is due to collagenase retained in the cell suspension, since inactivation of insulin by collagenase in the concentration used for perfusion (0.8 mg/ml) was only a few per cent of that in the hepatocyte suspension.

Uptake of $^{125}$I-Insulin—The time course of net uptake of $^{125}$I-insulin in concentrations from 36 pm to 1 nM by hepatocytes is shown in Fig. 3. At low insulin concentrations about 30 min were required to obtain steady state and this time decreased with increasing insulin concentrations.

Addition of 160 nM native insulin depressed B/F to about 5% of its maximum value demonstrating saturability of one part of the binding. The amount of bound $^{125}$I-insulin was constant from 30 to 60 min of incubation, suggesting that no significant disappearance of receptors takes place, i.e. the number of receptors can be considered as constant. Five per cent of the extracellular insulin was inactivated after 30 min...
In insulin was only used at higher concentrations (cf. Fig. 5). The mean values of $k$ were derived as $\ln 2/\tau_{1/2}$, where $\tau_{1/2}$ is the half-time for net uptake of receptor-bound insulin. Equation of the regression line is $k = 0.25 \, \text{nM}^{-1} \cdot \text{min}^{-1} \cdot 10^{-0.10 \, \text{min}^{-1}}$.

of incubation, and 10% after 60 min, i.e. the insulin concentration could be considered constant during the first 30 min, which was the time required to obtain steady state. The rate constants of net uptake ($k$) were calculated for the receptor-bound insulin from Fig. 3 and plotted versus the insulin concentration ($I$) according to Equation 2. As shown on Fig. 4 a straight line was obtained. In two experiments the mean values of the rate constants of dissociation ($k_{-1}$) and association ($k_{+1}$) were determined to be $0.12 \, \text{min}^{-1}$ and $0.23 \, \text{nmol}^{-1} \cdot \text{min}^{-1}$, respectively. The dissociation constant ($K_d$) of the binding reaction was $520 \, \text{pm}$ when calculated as the ratio between $k_{-1}$ and $k_{+1}$.

The concentration of receptors, $R_0$, was calculated as the initial rate of formation of receptor-bound insulin ($v$) divided by $I$ and $k_{+1}$ according to Equation 3. This is equal to the initial slope ($v/I$) of the binding curves in Fig. 3, corrected for nonspecific binding, divided by $k_{-1}$. In this calculation the number of receptors/cell was 16,100.

**Steady State Binding of $^{125}$I-Insulin—**Fig. 5 shows the binding of $^{125}$I-insulin at steady state. The curve was computed as the best fit (least square method) of Equation 6 to the experimental data. In the concentration range from 25 pm to 100 nm there is a close fit of the curve to the data and the value of $B/F$ decreases from 8 to 1, which represents the saturable receptor binding. At concentrations above 100 nm the ratio of $B/F$ is approximately constant representing nonspecific binding ($Q$) according to Equation 6. The presence of a group of very low affinity binding sites cannot be excluded since the computed curve deviated slightly from the experimental results above 100 nm. In 12 experiments, the binding was determined with $300 \, \text{pm}$ $^{125}$I-insulin and native insulin in concentrations from 25 pm to 2.5 pm. The dissociation constant, $K_d$, was $400 \pm 60 \, \text{pm}$ (S.E.) and the number of receptors was $15,200 \pm 1,400$/cell (S.E.). These values have been corrected for the presence of $300 \, \text{pm}$ $^{125}$I-insulin assuming that the binding affinities of moniodinated and native insulin are identical in hepatocytes (26). In three experiments $^{125}$I-insulin was used in concentrations from 12 pm to 28 nm, while native insulin was only used at higher concentrations (cf. Fig. 5). The mean values of $K_d$ and the receptor number/cell in these experiments were $600 \pm 100 \, \text{pm}$ (S.E.) and $22,300 \pm 2,400$ (S.E.), respectively.

The nonspecific binding increased about 10 times in the absence of albumin, but was constant in the range of albumin concentrations from 10 to 40 mg/ml. The receptor-bound insulin was not affected by variations in the albumin concentration. The receptor-binding characteristics ($K_d$ and $R_0$) were not changed in hepatocytes, which were incubated with 0.8 mg of collagenase/ml for 20 min following the perfusion of the liver during the cell isolation procedure.

**Specificity—**The specificity of insulin receptors in hepatocytes was investigated with chemically modified insulins and insulins from various animal species. As seen in Fig. 6 the binding of $300 \, \text{pm}$ $^{125}$I-insulin was inhibited by four insulin analogues with different affinity. The parallel binding curves suggest simple competitive binding. The binding affinity relative to pig insulin of nine insulin analogues is in agreement with that previously determined in adipocytes (Table I). Insulin from the Atlantic hagfish represents an exception. This insulin has a binding affinity relative to pig insulin of $6.9\% \pm 2.3\%$ (S.D., $n = 6$) in hepatocytes as compared with $23\% \pm 7\%$ (S.D., $n = 10$) in adipocytes (16). These differences were also found with Des-methionine B31 hagfish insulin.

**Dissociation of $^{125}$I-Insulin—**The decrease in $^{125}$I-insulin bound to hepatocytes following a 1000-fold dilution of the extracellular insulin is shown in Fig. 7A. The dissociation of the receptor-bound $^{125}$I-insulin is shown in Fig. 7B. In three experiments 88% of the receptor-bound $^{125}$I-insulin dissociated with a half-time of $5.6 \pm 0.6 \, \text{min}$ (S.E.). This corresponds to

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Relationship between the rate constant of net uptake ($k$) and the insulin concentration ($I$). From the data shown in Fig. 3, $k$ was derived as $\ln 2/\tau_{1/2}$ where $\tau_{1/2}$ is the half-time for net uptake of receptor-bound insulin. Equation of the regression line is $k = 0.25 \, \text{nM}^{-1} \cdot \text{min}^{-1} \cdot 10^{-0.10 \, \text{min}^{-1}}$.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Steady state binding of $^{125}$I-insulin. Hepatocytes (0.125%, v/v) were incubated 30 min with $^{125}$I-insulin in concentrations from 12 pm to 28 nm (○) or with 3 pm $^{125}$I-insulin plus native insulin in total concentrations from 3.6 nm to 4.1 pm (□). The ratio between bound and free $^{125}$I activity (see “Methods”) was plotted versus the log of the total insulin concentration ($I$). Each value represents mean of triplicate determinations and the standard deviation is shown as bars. The curve was computed as the best fit of Equation 6 to the experimental data by the least square method and the equation of the regression curve is $B/F = (5.8 \, \text{mmol/liter of packed cells})/(820 \, \text{pm} + I)) + 1.34$. The number of receptors/cell is equal to 26,700.
a value of $k_1$ of 0.12 min$^{-1}$ in agreement with the rate constant of dissociation determined in the uptake experiments. Accordingly, the kinetic behavior of the major part of receptor-bound $^{125}$I-insulin was consistent with one group of receptors with $K_0$ of 500 pm.

In order to examine for the presence of negative cooperative interactions of insulin receptors in hepatocytes the dissociation of receptor-bound $^{125}$I-insulin after dilution was followed in the presence and absence of native insulin (9, 30). As shown

**Table I**

<table>
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<th>Insulin analogue</th>
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<th></th>
<th>Adipocytes</th>
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<td>Pig</td>
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<td>S.D.</td>
<td>100</td>
<td>S.D.</td>
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<tr>
<td>Pig proinsulin</td>
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<td>2</td>
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<tr>
<td>A$_3$-acetil</td>
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<td>5</td>
<td>3</td>
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<tr>
<td>A$_3$-BOC</td>
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<td>3</td>
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<tr>
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<td>3</td>
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<tr>
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<td>Sea scorpion</td>
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<td>Shark</td>
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<tr>
<td>Hagfish</td>
<td>6.9</td>
<td>2.3</td>
<td>6</td>
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Unpublished data.

$^a$ p < 0.005.
of dilution using [hydroxymethyl-3H]inulin added prior to the cell exponential as assumed in the model (CL Equation 4), since a present. Thus, at 37°C insulin receptor binding in hepatocytes dissociation were identical whether or not native insulin was determined in 50-μl aliquots of buffer immediately after separation for unbound '251 activity at zero time by from the cells at the indicated times. The values have been corrected prebound '251-insulin as described in the legend of Fig. 7, the immuno-

Inactivation of Receptor-Bound 125I-Insulin—The 125I activity which appeared in the medium during dissociation of receptor-bound 125I-insulin (Fig. 7B) was tested for immunoreactivity as shown in Fig. 9. The fast dissociating fraction was released as 40 to 50% immunoactive 125I-insulin during the first 30 min. The slow dissociating fraction probably was nonimmunoreactive, since the immunoreactivity of the 125I activity which accumulated in the medium decreased to about 30% in the period from 30 min to 2 h. This was not due to extracellular inactivation of insulin, since a suspension of hepatocytes, which had been preincubated in the absence of insulin followed by washing as described in the legend of Fig. 7 did not inactivate more than 5% of 150 pM 125I-insulin during 90 min of incubation (data not shown). Furthermore, addition of 1 μM native insulin at time zero did not reduce the fraction of non-immunoreactive 125I activity released. This favors the idea that this inactivation follows receptor binding, since the extracellular inactivation of 125I-insulin is inhibited by 1 μM insulin (cf. Fig. 3).

FIG. 8. Dissociation of receptor-bound 125I-insulin in the absence and presence of native insulin. Hepatocytes (0.38, v/v) were incubated with 1 nM 125I-insulin for 15 min at 37°C or 130 min at 12°C followed by washing and resuspension in buffer as described in the legend of Fig. 7. The buffer contained either 100 nM native insulin (C) or no insulin ( ). After washing the receptor-bound 125I activity was determined at the times indicated and expressed in per cent of the value before washing. The experiments were performed in a thermostated room.

Parameters of insulin receptor binding to isolated rat hepatocytes at 37°C

Table II

<table>
<thead>
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<th>Method</th>
<th>Dissociation constant (pM)</th>
<th>Receptor number/cell</th>
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<tr>
<td>Kinetic analysis</td>
<td>520</td>
<td>16,100</td>
</tr>
<tr>
<td>Steady state analysis</td>
<td>490</td>
<td>15,200</td>
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</table>

in Fig. 8 the dissociation rate of 125I-insulin was accelerated by 100 nM native insulin at 12°C, whereas at 37°C the rates of dissociation were identical whether or not native insulin was present. Thus, at 37°C insulin receptor binding in hepatocytes was not influenced by negative cooperative interactions.

The decline of receptor-bound 125I-insulin was not monoeXponential as assumed in the model (cf. Equation 4), since a residual 12% of the receptor-bound 125I-activity was eluted with a considerably longer half-time (about 3 h). This biexponential time course was not influenced by addition of 100 nM native insulin (Fig. 8).

DISCUSSION

Properties of Receptor Binding—In the present study of binding of 125I-insulin to isolated hepatocytes at 37°C two different insulin binding sites could be distinguished in the concentration range from 12 pm to 4 μM. One binding site was saturated by 100 nM insulin and showed characteristics of a receptor, i.e. high affinity, limited capacity, and insulin specificity. The other site was not saturated at supraphysiological insulin concentrations and showed no insulin specificity. This nonspecific binding was probably due to adsorption of insulin, since it was dependent on the albumin concentration. Uptake and steady state experiments with insulin concentrations in the physiological range from 12 pm to 10 nM showed internal consistency (Table II) and were compatible with a model of a reversible bimolecular reaction between insulin and receptors with uniform affinity. However, 50% of the receptor-bound insulin dissociated from the cells in an inactivated form, confirming quantitatively the findings by Terris and Steiner (8) in hepatocytes at 30°C. In spite of this, the model of a reversible binding reaction could be used in the kinetic description of binding data, since the dissociation of 88% of the receptor-bound 125I-insulin, partly in an immunoreactive form and partly in an inactive form occurred with a rate constant of 0.12 min⁻¹ in agreement with that determined from uptake experiments. The residual 12% of the receptor-bound 125I-insulin was released at a far slower rate and only in a nonimmunoreactive form. This phenomenon is not included in the model and this leads to an overestimation of the calculated receptor number and dissociation constant by approximately 12%. The slow rate of dissociation of a fraction of the receptor-bound 125I-insulin was also observed by others (5, 7, 9, 31) and led to the assumption that a second class of receptors of very high affinity was present (5, 31) in spite of lack of internal consistency between the uptake and dissociation experiments.

Fig. 9. Properties of released 125I activity. During dissociation of prebound 125I-insulin as described in the legend of Fig. 7, the immunoreactive 125I-insulin (C) and the total released 125I activity ( ) were determined in 50-μl aliquots of buffer immediately after separation from the cells at the indicated times. The values have been corrected for unbound 125I activity at zero time by determination of the factor of dilution using [hydroxymethyl-3H]insulin added prior to the cell washing. The results are the mean of two experiments.
In our study the presence of a small number of receptors with a dissociation constant below 30 pM cannot be excluded (cf. Figs. 3 and 5). Alternatively, the slowly dissociating $^{125}\text{I}$ activity represents $^{125}\text{I}$-insulin which has been taken up by the cell and degraded following receptor binding. Such internalization has been suggested for $^{125}\text{I}$-insulin in isolated hepatocytes (32, 33) and for $^{135}\text{I}$-labeled epidermal growth factor in human fibroblasts (34).

In previous studies (5, 7-9, 31) insulin receptor binding at steady state over a wide range of concentrations has been consistent with either binding site heterogeneity (5, 7, 8, 31) or negative cooperativity (9, 30). The idea that insulin receptor binding is modified by negative cooperative interactions was based on the observation that the rate of dissociation of $^{125}\text{I}$-insulin was accelerated by addition of native insulin (30). Recently, Pollet et al. (35) reproduced this phenomenon in cultured lymphocytes at 15°C, but kinetic data were inconsistent with negative cooperativity and they concluded that the lymphocyte insulin receptors behave kinetically and at equilibrium as a homogenous class of binding sites with $K_d$ of 500 pM in the range of physiological insulin concentrations. At high insulin concentrations a low affinity binding was observed (35). In our study the phenomenon of an accelerated dissociation rate caused by excess of native insulin was present at 12°C, but not at 37°C (Fig. 8). A similar temperature dependence was observed by De Meyts et al. (9). At high insulin concentrations a low affinity binding site with a $K_d$ about 50 to 100 nM could not be excluded from our results (Fig. 5). The nature of this binding and its physiological importance is questionable, but it may represent binding of $^{125}\text{I}$-insulin to receptors for growth-promoting factors, e.g., somatomedin C, which have been described in isolated placental membranes (36), since insulin and somatomedin C cross-react at their respective receptors in placental and liver membranes (36, 37). In conclusion, at 37°C, insulin is bound to a homogenous class of noninteracting receptors in hepatocytes within the physiological insulin concentration range.

The dissociation constant of 500 pM is in accordance with the high affinity binding in purified liver plasma membranes (5) observed at 30°C, but the value is about 5 times lower than that reported for hepatocytes by other groups (6-8). Thus, a $K_d$ of 2 nM was found at 30°C (6) under conditions where degradation of insulin resulted in decreasing insulin concentrations during the experimental period. At 20°C native insulin showed half-maximal inhibition of $^{125}\text{I}$-insulin binding at a concentration of 4 nM (7), but this value represents an average $K_d$ of heterogenous insulin receptors. Similar results were obtained by Terris and Steiner at 30°C (8), who determined a $K_d$ of 3.5 nM as the concentration of insulin, which inhibits the binding of 500 pM $^{125}\text{I}$-insulin to its half-maximal value after 60 min of incubation. However, they observed that the binding declined after 30 min at insulin concentrations below 900 pM and the binding was not measured at $^{125}\text{I}$-insulin concentrations below 560 pM. Furthermore, the $K_d$ was calculated without correction for this concentration of tracer as well as the presence of 8% nonspecific binding. These differences explain the discrepancy with our $K_d$ value.

**Inactivation of Insulin**—The inactivating activity released to the extracellular medium was similar to that previously described in suspensions of adipocytes with respect to inhibition by pig and hagfish insulin (16). Hagfish insulin was an 8 times more potent inhibitor of the inactivation as compared to pig insulin. In contrast the receptor binding affinity of hagfish insulin was only 7% of that of pig insulin in hepatocytes. This value is in agreement with that of Terris and Steiner (8), who observed no discrepancy between the receptor binding affinity and the potency of hagfish insulin relative to pig insulin as inhibitor of the receptor-linked inactivation. This suggests that the mechanism of inactivation of insulin following receptor binding is different from that of the extra-cellular inactivation, which may be due to released activity of the cytosolic insulin-specific liver protease, which has a $K_m$ of 100 nM (38), whereas the microsomal glutathione-insulin transhydrogenase has a $K_m$ of 30 pM (39).

**Insulin Binding and Effect in Hepatocytes and Adipocytes**—Insulin receptors in hepatocytes are characterized by a higher binding affinity as compared with adipocytes (19) ($K_d$ 500 pM as compared to 3 nM), which is due to a higher rate constant of association ($k_a$, 0.23 as compared to 0.026 nM$^{-1}$ min$^{-1}$). The relative binding affinity of insulin from the Atlantic hagfish in hepatocytes is lower than the value determined in adipocytes (16), suggesting structural differences between the insulin receptors in these two tissues. This view is supported by unpublished results by Kahn and coworkers, who found that the anti-insulin receptor antibodies from patients with a syndrome of extreme insulin resistance and Acanthosis nigricans (40, 41) inhibits binding of insulin in adipocytes and purified liver plasma membranes to different degrees. The number of receptors/cell is lower in hepatocytes as compared to adipocytes (10) (15,000 as compared to 40,000), but expressed as receptors/unit of surface area the numbers are approximately similar.

Adipocytes are very sensitive to insulin and the sensitivity has partly been ascribed to a large excess of receptors (spare receptors) (10, 28, 42). Half-maximal and maximal stimulation of lipogenesis from glucose is obtained at insulin concentrations of 60 pm and 160 pm respectively (10, 43), which are within the physiological range in the peripheral blood (11). The corresponding occupancies of receptors with a $K_d$ of 3 nM are 2 and 5% (10). Metabolic effects of insulin in the perfused rat liver or in isolated hepatocytes: stimulation of glycogen synthesis (32, 44), stimulation of lipogenesis (45), inhibition of glucagon-stimulated gluconeogenesis (46), and activation of phosphodiesterase (47) occur at insulin concentrations in the range from 100 pm to 7 nM, which is in accordance with the range of concentrations of insulin in the portal blood (11). Even if degradation of insulin in some of these studies is taken into account the hepatocytes seem less sensitive to insulin than adipocytes. These observations indicate a closer relationship between the half-maximal receptor binding and effect of insulin in hepatocytes as compared to adipocytes. Thus the phenomenon of spare receptors with respect to the biological action of insulin seems less pronounced in hepatocytes than in adipocytes. One physiological implication of this difference is that the hepatic insulin effect may change rapidly with variations in the portal plasma insulin concentration, while in the adipose tissue the maximal effect is maintained for some time after exposure to high insulin concentrations (42, 48). However, direct studies of the relationship between receptor binding and effect are needed to prove this hypothesis.

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