Receptor-mediated insulin degradation and insulin-stimulated glycogenesis in cultured foetal hepatocytes

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Insulin-stimulated glycogenesis and insulin degradation were studied simultaneously at 37°C in cultured foetal hepatocytes grown for 2–3 days in the presence of cortisol. Degradation of cell-associated insulin, as measured by trichloroacetic acid precipitation, was significant after 4 min in the presence of 1–3 nM [125I]-labelled insulin. This process became maximal (30% of insulin degraded) after 20 min, a time when binding-state conditions were achieved. No insulin-degradative activity was detected in a medium that had been exposed to cells. At steady-state, the appearance of insulin degradation products in the medium was linearly dependent on time (1.5 fmol/min per 10⁶ cells at 1 nM [125I]-labelled insulin). Chloroquine (3–50 μM), bacitracin (0.1–10 mM) and NH₄Cl (1–10 mM) inhibited insulin degradation as soon as this became detectable and caused an increase in the association of insulin to hepatocytes after 20 min. Lidocaine and dansylcadaverine had similar effects, whereas N-ethylmaleimide, aproptinin, phenylmethanesulphonyl fluoride and leupeptin were found to be ineffective. Chloroquine, and also bacitracin, at concentrations that inhibited insulin degradation, decreased the insulin-stimulated incorporation of [14C]glucose into glycogen over 2 h. This effect of chloroquine was specific, since it did not modify the basal glycogenesis, or the glycogenic effect of a glucose load in the absence of insulin. It therefore appears that the receptor-mediated insulin degradation (or some associated pathway) is functionally related to the glycogenic effect of insulin in foetal hepatocytes.

It is now well established that, on interaction with its receptors in target cells, insulin is internalized and ultimately associates with lysosomes. Although the main function of this process is to mediate the degradation of the hormone and possibly also the removal of its receptors from the cell surface, it has been proposed that insulin internalization and/or degradation might be required for biological action (Steiner, 1977). The functional relationship between the degradation and short-term biological effects of insulin in adipocytes had been examined in the presence of the lysosomotropic agent chloroquine. These experiments indicated that insulin degradation was not a prerequisite for biological action (Suzuki & Kono, 1979; Hammons & Jarett, 1980; Marshall & Olefsky, 1980).

Cultured foetal hepatocytes have been shown to bind insulin specifically and to respond to the presence of the hormone by an enhanced ability to synthesize glycogen (Plas et al., 1979; Menuelle & Plas, 1981). In the present study, the degradation of insulin by these cells was characterized and found to be exclusively receptor-mediated. This culture system was also used to examine the effects of chloroquine and other drugs that inhibit the cellular processing of receptor-bound ligands on the binding, degradation and biological action of insulin. The results suggest that insulin's action, and its degradation, may be functionally related in foetal hepatocytes.

Materials and methods

Materials

Chloroquine (sulphate form) was a gift from Specia Laboratories, Paris, France. Lidocaine, dansylcadaverine, bacitracin, p-tosyl-L-arginine methyl ester, phenylmethanesulphonyl fluoride, N-ethylmaleimide and lima-bean trypsin inhibitor were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. Aprotinin was from Choay Laboratories, Paris, France, and leupeptin from Bachem, Budendorf, Switzerland. The sources of other materials...
were specified previously (Plas & Nunez, 1976; Rouer et al., 1980).

Culture procedure
Primary cultures of hepatocytes were obtained from 17- and 18-day rat foetuses (Sprague–Dawley) as described previously (Plas et al., 1973). Briefly, after mild trypsin treatment, the isolated cells were plated on a collagen substratum to which only the hepatocytes adhered, and after 18 h the non-adhering haematopoietic cells were removed. At this step, the culture medium (2 ml/dish) was replaced. This culture medium consisted of NCTC 109 medium (Evans et al., 1964) supplemented with 10% (v/v) foetal-calf serum, 10 µM-cortisol and 10 mM-Hepes \[\text{[4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.3.}\] Then it was supplemented daily with glucose (0.5 mg/dish). All the experiments were performed after 2 or 3 days of culture in the presence of cortisol, when the glycogenic effect of insulin is fully expressed (Plas & Nunez, 1976).

Insulin binding and degradation studies
Pig insulin was labelled with $^{125}\text{I}$ at a specific radioactivity of 130–170 Ci/g by a modification (Desbuquois et al., 1974) of the chloramine-T method. Incubations of $^{125}\text{I}$-labelled insulin (1–3 nM) with hepatocytes (about $1.3 \times 10^{6}$ cells/dish) were performed at 37°C and pH 7.3, in 2 ml of culture medium. On completion of the incubation, the medium was removed and the cells were washed with $4 \times 2.5$ ml of ice-cold Hanks solution. This washing step was performed within 12s. The cell-associated radioactivity was then extracted at $4^\circ\text{C}$ by three successive additions each of 0.5 ml of 8.5 mM-acetic acid containing 0.35 mM-betacrin. Unless otherwise stated, the recovery of radioactivity in the extract was about 95–98%; this value did not differ significantly from that observed when 0.1% (w/v) Triton X-100 containing 3 mM-acetic acid and 6 mM-urea was used in the extraction procedure of Terris & Steiner (1975) (Table 1). The small amount of radioactivity remaining was solubilized with 0.7 ml of 0.5 M-NaOH for 30 min at 75°C. Corrections were made for the non-specific association of $^{125}\text{I}$-labelled insulin to cells by performing parallel incubations in the presence of native insulin (3 µM).

The labelled material recovered from the cells was allowed to dry at 23°C in a vacuum desiccator containing solid NaOH. The residue was dissolved in 1 ml of 0.1 M-HCl and the solution was neutralized with 1 mM-Tris. This material was then tested for its ability to be precipitated by 5% (w/v) trichloroacetic acid and to bind specifically to rat liver membranes by the technique detailed previously (Desbuquois et al., 1974). The extract was also subjected to gel filtration on Sephadex G-75 equilibrated with 5 mM-acetic acid, and the labelled material, which was co-eluted with iodotyrosines, was characterized by paper chromatography (Rouer et al., 1980). It was verified that the extraction procedure did not modify the behaviour of $^{125}\text{I}$-labelled insulin for the three parameters mentioned above.

Glycogenesis studies
Glycogen labelling in the presence of [U-$^{14}$C]-glucose was determined as described previously (Plas et al., 1973). At day 2 or day 3 of the culture, [$^{14}$C]glucose (1 µCi/mg) was introduced in the medium together with insulin or the drug to be tested. The radioactivity present in glycogen was measured after 2 h of incubation. To express the results, a stimulation, or inhibition, index was used, defined as the following ratio: (nmol of [$^{14}$C]-glucose/h per mg of protein in treated cultures)/(nmol of [$^{14}$C]-glucose/h per mg of protein in control

### Table 1. Characterization of cell-associated radioactivity in hepatocytes incubated with $^{125}\text{I}$-labelled insulin

<table>
<thead>
<tr>
<th>Addition</th>
<th>Method</th>
<th>Radioactivity recovered in the extract (%)</th>
<th>Radioactivity eluded from Sephadex (%)</th>
<th>Radioactivity soluble in trichloroacetic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>94.7</td>
<td>Peak I 2.2</td>
<td>20.0</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>97.4</td>
<td>Peak II 1.5</td>
<td>—</td>
</tr>
<tr>
<td>Chloroquine (46 µM)</td>
<td>1</td>
<td>94.6</td>
<td>Peak III 1.5</td>
<td>22.0</td>
</tr>
<tr>
<td>Chloroquine (46 µM)</td>
<td>2</td>
<td>95.5</td>
<td></td>
<td>5.1</td>
</tr>
</tbody>
</table>

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cultures). One million hepatocytes corresponds to 380 µg of protein, or to about 2.7 mg of wet liver weight (Plas & Nunez, 1976).

Definitions

For each protocol, at least three experiments were performed with different cell preparations. Data are means ± S.E.M. for the numbers of experiments (n) in parentheses. Every symbol (in the Figures) and value (in the Tables) corresponds to a different culture dish.

Results

Characteristics of insulin binding, insulin degradation and insulin-stimulated glycogenesis

Hepatocytes were incubated with 1 nM-¹²⁵I-labelled insulin, a submaximal concentration for the glycogenic response (Plas et al., 1979), and the cell-associated radioactivity was extracted, quantified and characterized after various intervals. The association of ¹²⁵I-labelled insulin to cells occurred rapidly; binding steady-state was reached by 10 min and persisted for at least 3 h. At the steady-state, approx. 15 fmol of insulin/culture was bound. In the initial 4 min, this cell-associated radioactivity could not be distinguished from intact insulin (trichloroacetic acid precipitation and rebinding technique analysis). With time, the cell-associated insulin gradually lost integrity and by 20 min, degradation reached a steady-state and remained so for several hours. At the steady-state, 25–30% of the bound hormone was altered when examined by trichloroacetic acid precipitation, and 35% had lost its capacity to bind to liver membranes (Fig. 1). On gel filtration, 75–80% of the radioactivity was recovered as intact insulin; most of the remaining radioactivity was present as low-molecular-weight material and was co-eluted with monoiiodotyrosine. A small amount (1–2%) of radioactive material was also present in the void volume of the column (Table 1). The elution profile of the radioactivity was identical with that obtained when gel filtration was performed on Sephadex G-50 equilibrated with the urea/acetic acid/NaCl mixture as described by Tersis & Steiner (1975). When paper chromatography was performed, at least 80% of the low-molecular-weight material migrated with iodotyrosines, predominantly monoiiodotyrosine. These studies were repeated with various concentrations of ¹²⁵I-labelled insulin. Increasing this concentration from 0.1 to 3 nM did not change the percentage of cell-associated degradation (remaining constant at about 25%), but with 10 and 30 nM-insulin it fell (to respectively 15 and 10%), when tested by trichloroacetic acid precipitation.

When hepatocytes were incubated with ¹²⁵I-labelled insulin, no immediate degradation was detectable in the medium. It only appeared after 30 min, and then continued to increase regularly with time, with a velocity of 1.5 fmol/min per 10⁶ cells.

![Graph](image-url)

Fig. 1. Time course of association of insulin to hepatocytes and characterization of cell-associated hormone

At day 2 of the culture, 1 nM-¹²⁵I-labelled insulin was introduced in 2 ml of medium and hepatocytes were then incubated at 37°C for the times indicated. At the end of incubation, the total amount of cell-associated radioactivity was determined, and this material was characterized by trichloroacetic acid precipitation and rebinding techniques as described in the Materials and methods section. (a) Total (▲), trichloroacetic acid-precipitable (■) and trichloroacetic acid-soluble (□) cell-associated radioactivity are represented. (b) The percentage of total cell-associated radioactivity precipitable by trichloroacetic acid (○) and able to rebind to liver membranes (●) is represented. Each culture represents 1.6 × 10⁶ 18-day-foetal hepatocytes (▲) and 1.0 × 10⁶ 17-day-foetal hepatocytes (■).
Table 2. Effects of chloroquine on the degradation of cell-associated and medium insulin

At day 2 of the culture, 1 nM-125I-labelled insulin (2 pmol) was introduced in 2 ml of medium, and at the times indicated in the Table total binding and degradation of both cell-associated and medium insulin were determined. When tested, chloroquine was added at the same time as 125I-labelled insulin. Also 1 nM-125I-labelled insulin was added to 2 ml of medium in which hepatocytes had been grown for 30 h, but in the absence of cells (*). The fraction of the cell-associated radioactivity extracted by 8.5 M-acetic acid was 98% in the absence of chloroquine, whereas it was respectively 88, 79 and 76% after 2, 4 and 6 h of chloroquine presence. The percentage degradation of fresh 125I-labelled insulin (1.5%) was not subtracted.

<table>
<thead>
<tr>
<th>Time of extraction (h)</th>
<th>Addition</th>
<th>125I-labelled material bound (fmol/culture)</th>
<th>Cell-associated insulin degraded (% (fmol/culture))</th>
<th>Medium insulin degraded (%) (fmol/culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>—</td>
<td>30.0 7.5</td>
<td>12.4 248</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>—</td>
<td>33.3 7.8</td>
<td>21.3 426</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
<td>29.9 5.3</td>
<td>29.9 598</td>
</tr>
<tr>
<td>2 Chloroquine (46 μM)</td>
<td>76.8</td>
<td>9.0</td>
<td>6.9</td>
<td>4.9 98</td>
</tr>
<tr>
<td>4 Chloroquine (46 μM)</td>
<td>76.4</td>
<td>7.2</td>
<td>5.5</td>
<td>8.2 164</td>
</tr>
<tr>
<td>6 Chloroquine (46 μM)</td>
<td>63.8</td>
<td>8.4</td>
<td>5.4</td>
<td>12.0 240</td>
</tr>
<tr>
<td>2*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.2 24</td>
</tr>
<tr>
<td>2*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.6 32</td>
</tr>
</tbody>
</table>

(Table 2). The possibility that cell enzymes released into the medium contributed to the insulin-degrading activity was examined. No degradation of 125I-labelled insulin occurred when the hormone was incubated for 4 h in a medium in which hepatocytes had grown for 30 h (conditioned medium). Since, at the steady-state, equivalent amounts of degradation products were formed within the cell and released extracellularly, it was possible to calculate that within 1 min degradation affected approx. 7% of the cell-associated insulin.

Insulin-stimulated glycogenesis was measured by the incorporation of [14C]glucose into glycogen over a 2 h incubation with 3 nM-insulin; this concentration gives the maximal glycogenic effect (Plas et al., 1979). This incorporation was 70.6 ± 6.40 and 21.9 ± 1.61 (n = 6) nmol of [14C]glucose/h per mg of protein in the presence and absence of insulin respectively in 18-day-foetal hepatocytes grown for 3 days in the presence of 5 mM-glucose in the medium (insulin stimulation index, 3.2). A glucose load was an effective stimulator of glycogenesis in the absence of insulin. The incorporation of [14C]-glucose into glycogen was equal to 24.4 ± 1.48, 74.5 ± 5.77 and 92.8 ± 5.56 (n = 5) nmol of [14C]-glucose/h per mg of protein for final concentrations of 5, 15 and 35 mM-glucose respectively. Thus the stimulation of glycogenesis when the glucose concentration increased from 5 to 15 mM (glucose stimulation index, 3.1) was similar to that obtained by the addition of 3 nM-insulin.

Effect of various inhibitors on insulin binding and degradation

Agents that have been reported to inhibit degradation of peptide hormones by intact cells, particulate fractions and/or isolated enzymes were tested for their ability to affect the degradation and binding of 125I-labelled insulin in foetal hepatocytes. Chloroquine, NH₄Cl, bacitracin, lidocaine and dansylcadaverine decreased the degraded fraction of cell-associated radioactivity and increased the total bound radioactivity (Table 3). At the highest concentrations tested, these agents inhibited insulin degradation by 80–90%, and enhanced total binding by 30% (tests performed after 20 min of insulin and drug presence). A 50% inhibition was obtained with about 10 μM-chloroquine, 1–2 mM-bacitracin and 3–10 mM NH₄Cl. The concentrations required for half-maximal effects on binding were similar. In parallel experiments, leupeptin, N-ethylmaleimide, aprotinin, lima-bean trypsin inhibitor, p-tosyl-L-arginine methyl ester and glucagon were ineffective (Table 3) when used in a concentration range that is known to inhibit insulin degradation in other cell systems.

The effects of chloroquine and bacitracin on the time course of binding of 125I-labelled insulin and degradation of cell-associated radioactivity were also examined. The inhibitory effect of these drugs on insulin degradation occurred as soon as this process could be detected, i.e. after 4 min. It was more pronounced during the phase of insulin association than at the steady-state (Fig. 2). In contrast, no increase in total cell-associated radioactivity was detectable before 20 min. Later on this effect became more important, as a result of a slow but progressive increase in cell-associated radioactivity in the presence of the drugs. For example, the presence of 46 μM-chloroquine for 2–6 h increased the cell-associated radioactivity by more than 3-fold compared with control values (Table 2). In addition to
Table 3. Effect of different agents on the binding and degradation of 125I-labelled insulin

Hepatocytes were incubated at 37°C for 20 min with 3 nM-125I-labelled insulin. The agents tested were added together with 125I-labelled insulin at the concentrations indicated. At the end of incubation, the total amount and the percentage of trichloroacetic acid-soluble radioactivity in the cell extracts were determined as described in the Materials and methods section. Each culture represents 1.2 × 10^6, 1.4 × 10^6 and 1.3 × 10^6 18-day-foetal hepatocytes in 2 ml of culture medium for Expts. 1, 2, and 3 respectively. None of the agents listed in the Table affects the non-specific binding of insulin, which represents 0.1% of 125I-labelled insulin present in the medium. In parallel experiments, phenylmethanesulphonyl fluoride (0.1–0.5 mM), aprotinin (1–100 μM), lima-bean trypsin inhibitor (0.3–10 μM), p-tosyl-L-arginine methyl ester (0.2–2 mM) and glucagon (0.01 nM–1 mM) were found to be unable to modify binding and degradation of cell-associated insulin.

<table>
<thead>
<tr>
<th>Expt. 1</th>
<th>125I-labelled material bound (c.p.m./culture)</th>
<th>Radioactivity soluble in trichloroacetic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>13 880</td>
<td>27.6</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>14 450</td>
<td>25.0</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>14 930</td>
<td>22.3</td>
</tr>
<tr>
<td>0.7 mM</td>
<td>16 370</td>
<td>13.6</td>
</tr>
<tr>
<td>2.0 mM</td>
<td>16 090</td>
<td>6.9</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>13 680</td>
<td>27.5</td>
</tr>
<tr>
<td>3 mM</td>
<td>15 540</td>
<td>13.8</td>
</tr>
<tr>
<td>10 mM</td>
<td>17 200</td>
<td>6.0</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>No addition</td>
<td>13 140</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>13 970</td>
<td>28.8</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>14 500</td>
<td>23.6</td>
</tr>
<tr>
<td>1 mM</td>
<td>14 930</td>
<td>17.9</td>
</tr>
<tr>
<td>2 mM</td>
<td>14 880</td>
<td>14.8</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>12 340</td>
<td>34.0</td>
</tr>
<tr>
<td>5 μM</td>
<td>14 570</td>
<td>24.8</td>
</tr>
<tr>
<td>10 μM</td>
<td>15 400</td>
<td>17.7</td>
</tr>
<tr>
<td>20 μM</td>
<td>17 400</td>
<td>12.9</td>
</tr>
<tr>
<td>50 μM</td>
<td>17 520</td>
<td>6.7</td>
</tr>
<tr>
<td>Expt. 3</td>
<td>No addition</td>
<td>11 800</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>10 960</td>
<td>26.6</td>
</tr>
<tr>
<td>3 mM</td>
<td>11 760</td>
<td>24.2</td>
</tr>
<tr>
<td>10 mM</td>
<td>16 960</td>
<td>11.0</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>13 720</td>
<td>23.3</td>
</tr>
<tr>
<td>0.6 mM</td>
<td>13 680</td>
<td>13.4</td>
</tr>
<tr>
<td>2 mM</td>
<td>16 880</td>
<td>13.4</td>
</tr>
<tr>
<td>Dansylcadaverine</td>
<td>12 000</td>
<td>25.4</td>
</tr>
<tr>
<td>50 μM</td>
<td>16 120</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Affecting binding and degradation of 125I-labelled insulin, chloroquine decreased the rate at which cell-associated radioactivity was released into the medium, after removal of extracellular hormone (results not shown). Chloroquine and bacitracin also inhibited the degradation of insulin in the medium that occurred in the presence of cells. As this process is slow even in the absence of inhibitors, such effects were only detectable after 1 h. From 2 to 6 h after addition of 125I-labelled insulin, the rate of insulin degradation in the medium was inhibited by 60% in the presence of 46 μM-chloroquine (Table 2).

Effects of inhibitors of insulin degradation on basal and insulin-stimulated glyco genesis

Chloroquine, bacitracin and NH₄Cl were first examined for their effect on basal glyco genesis. In these experiments, [14C]glucose incorporation into glycogen was measured over a 2 h incubation period in the absence of insulin, and each drug was tested at concentrations that inhibited insulin degradation maximally. Basal glycogenesis was in general not affected by chloroquine concentrations below or equal to 100 μM (Fig. 3), but was decreased at higher concentrations. It was slightly enhanced by bacitracin at concentrations exceeding 1 mM (stimulation index for 2 mM-bacitracin 1.15–1.30). A marked inhibition was observed with NH₄Cl (inhibition index for 10 mM-NH₄Cl, 0.50). In view of these results, chloroquine and bacitracin were tested for their effect on the insulin-stimulated glyco genesis obtained during a 2 h pulse labelling in the presence of [14C]glucose. Chloroquine had a progressive, concentration-dependent, inhibitory effect
Fig. 2. Effect of chloroquine and bacitracin on the time course of insulin binding and insulin cellular degradation
Hepatocytes (18-day-foetal) were incubated at 37°C for the times indicated with 3 nM-125I-labelled insulin in the presence of 20 μM-chloroquine (a and c) or with 1 nM-125I-labelled insulin in the presence of 2 mM-bacitracin (b and d). Parallel incubations in the absence of these drugs were performed as controls. At the end of incubation, the total amount (∆ and A in a and b) and the trichloroacetic acid-soluble (○ and ● in c and d) cell-associated radioactivity were determined. ∆ and ○, Inhibitor present; A and ●, no inhibitor.

Fig. 3. Effect of chloroquine on insulin-stimulated glycogenesis, insulin binding and cellular insulin degradation
(a) Various amounts of chloroquine (indicated on each abscissa) were introduced in the medium, with (■) or without (□) 3 nM-insulin. At the same time, [14C]glucose was added (1 μCi/mg), and the radioactivity present in glycogen was determined 2 h later. (b and c) Various amounts of chloroquine were introduced together with 3 nM-125I-labelled insulin. The percentage of trichloroacetic acid-soluble (b) and the total (c) cell-associated radioactivity were determined after 1 h (○ and △) and 2 h (● and ▲) of incubation. Each culture represents 1.1 x 10⁶ 18-day-foetal hepatocytes.
Table 4. Effect of chloroquine on insulin- and glucose-stimulated glycogenesis
At day 2 or day 3 of the culture, insulin (3 or 10 nM) or a load of glucose (final glucose concentrations in the medium, 15 and 35 mM) were introduced in the medium together with chloroquine at the concentrations indicated. At the same time, [14C]glucose was added, and the radioactivity present in glycogen was determined 2 h later. In all experiments, the specific radioactivity of glucose in the medium was 1 μCi/mg. Each culture represents 1.3 x 10⁶ (Expt. 1) and 1.1 x 10⁶ (Expt. 2) 18-day-foetal hepatocytes in 2 ml of medium.

\[
\begin{array}{ccc}
\text{Expt. 1 (day 2)} & \text{No chloroquine} & 46 \mu M-\text{Chloroquine} \\
5 \text{mM-Glucose} & 30.6-30.3 & 29.2-28.1 \\
5 \text{mM-Glucose, 3 nM-insulin} & 69.1-68.6 & 39.4-36.4 \\
35 \text{mM-Glucose} & 119.5-122.2 & 115.9-126.8 \\
\text{Expt. 2 (day 3)} & \text{No chloroquine} & 68 \mu M-\text{Chloroquine} \\
5 \text{mM-Glucose} & 18.9 & 18.4 \\
5 \text{mM-Glucose, 10nM-insulin} & 66.9 & 32.3 \\
15 \text{mM-Glucose} & 63.4 & 63.8 \\
\end{array}
\]

on the ability of insulin to stimulate glycogenesis. In the experiment presented in Fig. 3, a total inhibition of the effect of 3 nM-insulin occurred with 100 μM-chloroquine, and the concentration required for a half-maximal effect was about 20 μM. It is noteworthy that the same concentration range of chloroquine was effective on insulin-stimulated glycogenesis as on insulin degradation and cell-associated radioactivity (Fig. 3). For 2 mM-bacitracin, the stimulation index in the presence of 3 nM-insulin was also decreased, from 3.1 in control cultures to 1.9 in the presence of the drug. To test the possibility that chloroquine might also intervene at some other specific step of the glycogenic pathway, tests were made on its effect on the glycogenic action of a glucose load. Chloroquine concentrations that were effective in inhibiting insulin degradation again impaired the insulin effect, but did not affect the glucose-load stimulated, or the basal, glycogenesis (Table 4). The effect of bacitracin on the glucose-load-stimulated glycogenesis was not tested.

Discussion
The functional relationship between insulin binding and insulin degradation was examined in foetal hepatocytes in culture. Insulin degradation was observed in the culture medium as well as within the cell itself. As no insulin-degradative activity was released extracellularly, this implies that the degradation products arose solely from the cells. The process of insulin degradation described in this paper would thus appear to be exclusively receptor-mediated. Insulin binding and insulin degradation have also been correlated in isolated (Terris & Steiner, 1975) and cultured (Caro & Amatruda, 1980) adult hepatocytes, and in perfused liver (Terris & Steiner, 1976). However, a non-receptor-mediated degradation has been found in other isolated hepatocyte preparations, especially at 37°C (Le Cam et al., 1975; Gammeltoft et al., 1978). This degradation process may be due to insulin-degrading proteinases released into the medium (Gammeltoft et al., 1978). There is evidence to show that receptor-mediated insulin degradation occurs intracellularly, and involves lysosomes. Internalization, and association of insulin with lysosomes or closely related structures, have been demonstrated morphologically in isolated hepatocytes (Carpentier et al., 1979a) and intact liver (Bergeron et al., 1979; Carpentier et al., 1979a). Biochemical studies with the latter system have shown that internalized insulin, unlike insulin bound to cell-surface membranes, is, in part, degraded hormone (Desbuquois et al., 1979; Posner et al., 1980). The presence of cell-associated degradation products in cultured foetal hepatocytes suggests a process of insulin internalization. Indeed, any product formed at the cell surface, by virtue of its inability to bind to the receptors, would not be expected to remain associated to cells.

In the present study, the lysosomotropic amine chloroquine was found to inhibit degradation of both cell-associated insulin and medium insulin, and concomitantly to increase cell-associated radioactivity. NH₄Cl and lidocaine produced similar effects, whereas leupeptin was ineffective; these results confirm those obtained with adult hepatocytes and hepatoma cells (Terris et al., 1979). The enhanced binding caused by chloroquine presumably reflects the accumulation of undegraded insulin within the cell, rather than an increase in the number and/or the affinity of the receptors (Marshall &
Olesky, 1979). Lysosomotropic amines are generally believed to inhibit lysosomal proteolysis by their ability to increase lysosomal pH; they may also directly affect the activity of some lysosomal proteinases, like cathepsin B1 (De Duve et al., 1974). Chloroquine has been found to enhance the association of insulin to adipocytes (Hammons & Jarett, 1980) and of epidermal growth factor to fibroblasts (McKanna et al., 1979); in both cases, morphologically identified lysosomes were involved. These findings do not rule out the possibility that chloroquine may also interfere with earlier steps in the processing of receptor-bound ligands.

In cultured foetal hepatocytes, the polypeptide antibiotic bacitracin was found to modify insulin binding and degradation in the same way as chloroquine. Bacitracin is known to inhibit insulin and glucagon degradation in broken and intact liver cell preparations (Desbuquois et al., 1974; Carpentier et al., 1979b; Rouer et al., 1980), as well as liver glutathione–insulin transhydrogenase [protein–disulphide reductase (glutathione); Roth, 1981]. This drug, and dansylcadaverine, which is also an effective inhibitor of insulin degradation in the present study, have been reported to block epidermal growth factor receptor-mediated endocytosis, and to inhibit transglutaminase (Maxfield et al., 1979; Haigler et al., 1980). However, as bacitracin has been shown to enhance binding, and to inhibit degradation of glucagon in liver membranes (Desbuquois et al., 1974), it is not certain that this drug acts at intracellular sites. Furthermore, bacitracin is not known to accumulate in lysosomes and/or to inhibit lysosomal enzymes. It is of interest to note that bacitracin, which inhibits non-receptor-mediated insulin degradation in isolated adult hepatocytes (Carpentier et al., 1979b; Rouer et al., 1980) is also an effective inhibitor of the receptor-mediated process in cultured foetal hepatocytes.

Polypeptides that were chemically unrelated to insulin, several inhibitors of trypsin or related enzymes and N-ethylmaleimide failed to inhibit insulin cellular degradation in the present study. Since some of these agents can inhibit insulin degradation by liver membranes (Freychet et al., 1972) and purified proteolytic enzymes (Burghen et al., 1972; Varandani et al., 1975), their effectiveness in foetal hepatocytes may be due to their inability to have access to the insulin-inactivating system(s). When these inhibitors block insulin degradation in isolated hepatocytes (Le Cam et al., 1975; Carpentier et al., 1979b) and circulating granulocytes (Fussanger et al., 1976), they may do so by acting on non-receptor-mediated degradation processes. The effects of the inhibitors described here do not provide further information on the nature of the enzyme(s) involved in cell-associated insulin degradation in hepatocytes. Two potential candidates are the enzymes glutathione–insulin transhydrogenase (Varandani et al., 1975) and insulin-specific proteinase (Burghen et al., 1972). Although these enzymes are not primarily associated with lysosomes, both have been identified in liver membranes (Varandani, 1973; Duckworth, 1979); this finding is consistent with the suggestion (Terris et al., 1979) that an early degradative event may affect insulin at the cell surface before, or during, endocytosis of the ligand–receptor complex.

One major finding of the present study was that chloroquine and bacitracin can inhibit insulin-stimulated glycogenesis. It is noteworthy that their effect on insulin degradation and hormonal action occurred with the same concentration range of the drug. The effect of chloroquine cannot be attributed to its toxicity since both basal and glucose-stimulated glycogenesis remained unaffected. Bacitracin, which has itself a glycogenic effect, was not tested for its interference with the glycogenic effect of a glucose load. While this investigation was in progress, a report by Hofmann et al. (1980) appeared, which described a partial inhibition of both insulin-stimulated glycogenesis and insulin degradation by chloroquine, and also by vinblastine, in cultured hepatoma cells. By contrast, no relationship could be established between the inhibition of insulin degradation by chloroquine, and such short-term biological insulin effects as stimulation of glucose transport and oxidation, and inhibition of adrenaline-stimulated lipolysis in isolated adipocytes (Suzuki & Kono, 1979; Hammons & Jarett, 1980; Marshall & Olesky, 1980). Whether these results reflect differences in fundamental mechanisms by which insulin elicits its various effects is as yet unknown.

At first sight, the ability of chloroquine and bacitracin to inhibit insulin degradation and insulin-stimulated glycogenesis support the previous proposal (Steiner, 1977) that insulin fragments released intracellularly might mediate some effects of the hormone. Several observations are consistent with this proposal; synthetic peptides with the sequence B23–B26 have insulin-like action (Weitzel et al., 1975), antibodies against glutathione–insulin transhydrogenase suppress phosphodiesterase activation by insulin in isolated adipocytes (PhePS & Varandani, 1977) and IM4 lymphocytes, which failed to show biological responsiveness to insulin, do not degrade receptor-bound insulin (Sonne & Gliemann, 1980). However, because anti-(insulin receptor) antibodies mimic short-term (Kahn et al., 1977) and long-term (Van Obberghen et al., 1979) effects of insulin, this mechanism is now regarded as unlikely. For example, anti-(insulin receptor) antibodies have been shown to both mimic the glycogenic effect of insulin as well as to inhibit insulin binding in isolated preparations of muscle (Le Marchand-Brustel et al., 1982).
1978) and adult hepatocytes (Baldwin et al., 1980). The results presented here, and those of Hofmann et al. (1980), are compatible with the concept that degradation of a cellular, perhaps membrane-associated, component may be involved in insulin action. It is significant that insulin has been shown to trigger the proteolytic release of a peptide material in isolated adipocyte membranes that activates pyruvate dehydrogenase (Seals & Czech, 1980; Seals & Jarett, 1980). This material may be identical with the chemical mediator generated by insulin in muscle (Larner et al., 1979; Jarett & Seals, 1979).

In this context, the relationship between insulin degradation and its glycogenic effect in cultured foetal hepatocytes could be explained if we postulate that insulin degradation, and the proteolytic process implied in the generation of the chemical mediator of insulin, are functionally related, or just correlated. An alternative possibility would be that the insulin-degrading activity, by liberating the insulin receptor, could modify the fate of the receptor in the cell, thus influencing the number, and the rate of renewal, of insulin receptors at the cell surface.

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References

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