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Insulin binding to cultured adult hepatocytes

Effects of bacitracin and chloroquine on the nature of cell-associated radioactivity

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Sephadex (G-50 fine grade)-gel chromatography and trichloroacetic acid (TCA) precipitation were used to investigate the effects of chloroquine and bacitracin on the nature of cell-associated radioactivity in studies on the binding and degradation of ^{125}I-insulin in cultured rat hepatocytes. Sephadex peak I, eluted with the void volume, increased with hepatocyte incubation time and comprised 6% of total cell-bound radioactivity at 120 min. However, all radioactivity in this peak was due to unspecific binding. Peak II, corresponding to intact insulin, represented 95% of specifically cell-associated label at 5 min and decreased to 77% at 120 min. Peak III, containing the final low-M_r degradation products, increased with incubation time (22% of specifically bound label at 120 min). The TCA-precipitable and TCA-soluble fractions of hepatocytes extracted with 0.1% SDS were within 4–7% of the proportions of radioactivity in peaks II and III respectively. Scatchard plots based on insulin-binding data from Sephadex chromatography or TCA precipitation were identical. Dissociation studies revealed that at least 75% of the intact insulin associated with the hepatocytes was bound to receptors at the cell surface. Bacitracin increased the proportion of cell-associated intact hormone and decreased that of ligand degraded when analysed by either Sephadex chromatography or TCA precipitation. The proportion of surface-bound to internalized intact hormone remained unaltered, indicating that bacitracin acted predominantly at the cell surface. In the presence of chloroquine, which dramatically increased the contribution of peak I to specific binding, 'intact' insulin was substantially overestimated when determined as the TCA-precipitable fraction. In addition, all peak I material and 50% of cell-associated label in peak II was trapped intracellularly, thereby pointing to the lysosomal or pre-lysosomal site of action of this drug.

INTRODUCTION

In studies on ^{125}I-insulin binding to cells capable of degrading the hormone under appropriate incubation conditions, a substantial amount of the radioactivity associated with the cells may represent degraded rather than intact tracer insulin (Caro & Amatruda, 1980; Caro *et al.*, 1982; Assoian & Tager, 1982; Donner, 1982). Although the error introduced by including the labelled degradation products in the calculation of cell-bound hormone, potentially leading to non-linearity of the Scatchard plot, is controversial (Caro & Amatruda, 1980; Davidson & Venkatesan, 1982), subtraction of degraded tracer from 'bound' hormone is essential for the correct determination of receptor numbers. Knowledge about the nature of cell-associated radioactivity is even more important when substances affecting hormone degradation are investigated. We have therefore used the extraction of cells with acetic acid, urea and detergents and subsequent chromatography on a Sephadex column (Kahn & Baird, 1978; Hammons & Jarett, 1980; Caro & Amatruda, 1981; Davidson & Venkatesan, 1982), as well as the precipitation of cell extracts by trichloroacetic acid (TCA) as described in adipocytes (Hammons & Jarett, 1980), to examine the potential effects of bacitracin and chloroquine on the nature of cell-associated radioactivity in studies on the binding and degradation of ^{125}I-insulin in cultured adult rat hepatocytes.

MATERIALS AND METHODS

Materials

Plastic culture dishes were from Nunc, Wiesbaden, Germany. Stock media for hepatocyte culture, glutamine and antibiotics were purchased from Biochrom, Berlin, Germany. Monoiodinated pig [^{125}I-Tyr^{A14}]insulin and unlabelled purified pig insulin were kindly given by Dr. Bruce Frank through the courtesy of Dr. F. H. Enzmann, Eli Lilly and Co., Bad Homburg, Germany. Labelled insulin was purified by chromatography on Sephadex G-50 (fine grade) before use. Bovine serum albumin, from Behringwerke, Marburg, Germany, was dialysed extensively against Earle's balanced salt solution before addition to the medium. Sephadex G-50 was obtained from Pharmacia, Freiburg, Germany. All other chemicals were analytical grade.

Cell culture

Hepatocytes were isolated from fasted male Wistar rats (Chbb strain; Thomae, Biberach, Germany), weighing 150–180 g, by use of a modification (Fleig *et al.*, 1984*a*) of the procedure of Berry & Friend (1969). After a 4 h seeding period with serum-supplemented Dulbecco's minimal essential medium, hepatocyte monolayers were maintained under serum- and hormone-free conditions as detailed previously (Fleig *et al.*, 1984*b*). Dulbecco's minimal essential medium was supplemented with

Abbreviation used: TCA, trichloroacetic acid.

10 mM-glucose, 2 mM-lactate, 2 mM-glutamine, essential and non-essential amino acids, 40 mM-$NaHCO_3$ [pH 7.4 at 37 °C under CO_2/air (2:23)], 50 μunits of penicillin/ml, 50 μg of streptomycin/ml and 1 mg of albumin/ml. Medium was changed at 24 h. After 48 h, the monolayers were washed three times with serum- and hormone-free medium and then used for insulin-binding experiments.

Incubation protocols and sample preparation

Hepatocytes were incubated for various periods of time as indicated in the medium detailed above at 37 °C with 50 pM-^{125}I-insulin with or without unlabelled insulin and with or without chloroquine or bacitracin. Unspecific binding was determined in the presence of excess unlabelled insulin (1 μM) and represented 10–15% of total insulin bound. Four to five dishes of each experimental condition were run in parallel. After the incubation, the medium was aspirated and analysed for TCA-soluble and TCA-precipitable radioactivity as described by Fleig *et al.* (1984*b*). The monolayers were washed five times with ice-cold phosphate-buffered saline within 20 s. Then they were either dissolved in 1 ml of 0.1% SDS in the cold or extracted into 1 ml of 4 M-urea/1 M-acetic acid/0.1% (v/v) Triton X-100. For dissociation experiments, monolayers incubated with 50 pM-^{125}I-insulin with or without 1 μM unlabelled hormone and with or without chloroquine or bacitracin for 120 min were washed as described above. Thereafter, dishes were either immediately analysed as detailed above or incubated for another 60 min in 3 vol. of the same medium as above, except that labelled hormone was omitted and all dishes received 1 μM unlabelled insulin to allow surface-bound insulin to dissociate or to be processed by the cells. Dissociation medium and monolayers were then processed as detailed above. Alternatively, monolayers incubated with ^{125}I-insulin for 120 min were washed, treated with cold (4 °C) 0.5 M-NaCl/0.2 M-acetic acid (pH 2.5) for 6 min (Haigler *et al.*, 1980) to dissociate surface-bound label and then processed as usual.

Analysis of cell-associated radioactivity

Monolayers solubilized in SDS were precipitated with TCA (final concn. 10%, w/v). After centrifugation, soluble and precipitable radioactivity was determined. Hepatocyte extracts in 4 M-urea/1 M-acetic acid/0.1% Triton X-100 were counted for total cell-bound radioactivity and then chromatographed on a column (1.8 cm × 30 cm) of Sephadex G-50 (fine grade) equilibrated and eluted with the same buffer at a flow rate of 1 ml/min; 1 ml fractions were collected and counted for radioactivity. Counts were corrected for ^{125}I decay, since analysis of a single experiment lasted several days. All binding data reflect specific binding. Similarly, proportions of TCA-soluble and TCA-precipitable radioactivity and of label in peaks I–III from Sephadex chromatography were obtained by correcting total radioactivity in the respective fractions for the amount of label detected in the presence of 1 μM unlabelled insulin.

DNA was extracted from parallel monolayers as detailed previously (Fleig *et al.*, 1984*a*) and quantified by the diphenylamine reaction (Burton, 1956).

RESULTS

In a first series of experiments we followed the time course of ^{125}I-insulin binding to the hepatocyte monolayers between 5 and 120 min of incubation. The amount of specifically bound insulin, normalized to cellular DNA, is given in Table 1. In addition, TCA-soluble and TCA-precipitable proportions of cell-associated radioactivity were compared with the different labelled fractions eluted from the Sephadex column. Two representative elution profiles of hepatocyte extracts after 5 and 120 min of incubation are presented in Fig. 1. Peak I, eluted with the void volume, represents label incorporated in or associated to a high-M_r compartment. Its contribution to total cell-associated radioactivity increased with incubation time, but did not exceed 6% of total even after 2 h. However, label appearing in peak I was completely due to unspecific binding, because this peak contained the same amount of radioactivity also in the presence of 1 μM unlabelled insulin. Peak II appeared at the position of intact ^{125}I-insulin. The relative proportion of this peak in specifically cell-bound radioactivity decreased from 95.5% at 5 min to 76.9% at 120 min. In contrast, peak III, co-migrating with ^{125}I-tyrosine and representing the

Table 1. Analysis of cell-associated ^{125}I radioactivity by precipitation with TCA and by Sephadex chromatography: time course of insulin binding

Hepatocytes cultured under control conditions for 48 h were incubated with ^{125}I-insulin (50 pM) in the absence or presence of 1 μM unlabelled insulin for the indicated times and processed as described in the Materials and methods section. Percentages given characterize the nature of ^{125}I radioactivity specifically bound to the cells. Values are means ± 1 S.E.M. for five plates and are representative of three identical experiments. Abbreviation: n.d., not detectable.

Incubation time (min)	^{125}I-insulin specifically bound (fmol/μg of DNA)	Nature of cell-bound label (% of specifically bound radioactivity)				
		TCA		Sephadex peak		
		Precipitate	Soluble	I	II	III
5	0.231 ± 0.007	90.98 ± 0.48	9.03 ± 0.48	n.d.	95.45 ± 2.46	3.44 ± 0.40
15	0.389 ± 0.009	85.76 ± 0.52	14.24 ± 0.52	n.d.	91.34 ± 1.08	7.69 ± 0.91
30	0.414 ± 0.013	79.52 ± 1.59	20.48 ± 1.59	0.74 ± 0.55	83.35 ± 3.72	13.78 ± 3.43
120	0.335 ± 0.022	73.24 ± 2.05	26.76 ± 2.05	n.d.	76.94 ± 1.31	22.03 ± 1.15

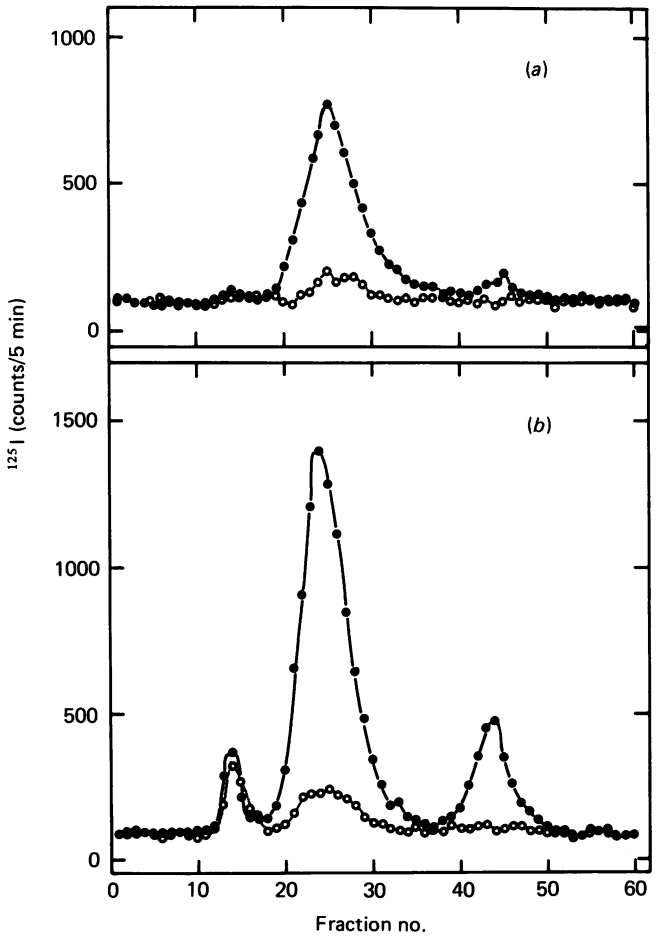

Fig. 1. Sephadex chromatography of hepatocyte extracts in ^{125}I-insulin binding: time course

Cells cultured under control conditions for 48 h were incubated with 50 pM-^{125}I-insulin without (●) or with (○) 1 μM unlabelled insulin for (*a*) 5 or (*b*) 120 min. Cells were extracted as described in the Materials and methods section and chromatographed on Sephadex G-50 (fine grade) with 4 M-urea/1 M-acetic acid/0.1% Triton X-100 as the eluent.

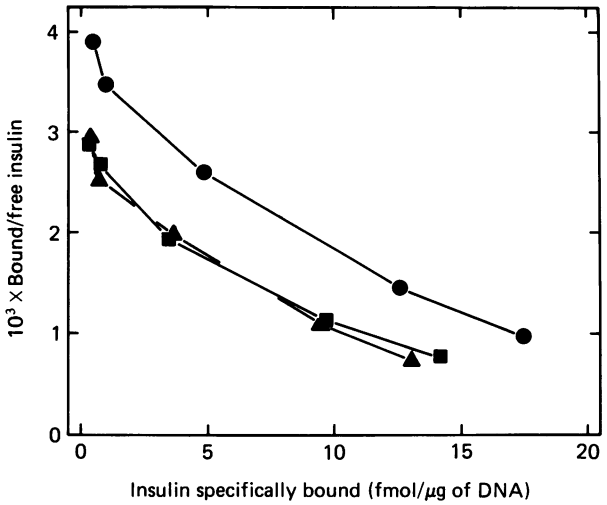

Fig. 2. Scatchard analysis of ^{125}I-insulin binding to cultured hepatocytes: correction for degraded hormone

Cells cultured for 48 h were incubated with 50 pM-^{125}I-insulin and various concentrations of unlabelled insulin for 30 min. Then parallel monolayers were either dissolved in 0.1% SDS and processed by TCA precipitation or extracted into 4 M-urea/1 M-acetic acid/0.1% Triton X-100 and chromatographed on Sephadex G-50 (fine grade) with the same buffer. Specific binding was either uncorrected for degraded hormone associated with the cells (●) or corrected by using TCA-precipitable radioactivity (▲) or label in Sephadex peak II (■). Data give means of five plates and are one representative of three experiments. For clarity, S.E.M. values of bound and of bound/free hormone (<5% of means) are not given in the plot.

low-M_r degradation products, increased with time, representing as much as 22% of radioactivity specifically associated with the cells at 120 min of incubation.

TCA-precipitability of peaks I–III could not be determined, owing to the presence of urea and Triton X-100, which extremely stabilized the solution. It was only after the addition of 30 mg of albumin/ml as a carrier that more than 95% of the radioactivity in peaks I and II was precipitated by TCA, whereas radioactivity in peak III remained soluble. Radioactivity in peak II comprised only 3.7–6.6% more of cell-associated radioactivity than was precipitated by TCA. Therefore the TCA-precipitable fraction was a good estimate of intact insulin that was eluted as peak II from the Sephadex column. On the other hand, TCA-soluble radioactivity represented 4–7% more of cell-associated radioactivity than was detected in peak III.

Fig. 2 demonstrates the effect on the Scatchard analysis of correcting the binding data from a 30 min incubation with ^{125}I-insulin and various concentrations of unlabelled insulin for degraded hormone. As was to be expected from the previous experiments, both methods of analysing cell-bound radioactivity revealed virtually identical plots. Correction for degraded hormone significantly decreased the receptor quantity compared with uncorrected data, but ligand affinity and the curvilinearity of the plot were not affected.

A consideration of the association of degraded hormone with cells might be even more important in experiments with agents that interfere with hormone degradation. We have therefore applied both methods of analysing cell-bound radioactivity to incubations of hepatocytes with labelled insulin in the presence of bacitracin, an inhibitor of glutathione-insulin transhydrogenase (Roth, 1981) and of the lysosomotropic agent chloroquine. As demonstrated in Table 2, label specifically bound to the cells was increased dramatically over control conditions by both chloroquine and bacitracin, in a dose-dependent fashion. As in control cells, the TCA-precipitable fraction correctly reflected radioactivity in peak II of gel filtration also in bacitracin-treated hepatocytes. In monolayers incubated in the presence of 50 μM- or 200 μM-chloroquine, however, the amount of 'intact' hormone was substantially overestimated by the TCA-precipitable fraction. This was the consequence of up to 22% of the specifically bound counts being eluted with peak I from the Sephadex column (Fig. 3), which were also precipitated by TCA. Such a phenomenon was not observed in the presence of bacitracin. Both drugs

Table 2. Modulation of specific insulin binding and of the nature of cell-associated ^{125}I radioactivity by bacitracin and chloroquine

Cells cultured under control conditions for 48 h were incubated for 120 min with ^{125}I-insulin (50 pM) with or without chloroquine or bacitracin in the presence or absence of 1 μM unlabelled insulin, and processed as described in the Materials and methods section. Data are presented as the means ± 1 S.E.M. for five dishes (one representative example of three different experiments) and characterize the nature of label specifically bound to the hepatocytes. Abbreviation: n.d., not detectable.

Incubation conditions	^{125}I-insulin specifically bound (fmol/μg of DNA)	Nature of cell-bound label (% of specifically bound radioactivity)				
		TCA		Sephadex peak		
		Precipitate	Soluble	I	II	III
Control	0.373 ± 0.022	77.34 ± 0.35	22.66 ± 0.35	n.d.	76.82 ± 1.37	22.16 ± 1.43
Chloroquine (50 μM)	0.718 ± 0.024	83.39 ± 0.56	16.61 ± 0.56	11.87 ± 0.44	75.04 ± 1.07	12.01 ± 0.63
Chloroquine (200 μM)	1.089 ± 0.043	87.04 ± 1.25	12.94 ± 1.24	21.97 ± 0.99	68.76 ± 1.33	9.10 ± 0.71
Bacitracin (0.2 mM)	0.537 ± 0.031	83.15 ± 0.90	16.84 ± 0.91	1.04 ± 0.61	82.76 ± 2.22	13.53 ± 2.18
Bacitracin (1.0 mM)	0.668 ± 0.031	86.47 ± 1.06	13.53 ± 1.06	n.d.	88.75 ± 1.22	10.05 ± 1.24

significantly decreased the proportion of peak III in cell-associated radioactivity.

In order to characterize cell-associated label further, experiments were performed to dissociate surface-bound radioactivity from the monolayer. As shown in Table 3, three-quarters of the cell-bound intact label was released into the dissociation medium as the intact polypeptide under control conditions and in the presence of bacitracin, whereas half of the cell-bound TCA-precipitable radioactivity remained with the hepatocytes in the presence of chloroquine, indicating that they were sequestered intracellularly. Similarly, only one-tenth of Sephadex peak II material remained with control and bacitracin-treated cells, whereas virtually nothing of peak I and only half of peak II material dissociated from the monolayer in the presence of chloroquine. Similar results were obtained when surface-bound label was removed by incubation at pH 2.5 for 6 min (results not shown).

DISCUSSION

The present study clearly established the importance of correcting binding data obtained from incubations at 37 °C for degraded ^{125}I-insulin radioactivity associated with hepatocytes. This correction results in an estimate of those receptors to which intact insulin is actually associated. We have also demonstrated that, under routine conditions, analysis of cell-bound radioactivity

Table 3. Dissociation of cell-associated ^{125}I-insulin from cultured hepatocytes

Hepatocytes were incubated as described in Table 2 and washed. Then parallel dishes were either immediately analysed as detailed in the Materials and methods section or incubated in a 3-fold volume of dissociation medium containing 1 μM unlabelled insulin with or without chloroquine or bacitracin as during the association period. After 60 min, dissociation medium was analysed for dissociated TCA-soluble and precipitable radioactivity, and monolayers were washed and processed as usual. Data are presented as the means ± 1 S.E.M. for five dishes (one representative example of five different experiments) and characterize the nature of label released into the medium or remaining with the cells.

Incubation conditions	^{125}I-insulin specifically bound (pre-dissociation) (fmol/μg of DNA)	Nature of cell-bound and released label after 60 min dissociation			
		TCA-precipitable radioactivity		Cell-bound radioactivity in Sephadex peak	
		In medium*	In cells*	I†	II‡
Control	0.316 ± 0.019	74.2 ± 0.9	11.4 ± 0.8	—	13.7 ± 0.5
Chloroquine (200 μM)	1.186 ± 0.050	48.1 ± 0.7	49.1 ± 0.8	91.7 ± 1.3	43.4 ± 1.8
Bacitracin (1.0 mM)	0.756 ± 0.043	77.7 ± 1.2	12.8 ± 0.9	—	10.5 ± 1.4

* % of pre-dissociation cell-associated TCA-precipitable radioactivity.
† % of pre-dissociation cell-associated radioactivity in peak I.
‡ % of pre-dissociation cell-associated radioactivity in peak II.

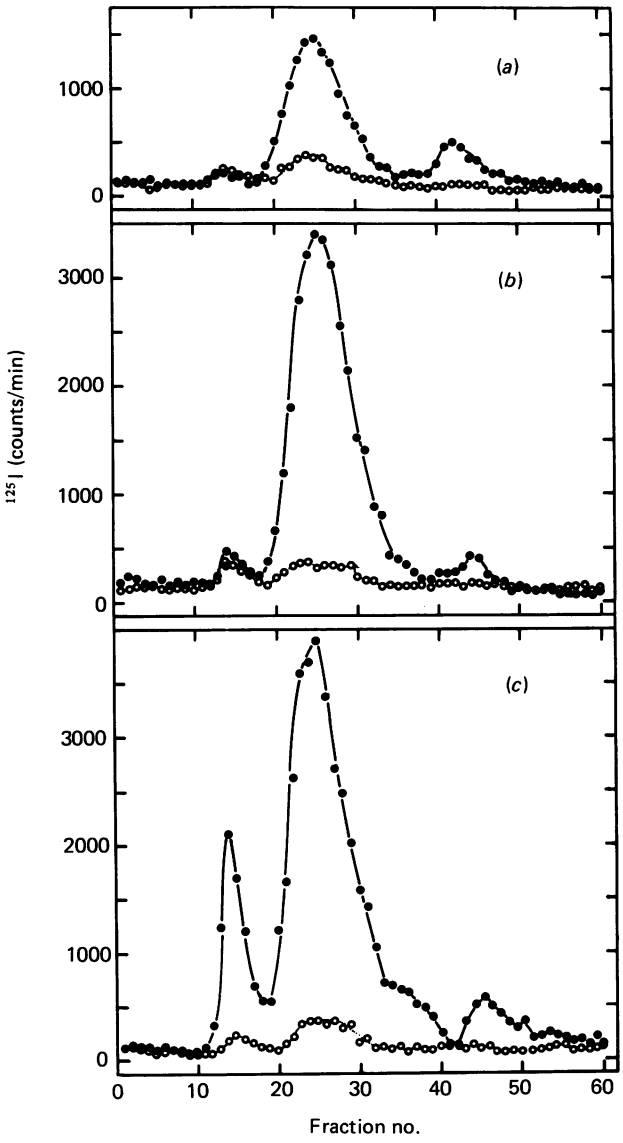

Fig. 3. Sephadex chromatography of hepatocyte extracts in ^{125}I-insulin binding: effects of bacitracin and chloroquine

Hepatocytes cultured for 48 h were incubated with 50 pM-^{125}I-insulin in the absence (●) or presence (○) of 1 μM unlabelled insulin under control conditions (*a*), with 1 mM-bacitracin (*b*) or with 200 μM-chloroquine (*c*) for 120 min. Cells were extracted and chromatographed on Sephadex G-50 (fine grade) as described in the Materials and methods section.

by Sephadex chromatography or by TCA precipitation gave identical results. Therefore the TCA-precipitable fraction of cell-associated label represents an accurate estimate for intact insulin under such conditions.

To date, precipitation of cell extracts with TCA and analysis by gel chromatography were only performed in insulin-binding studies with isolated adipocytes (Hammons & Jarett, 1980) and cultured foetal hepatocytes (Plas & Desbuquois, 1982). Unfortunately, the results derived from the different methods were not directly compared in the work on adipocytes, since quantitative data from gel-filtration analysis were not given. Interestingly, chloroquine did not affect the radioactivity eluted with peak I in adipocytes (Hammons & Jarett, 1980) as well as in cultured foetal hepatocytes (Plas & Desbuquois, 1982), much in contrast with the results in adult hepatocytes presented here and by others (Varandani *et al.*, 1982*b*). Foetal hepatocytes also differ from adult cells in that they are incapable of down-regulating the insulin receptor in response to insulin itself (Pringault *et al.*, 1985; Fleig *et al.*, 1985). An analysis by Sephadex chromatography of the radioactivity associated with adult hepatocytes has been performed by various groups, with conflicting results (Caro & Amatruda, 1981; Caro *et al.*, 1982; Varandani *et al.*, 1982*a*). Cell-bound radioactivity eluted with the void volume was significantly greater in the experiments of Caro & Amatruda (1981) on isolated cells as compared with the present study. They found as much as 39% of total cell-associated label in this fraction after 2 h of incubation at 30 °C. This more than 6-fold difference is readily explained by the 30-fold lower albumin concentration in the medium in our experiments (1 mg/ml), since albumin in the medium appears to be positively correlated with the occurrence of ^{125}I radioactivity in the high-M_r fraction (Caro & Amatruda, 1981). In addition, cultured rather than freshly isolated hepatocytes were used in the present study at 37 °C instead of 30 °C. Our data are supported by the detection of about 8% of total cell-bound label in peak I from Sephadex G-75 chromatography in a similar culture system after 4 h of incubation (Varandani *et al.*, 1982*a*).

When specific rather than total binding is considered, peak I did not contribute detectably to cell-associated radioactivity (Fig. 1, Table 1). Unfortunately, previous studies (Caro & Amatruda, 1981; Caro *et al.*, 1982; Varandani *et al.*, 1982*a*) have failed to correct the data derived from gel chromatography for unspecific binding. To our best knowledge, the experiments presented above are the first to demonstrate that the nature of cell-associated radioactivity is strikingly different under conditions of 'specific' and 'unspecific' binding. Processing of insulin to high-M_r material contained in peak I and interpreted as randomly cross-linked A- and B-chains (Varandani *et al.*, 1982*a*) occurred under conditions of unspecific binding, whereas metabolism to low-M_r products appeared to be restricted to hormone bound to specific receptors. This emphasizes the necessity to correct results of Sephadex chromatography for unspecific binding.

Cell-bound radioactivity eluted with the low-M_r degradation products (peak III) was nearly identical in this study and in the experiments on isolated cells (Caro & Amatruda, 1981). In contrast, others (Varandani *et al.*, 1982*a*) failed to detect any cell-associated radioactivity in the low-M_r fraction from cultured hepatocytes incubated with ^{125}I-insulin for 4 to 24 h. Since such degradation products accumulated readily in the medium, this could only be explained by their immediate and complete extrusion from the cells, a conclusion interfering with the findings of the present and other studies (Kahn & Baird, 1978; Hammons & Jarett, 1980; Caro & Amatruda, 1981; Davidson & Venkatesan, 1982). Analysis of specifically cell-bound radioactivity by precipitation with TCA slightly overestimated these low-M_r degradation products, because up to 5% of label in peak II was not precipitated with TCA.

When drugs capable of interacting with hormone degradation were included in the medium, precipitation

with TCA led to erroneous estimates, owing to an increase in the amount of label in peak I. For bacitracin, which did not affect the absence of peak I in specifically bound radioactivity, precipitability and solubility in TCA still correctly recognized 'intact' and 'degraded' insulin. After incubation with the lysosomotropic agent chloroquine, however, precipitation with TCA substantially overestimated 'intact' hormone, and was therefore a poor alternative to gel filtration as the analytical procedure.

Because three-quarters of cell-associated intact insulin dissociated from control and bacitracin-treated cells into the medium as intact hormone, at least this proportion was 'surface-bound' at the end of the incubation period (Table 3). This suggests that bacitracin inhibited the degradation of insulin predominantly at the cell surface, but did not impair internalization. In contrast, all radioactivity in the high-M_r fraction and about one-half of the label in peak II (intact hormone) were trapped intracellularly in the presence of chloroquine, indicating that less than 50% of cell-bound TCA-precipitable label represented insulin bound to surface receptors. Whether this cell-bound non-dissociable radioactivity in Sephadex peak I represents randomly cross-linked A- and B-chains (Varandani *et al.*, 1982*a*) or undissociated receptor–ligand complexes accumulating in the prelysosomal compartment (Brown *et al.*, 1983) remains to be investigated.

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