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CELLULAR BINDING SITES FOR INSULIN IN RAT LIVER

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SUMMARY

A study of the sites of insulin binding in subcellular fractions of rat liver is reported. A method for the isolation of liver plasma membranes, which permits one to follow quantitatively the distribution of all the parameters of interest, was modified and applied to the study of the cellular topography of insulin binding. The insulin-binding capacity did not follow closely the enzyme marker (5′-nucleotidase) for plasma membranes when differential centrifugation schemes were used, and the divergence from this marker was more prominent when separations were performed on discontinuous sucrose gradients. A significant amount of insulin binding capacity was always present in fractions with higher density than those containing the majority of 5′-nucleotidase. Results of studies on linear sucrose gradients have disclosed in some of the purified membrane fractions small but consistent differences in density of the insulin binding, and plasma membrane particles. It is suggested that there may be several types of intracellular membranes to which insulin can bind besides the plasma membranes.

INTRODUCTION

In recent years, a number of investigators have been concerned with the elucidation of the site of action of insulin in a variety of cells [1–11]. While there seems to be a general agreement that the binding site is the plasma membrane [1, 2, 9, 12–14], detailed studies of the cellular distribution of insulin binding have not been reported.

The current concept that the insulin binding sites are located solely on the cell surface originated from the work of Cuatrecasas [15] showing that when equal concentrations of free or Sepharose-bound insulin were added to isolated fat cells, the same degree of stimulation of glucose utilization was obtained. This concept gained support from in vitro binding studies on crude [16], but also highly purified plasma membrane fractions, and finally led to the conclusion that there is no significant specific insulin binding to intracellular membrane structures [17]. The validity of some of these results [15], however, has been questioned by a number of investigators [18–20].

The study of the cellular localization of the binding site of any extracellular material must take cognizance of certain principles of cellular fractionation, as was clearly delineated by de Duve [21]. Assignment of the specific localization of a compound to a subcellular fraction requires the determination of the distribution of that compound in all fractions obtained with a particular method. So far, no such complete distribution pattern of insulin binding has been published.

In the present investigation the distribution of the binding ability of insulin in all subcellular fractions was studied and it was compared to that of the marker enzymes characteristic for these fractions. The results suggest that the localization of the specific binding site(s) may not reside exclusively in the plasma membranes, but possibly in other cellular structures as well.

MATERIALS AND METHODS

Preparation of cellular fractions

Male Sprague-Dawley rats (130–150 g) were fasted for 16–18 h, and killed by decapitation. The livers were excised, and perfused with ice-cold buffered 0.25 M sucrose (5 mM in Tris · HCl, pH 8.0). Homogenization and fractionation were performed as described by Touster et al. [22], except that the SW 27 swinging bucket rotor was utilized for the flotation of the plasma membranes. In some experiments an additional layer of sucrose (42 % $\varrho = 1.187$) was introduced into the discontinuous gradient. In three additional experiments the isolated plasma membrane fractions were further subfractionated on a linear sucrose gradient. Details are given in the figure legends of particular experiments.

Insulin binding assay

The binding of insulin to the various subcellular fractions was performed essentially according to the method described by Cuatrecasas [1]. ^{125}I-labelled insulin was incubated with the individual fractions, and after filtration and washing on EGWP Millipore filter (Millipore Corp., Bedford, Mass.) the filter discs were dried under infrared light and the radioactivity was measured in a Packard Tri-Carb scintillation counter. The efficiency of counting of ^{125}I was not affected by the presence of the components of the material analyzed. ^{125}I-labelled insulin was purchased from New England Nuclear Corp., Boston, Mass. (specific activity between 80 and 100 Ci/g). Occasionally, bovine insulin obtained from Eli Lilly & Co. (Indianapolis, Ind.) was purified [23], six times recrystallized, and then iodinated with Na^{125}I in our laboratory [1].

Enzyme assays

Glucose-6-phosphatase (EC 3.1.3.9) was determined according to the method of de Duve et al. [24], and 5'-nucleotidase (EC 3.1.3.5) as described by Touster et al. [22]. In both assays, the inorganic phosphate released was measured by the method of Lowry and Lopez [25]. *N*-acetyl-β-D-glucosaminidase (EC 3.3.1.30) was assayed as described by Horvat and Touster [26]. Alkaline phosphatase (EC 3.1.3.1) was measured in an incubation mixture of 1.0 ml which was 2 mM in *p*-nitrophenylphosphate, 10 mM in Mg^{2+}, and 200 mM in Tris · HCl buffer, pH 8.9. Subcellular fractions were added in such amounts that the absorbance of the reaction product

did not exceed 0.5 absorbance units. After incubating for 20 min at 37 °C, the reaction was stopped by the addition of 1.2 ml of 0.75 M Na_2CO_3. The amount of *p*-nitrophenol released was determined by the absorbance at 400 nm in a Zeiss spectrophotometer. Protein concentrations were assayed by the method of Miller [27] using bovine serum albumin as standard (Armour). Doubly distilled water was used throughout.

All enzyme assays were performed under conditions of optimum substrate concentration, and linearity with regard to enzyme concentration and time of incubation.

Enzyme units are expressed as μmol of product formed per min. Specific activity corresponds to units/mg of protein. Specific activity of insulin binding refers to fmol of ^{125}I-labelled insulin bound/mg protein.

RESULTS

The insulin-binding capacity, the activities of three marker enzymes and the protein content in four major subcellular fractions are shown in Table I and Fig. 1. Preliminary experiments had indicated that there was no binding of insulin to either mitochondria or lysosomes. Therefore, in all experiments a combined mitochondrial-lysosomal fraction was isolated and identified by the lysosomal marker enzyme. The marker enzymes chosen are: 5′-nucleotidase for plasma membranes, *N*-acetyl-β-D-glucosaminidase for lysosomes, and glucose-6-phosphatase for microsomes. As seen in Fig. 1, two subcellular fractions, the nuclear (N) and the microsomal (P), show an enrichment in 5′-nucleotidase. This distribution is characteristic for plasma membrane

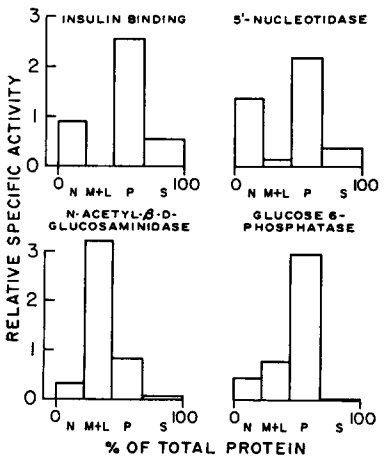

Fig. 1. Distribution of insulin-binding activity and three marker enzymes in subcellular fractions of rat liver. Fractionation, enzyme assays and insulin binding were performed as described in Materials and Methods, and in legend to Table I. Relative specific activity is percentage of total activity/percentage of total protein in each fraction. On the abscissa, the fractions are represented by their relative protein content, cumulatively from left to right in the order in which they are obtained. N, nuclear fraction; M+L, mitochondrial+lysosomal fraction; P, microsomal fraction; S, soluble fraction.

TABLE I

INSULIN-BINDING AND ENZYMATIC ACTIVITIES IN SUBCELLULAR FRACTIONS OF RAT LIVER

Fractionation of the liver and the enzyme assays were performed as described in Materials and Methods. The insulin binding assay was performed in a 0.2 ml incubation mixture for 40 min at 24 °C and $5 \cdot 10^{-10}$ M ^{125}I-labelled insulin. Non-specific binding of insulin was determined by incubating another aliquot of the particular fraction in the presence of an excess (1.6 nmol) of bovine insulin. All assays represent the average of duplicate incubations. Recovery is based on analyses of the various fractions, the values for the homogenate being 100 %.

Fraction	Proteins	Insulin binding		5'-Nucleotidase		*N*-Acetyl-β-D-glucosaminidase		Glucose-6-phosphatase	
	(mg/g liver)	Binding activity (pmol/g liver)	Spec. act. (fmol/mg protein)	Activity (units/g liver)	Spec. act. (units/mg protein)	Activity (units/g liver)	Spec. act. (units/mg protein)	Activity (units/g liver)	Spec. act. (units/mg protein)
Homogenate	200.5	2.076	10	11.8	0.058	5.0	0.025	19.03	0.095
Nuclei	42.4	0.365	9	3.9	0.093	0.34	0.008	1.96	0.046
Mitochondria + Lysosomes	42.4	0.128	3	0.5	0.012	3.2	0.075	3.43	0.081
Microsomes	47.4	1.112	23	7.1	0.149	0.93	0.020	14.32	0.302
Supernatant	61.7	0.316	5	1.5	0.025	0.08	0.001	0.11	0.002
Recovery (%)	97	92		110		91		104	

enzymes fractionated in 0.25 M sucrose (for discussion see ref. 22). The distribution of insulin binding resembles that of 5′-nucleotidase but is not identical. The dissimilarity becomes even more prominent when the separation of plasma membranes is achieved by fractionation of the nuclear and microsomal pellets on a discontinuous sucrose gradient, as indicated in Fig. 2A. In Table II, the distribution of insulin binding activity is compared with that of the plasma membrane marker enzyme. Fractions N_I and N_{II} (Fig. 2A), representative of light plasma membranes (see below), contain 58 % of the 5′-nucleotdiase, but only 33 % of the total insulin-binding activity applied to the gradient. On the other hand, Fraction N_{IV} has 32 % of insulin binding and only 13 % of the 5′-nucleotidase activity. These differences are even more striking in the fractions from the microsomal gradient. In Fraction P_{II} (plasma membranes) 51 % of 5′-nucleotidase and only 23 % of the insulin binding activity are recovered, while Fraction P_{IV} contains more than half of the latter (54 %) and only 29 % of the enzyme activity applied to the gradient. The specific activity of the plasma membrane marker is higher in Fraction N_{II} (1.98) than in Fraction P_{II} (1.13), as is the specific insulin binding activity (380 fmol/mg protein in Fraction N_{II} and 160 fmol/mg protein in Fraction P_{II}).

It has been reported [28] that two kinds of plasma membrane preparations can be obtained from rat liver, (a) low density, $\varrho = 1.12$, and (b) high density plasma membranes, $\varrho = 1.18$. In the present investigation, Fractions N_{II} and P_{II} correspond to the lighter plasma membranes, while the high density membranes remain at the interphase between the 57 % and 37 or 34 % sucrose solutions, as shown in Fig. 2A,

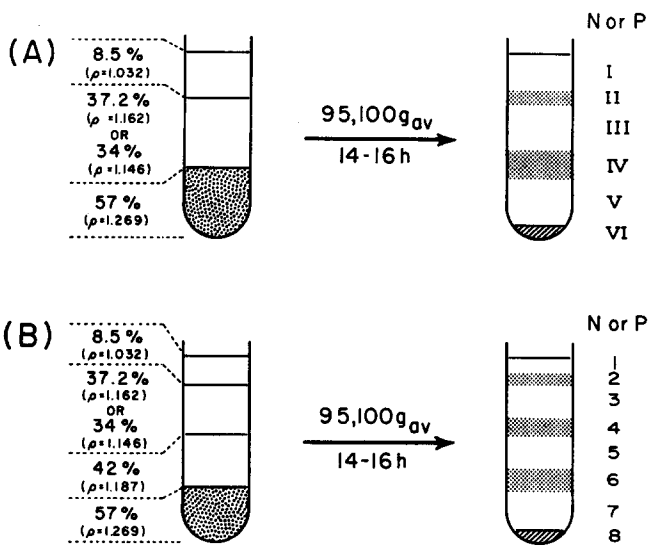

Fig. 2. Patterns of distribution of membrane particles on a discontinuous sucrose gradient. Nuclear (N) and microsomal (P) fractions were isolated as in Fig. 1. These fractions were each suspended in 57 % sucrose and subjected to gradient centrifugation (A) according to Touster, et al. [23], or (B) with the introduction of an additional layer of sucrose. When the B type of gradient was applied with the nuclear fraction, 8 fractions were collected, and with the microsomal fraction only 7, since the pellet (Fraction P_8) was very small and represented only aggregated particles.

TABLE II

DISTRIBUTION OF INSULIN BINDING AND ENZYMATIC ACTIVITIES THROUGHOUT A DISCONTINUOUS SUCROSE GRADIENT FROM ISOLATED NUCLEAR AND MICROSOMAL FRACTIONS OF RAT LIVER HOMOGENATE (GRADIENT TYPE A IN FIG. 2)

Total = sum of individual fractions. Recovery is based on analyses of the individual fractions, the total values of the nuclear and microsomal fractions applied to the gradient being 100%. The nuclear (N) and the microsomal (P) fractions of Table I and Fig. 1 were further fractionated on a discontinuous sucrose gradient as described under *Materials and Methods*. Fractions N_I–N_{VI} and P_I–P_{VI} correspond to those shown in Fig. 2A. The concentration of ^{125}I-labelled insulin in the incubation mixtures was $3 \cdot 10^{-10}$ M.

Fraction	Proteins	Insulin binding		5′-Nucleotidase		*N*-Acetyl-β-D-glucosaminidase		Glucose-6-phosphatase	
	(% of total)	(% of total)	(fmol/mg protein)	(% of total)	(units/mg protein)	(% of total)	(units/mg protein)	(% of total)	(units/mg protein)
N_I	1.2	4.5	210	14.5	1.71	0.4	0.003	0.7	0.020
N_{II}	5.8	28.0	384	42.9	1.98	1.9	0.007	2.8	0.032
N_{III}	6.7	8.9	52	8.4	0.20	0.9	0.002	4.8	0.036
N_{IV}	24.8	32.0	53	13.3	0.08	28.4	0.012	42.6	0.062
N_V	22.9	6.8	80	5.0	0.03	15.2	0.004	26.6	0.043
N_{VI}	38.5	19.7	140	16.0	0.06	53.1	0.823	22.6	0.021
Recovery (%)	86	103		87		103		86	
P_I	0.6	0.2	6	1.7	0.49	0.3	0.009	0.1	0.058
P_{II}	7.1	22.8	160	50.8	1.13	3.4	0.009	4.2	0.195
P_{III}	7.7	9.9	24	15.3	0.32	2.9	0.007	6.2	0.268
P_{IV}	60.7	53.6	17	28.8	0.07	64.1	0.019	76.2	0.415
P_V	20.7	5.6	5	3.0	0.02	25.6	0.023	12.4	0.198
P_{VI}	3.3	7.8	25	0.3	0.02	3.8	0.022	0.8	0.086
Recovery (%)	82	76		87		80		89	

together with other membranous particles. In order to resolve this heterogeneous membrane fraction, an additional layer of sucrose (42 %, $\varrho = 1.19$) was introduced, as shown in Fig. 2B. Membranes lighter than 1.19 should float on top of this sucrose layer. Typical results of such fractionations with the nuclear and microsomal pellets are shown in Table III. Fractions N_2 and N_4, and P_2 and P_4, respectively, correspond to the light and heavy plasma membranes. The distribution (% of total activities applied to the gradient) of insulin binding and 5'-nucleotidase is very similar in the fractions obtained from the nuclear pellet. In the light membranes (Fraction N_2), the specific activity of insulin binding is 3.5 times higher, and of the 5'-nucleotidase 5 times higher than in the heavy membranes (Fraction N_4). The heavy membranes also contain more of the microsomal enzyme marker, glucose-6-phosphatase, than the light membranes (not shown in Table III).

In fractions of the gradient obtained from the microsomes the distribution of the two activities does not follow the same pattern. While a large percentage of insulin binding activity resides in the high density membranes (30 % of the total as contrasted with 16 % in the light fraction), more of the 5'-nucleotidase is present in the light membrane fraction (39 % as compared to 25 % in the heavy membranes). Moreover, 26 % of the insulin binding capacity remains in Fraction P_6, which contains about 70 % of glucose-6-phosphatase (not shown in Table III) but only 7 % of 5'-nucleotidase activities.

House et al. [29] have reported a difference in the distribution of 5'-nucleotidase and alkaline phosphatase in the two types of plasma membrane fractions. The former is predominantly present in the light particles and the latter in the heavy membranes. Indeed, our results (Table III) show that the pattern of distribution of alkaline phosphatase is not identical to that of 5'-nucleotidase, but is more closely similar to the distribution of insulin binding activity. In particular, Fraction P_6 contains significant insulin binding and alkaline phosphatase activities, but very little of 5'-nucleotidase.

To investigate further the differences in the light (Fraction N_2 or P_2) and heavy (Fraction N_4 or P_4) plasma membranes, these fractions were individually subjected to isopycnic centrifugation on a linear sucrose gradient (Fig. 3). As seen in Fig. 3A, Fraction N_2 is homogeneous, with a median density of $\varrho = 1.159$ for all the parameters studied. Fig. 3B shows that in Fraction P_2 there is a divergence in the density of the particles containing insulin binding, 5'-nucleotidase and glucose-6-phosphatase activities. The differences are small but very reproducible. In three different experiments, the median density for 5'-nucleotidase was $\varrho = 1.124$, for insulin binding $\varrho = 1.132$, and for glucose-6-phosphatase $\varrho = 1.127\ g \cdot cm^{-3}$. Although the peak of 5'-nucleotidase is in Tube 11, the highest specific activity is in Tube 12, representing a 1.7 fold increase over the original value in Fraction P_2. The peak for insulin binding and the highest specific activity is in Tube 10, representing a 1.5 fold purification.

As opposed to the above two fractions, Fraction P_4 turned out to be composed of two discrete groups of particles, with two different mean densities, visibly separated from each other as two discrete bands on the gradient (Fig. 3C). In the higher density band, the peak for both 5'-nucleotidase and insulin binding occurs at $\varrho = 1.176$ $g \cdot cm^{-3}$, and there is a shoulder at that density in the activity of glucose-6-phosphatase. In the lower density band, the peak of the first two activities, 5'-nucleotidase and insulin binding, is at $\varrho = 1.158$, while that for glucose-6-phosphatase is at $\varrho = 1.153$. The peak of protein is also at $\varrho = 1.153$. One should note again the divergence in the

TABLE III

INSULIN BINDING AND ENZYMATIC ACTIVITIES IN FRACTIONS OF A DISCONTINUOUS SUCROSE GRADIENT FROM ISOLATED NUCLEAR AND MICROSOMAL PARTICLES OF RAT LIVER HOMOGENATE (GRADIENT TYPE B IN FIG. 2)

Total = sum of individual fractions. Fractionation of the liver and the enzyme assays were performed as described under Materials and Methods. Fractions N_1–N_8 and P_1–P_7 correspond to those shown in Fig. 2. In different experiments recoveries for proteins were 90–98 %, for insulin binding, 75–90 %, and for 5'-nucleotidase, 98–100 %.

Fraction	Protein (% of total)	Insulin binding (% of total)	(fmol/mg protein)	5'-Nucleotidase (% of total)	(munits/mg protein)	Alkaline phosphatase (% of total)	(munits/mg protein)
N_1	0.5	2.0	33	1.0	360	1.5	5
N_2	2.2	38.2	211	48.5	1504	30.9	30
N_3	4.0	7.0	59	10.0	315	5.0	10
N_4	3.2	13.3	58	12.2	295	9.6	6
N_5	7.2	6.5	17	4.4	53	6.1	4
N_6	36.5	15.2	18	7.4	43	24.8	2
N_7	12.4	2.5	3	1.8	12	8.7	2
N_8	34.0	17.2	8	14.8	38	13.1	1
P_1	0.7	0.9	74	3.0	973	1.3	14
P_2	4.1	16.2	159	38.6	1521	26.1	33
P_3	2.8	8.5	124	14.3	835	9.2	17
P_4	12.2	30.4	95	24.5	354	18.2	9
P_5	10.9	10.4	48	7.6	95	14.1	6
P_6	45.3	26.5	29	7.2	41	20.4	3
P_7	24.0	7.0	15	4.8	34	10.8	3

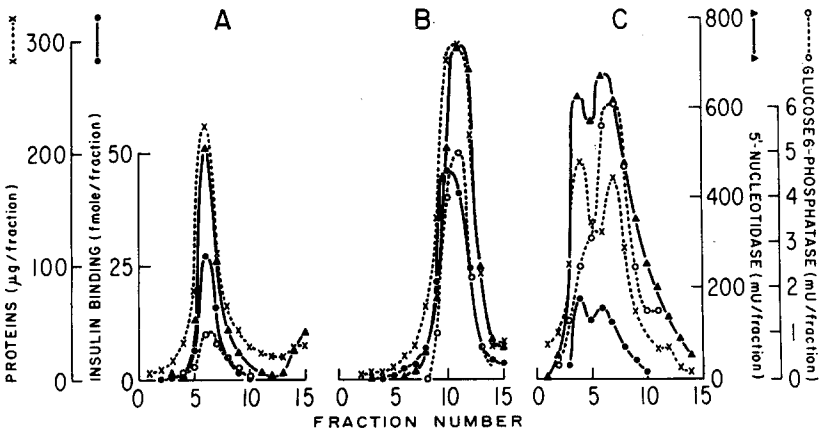

Fig. 3. Subfractionation of isolated plasma membrane fractions from rat liver. Fractions N_2 (A), P_2 (B), and P_4 (C) obtained from the discontinuous gradient (Fig. 2B) were diluted with 0.25 M sucrose/5 mM Tris · HCl, pH 8.0, and centrifuged in the Ti 60 rotor (Spinco) for 60 min at 246 000 g_{av}. The pellets were resuspended in a fresh sucrose solution and made up to the desired volume. These concentrated suspensions were then layered on top of a linear sucrose gradient (from $\rho = 1.19$ to $\rho = 1.10$) and centrifuged in the SW 50.1 Spinco rotor at 270 000 g_{av} for 14 h. 0.3 ml fractions were collected. Insulin binding was determined at a $3 \cdot 10^{-10}$ M concentration of labelled insulin. Since in Fraction P_4 the total activity of 5'-nucleotidase was very low, the values plotted in Fig. 3C were obtained by multiplying the actual units by a factor of 10. Insulin binding in fmol/fraction (●—●), 5'-nucleotidase in munits/fraction (▲—▲), glucose-6-phosphatase in munits/fraction (○...○) protein in μg/fraction (×...×). Density decreases from left to right.

distribution of the total insulin binding and 5'-nucleotidase activity between the two density peaks: there is more insulin binding activity in the heavier fraction while the opposite is true for the plasma membrane marker. There are also differences in the pattern of distribution of specific activities. 5'-Nucleotidase shows two peaks of specific activity which coincide with the positions of total activity: the first peak is in Tube 6 and the second in Tube 9, but neither of these peaks represents any degree of purification with regard to the sample applied to the gradient. On the other hand, the specific activity of insulin binding shows only one peak, in Tube 6, and this represents a 1.4 fold purification. Although in some experiments there were differences in the occurrence of insulin binding and 5'-nucleotidase activities in Fraction N_4, this fraction was not subjected to further analyses on a linear sucrose gradient.

DISCUSSION

Studies on intracellular localization of cellular components are performed on various fractions of cell homogenates obtained by different centrifugation schemes. The characterization of these fractions is based either on morphological or biochemical principles (enzyme markers). The separation of the different organelles is a relatively easy task today, while the separation and characterization of the membranous components of the cell are still equivocal and dependent on the particular method used.

The method we have selected for the study of the distribution of the insulin binding component(s) of the cells allows us not only the isolation of a particular

fraction with a high specific activity, but also a complete accounting of the amounts of insulin bound by the various fractions. In the first steps of this method (differential centrifugation) the plasma membranes, as judged by the distribution of the enzyme marker 5'-nucleotidase, are recovered in the nuclear and in the microsomal fractions which also contain the bulk of the insulin binding capacity. Even in this rough fractionation procedure (Table I and Fig. 1), one can notice a divergence in the distribution pattern of the marker enzyme and the insulin binding capacity. On further fractionation, the plasma membrane fractions obtained (Tables II and III) show a higher ratio of 5'-nucleotidase to insulin binding activity in the light plasma membrane fractions and the reverse in the heavy membrane fractions. This is particularly striking in the gradient derived from the microsomal pellet (Table III) where the highest insulin-binding activity resides in Fraction P_4 ($\varrho \approx 1.18$) while the highest percentage of 5'-nucleotidase activity is present in the lighter membrane fraction, Fraction P_2 ($\varrho < 1.15$). This finding is different from that reported by House and Weidemann [6]. These workers found the highest insulin binding activity to coincide with the highest percentage of 5'-nucleotidase in the low density plasma membrane fraction.

It should be pointed out that the percent binding activity remaining at the interphase of the 57 % and 42 % sucrose (Fraction P_6) varied to some extent from experiment to experiment. However, the occurrence of the highest insulin binding activity in the $\varrho \approx 1.18$ density sucrose (Fraction P_4) was not altered by this variability. It is also noteworthy that in the experiment shown in Table III, 25 % of the insulin binding activity remains in Fraction P_6 while membrane fragments containing the marker enzyme have largely migrated into lower density sucrose solutions (Fractions P_1–P_4). This type of dissimilarity was generally observed in all fractionation experiments.

Recently Bergeron et al. [30], reported the presence of insulin binding activity in the Golgi fraction of rat liver. In studies on the isolation of the Golgi apparatus [31, 32] it was demonstrated that the Golgi membranes have a density lower than 1.15. Therefore, all the Golgi membranes, representing about 7 % of the total microsomal membranes [32], are included in our P_2 fraction.

What is the basic difference between light and heavy membranes? Evans [28] has isolated two types of plasma membranes and has postulated, mostly on morphological grounds, that the two types of membranes originate from different parts of the hepatocyte. House and Weidemann [6] confirmed the existence of these two types of membranes and have further shown a specific distribution of alkaline phosphatase in them with the highest specific activity in the membrane fraction with a density of $\varrho = 1.18$. In our experiments, the distribution of total activity of alkaline phosphatase is similar to that of insulin binding (Table III), although the specific activity of this enzyme displays a pattern similar to that of 5'-nucleotidase, i.e. shows the highest specific activity in the lighter membrane fractions.

Such discrepancies enhance the importance of accounting for the total activities recovered in all cellular fractions for all parameters studied, rather than relying mainly on following those fractions having the highest specific activities. Thus, for example, when Fraction P_4 was subfractionated on a linear sucrose gradient, two distinct peaks of insulin binding activity were obtained (Fig. 3C), indicative of the presence of two distinct populations of particles. Specific activity calculation indicates the presence of only one peak.

It should be pointed out that a significant amount of the insulin-binding activity was always associated with the nuclei (Fraction N_6 in Table II and Fraction N_8 in Table III). Approximately the same percentage of 5'-nucleotidase was also found in this fraction. This could be due to a contamination of the nuclei with plasma membranes, but it could also represent an intrinsic property of the nuclear envelope. The similarity in the concentration of these two activities in the nuclear fraction, as opposed to the dissimilarities observed in the various membrane fractions, makes it tempting to speculate that both of these activities are part of the nuclear membrane. Widnell [33] has shown histochemically the presence of 5'-nucleotidase in the endoplasmic reticulum of the rat hepatocyte. The continuity of the nuclear evnelope with the endoplasmic reticulum is an established fact. Studies to verify this hypothesis are currently in progress.

The general trend displayed by the insulin binding membranes in the various centrifugation schemes indicated that the insulin-binding activity resides in higher density particles than the 5'-nucleotidase activity. The lightest membranes are in Fraction P_2 which, upon isopycnic centrifugation, banded around a median density of 1.125–1.135 (Fig. 3B). Originally [22], this fraction was described as a plasma membrane fraction, but its very low density suggests that it may be Golgi membranes which exhibit this same low density [32]. The second type of insulin-binding membranes have a density of 1.158 (Fraction N_2 and the lighter band of Fraction P_4 in Fig. 3C). The third type of membranes are those with a density of 1.176 (heavy band of Fraction P_4 in Fig. 3C) and there is a significant percentage of the total insulin binding capacity of rat liver having a higher density than those mentioned above.

The results of the work reported here indicate that insulin binding activity does not follow closely the pattern of distribution of plasma membrane markers, and imply that insulin-binding sites may be present in some other membrane structures as well. However, whether all these binding sites represent true biological receptors is not as yet clear.

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