JCI The Journal of Clinical Investigation

Demonstration of the insulin receptor in vivo in rabbits and its possible role as a reservoir for the plasma hormone.

A J Zeleznik, J Roth

J Clin Invest. 1978;61(5):1363-1374. https://doi.org/10.1172/JCI109054.

Research Article

Based on studies of the interaction of insulin with its receptors in vitro, we calculated that a receptor compartment should be measurable directly in vivo. For this purpose, rabbits were injected intravenously with a labeled insulin that has low affinity for receptors in combination with a radioiodinated insulin that has high affinity for receptors. Plasma concentrations of labeled insulins were measured at selected intervals after injection. Apparent volumes of distribution were calculated by extrapolation of plasma distribution were calculated by extrapolation of plasma disappearance curves; high affinity insulins consistently distributed into spaces that were two-three times greater than those of the low affinity insulins. Injections of unlabeled pork insulin before tracer insulins decreased the distribution space of the high affinity insulin in a dose-dependent manner while having little or no effect on the distribution space of the low affinity labeled insulin. When unlabeled insulin was injected after the tracer insulins, there was an immediate rise in the plasma concentration of the high affinity insulin with only a slight change in the plasma concentration of the low affinity insulin. These results demonstrate that high affinity insulins distribute into a body compartment which has many properties of the insulin receptor previously studied in vitro. This receptor compartment: (a) recognizes insulins based on their biological potencies; (b) is saturated by elevated concentrations of […]

Demonstration of the Insulin Receptor In Vivo in Rabbits and its Possible Role as a Reservoir for the Plasma Hormone

ANTHONY J. ZELEZNIK and JESSE ROTH, *Diabetes Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014*

ABSTRACT Based on studies of the interaction of insulin with its receptors in vitro, we calculated that a receptor compartment should be measurable directly in vivo. For this purpose, rabbits were injected intravenously with a labeled insulin that has low affinity for receptors in combination with a radioiodinated insulin that has high affinity for receptors. Plasma concentrations of labeled insulins were measured at selected intervals after injection. Apparent volumes of distribution were calculated by extrapolation of plasma disappearance curves; high affinity insulins consistently distributed into spaces that were two–three times greater than those of the low affinity insulins.

Injections of unlabeled pork insulin before tracer insulins decreased the distribution space of the high affinity insulin in a dose-dependent manner while having little or no effect on the distribution space of the low affinity labeled insulin. When unlabeled insulin was injected after the tracer insulins, there was an immediate rise in the plasma concentration of the high affinity insulin with only a slight change in the plasma concentration of the low affinity insulin.

These results demonstrate that high affinity insulins distribute into a body compartment which has many properties of the insulin receptor previously studied in vitro. This receptor compartment: (*a*) recognizes insulins based on their biological potencies; (*b*) is saturated by elevated concentrations of insulin; and (*c*) insulin bound to receptors is in equilibrium with free hormone in plasma. Further, the bound to free ratios for hormone, calculated from these data, suggest that in vivo >50% of the extrapancreatic insulin is bound to receptors during normal physiological states.

INTRODUCTION

While early attempts to study receptors for peptide hormones utilized both in vivo and in vitro model systems (2–4), in recent times in vitro systems have been used almost exclusively[1] to demonstrate the interaction of hormones with their specific receptors on target cells (8–10). By the use of radioactively labeled hormones that retain affinity for receptors, it has been possible with these in vitro systems to measure rather precisely the concentration and the affinity of receptors for specific hormones.

From measurements in vitro of the affinity and the concentration of insulin receptors on liver cells, heart muscle, lymphocytes, adipocytes, and other tissues, we estimate that in vivo about one-half of the extrapancreatic hormone is actually bound to receptors[2,3]

Received for publication 4 October 1976 and in revised form 15 December 1977.

[1] The receptor for luteinizing hormone and human chorionic gonadotropin in ovaries has been extensively studied by both in vivo and in vitro procedures (5–7).

[2] For our calculations, the hepatocyte has a volume of $\cong 1.2 \times 10^4\ \mu m^3$ and has 2.3×10^5 surface insulin receptors per cell (11). In the whole body if the cells occupy 60% of the volume and extracellular fluid 40%, then the "concentration" of receptors in the extracellular fluid would be $\cong 2$ nM. Due to negative cooperativity, the affinity of insulin for the insulin receptor is inversely related to the degree of occupancy of receptor; at resting levels of insulin (0.1 nM) for the reaction of hormone (H) plus receptor (R) $\rightleftarrows$ hormone-receptor complex (HR), the equilibrium constant (K_a) is about $(2\ nM)^{-1}$ at 24°C (12), or $K_d = 2$ nM. For a simple bimolecular reaction, where total receptor concentration (R_0) = unoccupied receptors (R) plus occupied receptors (HR), the bound/free (B/F) of insulin $= K_a(R_0 - HR)$; since $HR \ll R_0$, $B/F \cong K(R_0) \cong 1$, i.e., 50% of the hormone in the extracellular fluid is receptor bound. In interpreting these calculations, the reader should appreciate that the number of insulin receptors per unit of cell surface, when we assume cells to be simple geometric forms, is quite similar over a very wide range of cell types (13); therefore if the volume of the average cell in vivo is less than that of the hepatocyte, the B/F would be higher. The value for K_a in the calculation was obtained at 24°C and was not obtained at truly limiting values for the concentration of tracer; we estimate that the effect of correction to 37°C and correction to the limiting value for K_a would about cancel one another.

[3] *Abbreviations used in this paper*: B/F, bound free ratio; H, hormone; R, receptor; TCA, trichloroacetic acid.

and that a receptor compartment should be demonstrable in the whole animal in vivo. In the present study we show that insulins with high affinity for receptor (pork insulin and chicken insulin) distribute into a body compartment that is two to three times larger than that of low affinity insulins (guinea pig insulin and pork proinsulin). Further, we show that this extra compartment has the fundamental properties of the insulin receptor studied in vitro.

METHODS

Animals. Male New Zealand white rabbits (2–3 kg) were purchased from Dutchland Laboratory Animals, Inc., Denver, Pa., and housed individually in temperature and light controlled animal quarters. Standard rabbit chow and water were provided ad libitum. Animals were fasted for 16 h before each experiment.

Insulins. Monocomponent pork insulin (lot 615-D63-10) and pork proinsulin (lot 615-1112B-84-C) were generously provided by Dr. Ronald Chance, Eli Lilly & Co., Indianapolis, Ind. Guinea pig insulin was a gift of Dr. Cecil Yip, University of Toronto, Toronto, Canada. Chicken insulin was provided by Dr. Simon and Dr. Freychet, Institut National de la Santé et de la Récherche Médicale, Nice, France.

Insulins were iodinated with Na^{131}I or Na^{125}I (Amersham/Searle Corp., Arlington Heights, Ill.) by a modification (14) of the chloramine-T method (15). ^{125}I-insulin was separated from unreacted iodide by filtration on Sephadex G-50 (0.9 × 55 cm) in phosphate-buffered saline, pH 7.35, in 0.1% bovine serum albumin. ^{125}I-insulins were prepared at sp act 150–180 μCi/μg (0.4–0.5 iodine atoms per insulin molecule) whereas ^{131}I-insulins were prepared at sp act 200–270 μCi/μg (0.07–0.1 atoms of ^{131}I per insulin molecule).[4] The labeled insulins were stored in phosphate-buffered saline, pH 7.35, with 0.1% bovine serum albumin at −20°C. At the beginning of each experiment, >90% of the iodinated insulin was precipitable in trichloroacetic acid.

Binding of ^{125}I-insulins to cultured human lymphocytes in vitro. Cultured lymphocytes of the IM-9 line (17) were grown in continuous culture as described elsewhere (18). ^{125}I-guinea pig insulin, ^{125}I-pork insulin, and ^{125}I-chicken insulin were incubated with lymphocytes as described in the legend to Table I. Binding assays were performed under conditions such that B/F was directly proportional to the affinity constant (K). Table I shows that the B/F ratios of ^{125}I-guinea pig, ^{125}I-pork, and ^{125}I-chicken insulins were 0.05, 1.0, and 3.0, respectively. These values are in close agreement with previously reported data with respect to both biopotencies of the individual insulins and receptor affinities (19–21).

Degradation of ^{125}I-insulins by membrane fraction from rabbit liver. A membrane fraction from rabbit liver was prepared as described in reference (22) inclusive to step five. ^{125}I-insulins were incubated with this membrane fraction and the results are shown in Table I. ^{125}I-guinea pig, pork, and chicken insulins were degraded at the same rate. Further, unlabeled pork insulin competed with all three radioiodinated insulins in a concentration-dependent fashion.

[4] In commercial preparations of "carrier-free" ^{131}I, the ^{131}I may represent only 10–30% of the total iodine (^{131}I plus ^{127}I) (16). Therefore, the total iodine atoms per insulin molecule, including corrections for radioactive decay, may be 3–20 times greater than the ^{131}I content.

TABLE I
Properties of ^{125}I-Insulins Used in In Vivo Studies

Insulin	Binding to cultured human lymphocytes*	Percentage of ^{125}I-insulin degraded by rabbit liver membranes‡		Concentration of pork insulin that inhibited degradation by 50%§
		15 min	*60 min*	
Chicken	3.0	8	19	1.1 μM
Pork	1.0	6	18	1.1 μM
Guinea pig	0.05	11	21	3.1 μM

* ^{125}I-chicken, -pork, and -guinea pig insulins (0.1 nM) were incubated with IM-9 lymphocytes (5 × 10^6 cells/ml) in the absence and presence of unlabeled pork insulin (50 μg/ml) at pH 7.8 (Hepes-lymphocyte buffer) in a total volume of 0.5 ml at 15°C for 90 min. At the end of the incubation, aliquots (100 μl) were transferred to plastic tubes containing 200 μl fresh buffer and centrifuged for 1 min in a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, Calif.); the supernates were aspirated and discarded, and the tips of the tubes, which contained the cell pellets, were excised and counted for ^{125}I radioactivity. Specific binding of ^{125}I-insulins was calculated by subtracting the counts associated with cell pellets in the presence of unlabeled insulin from the counts associated with cell pellets in the absence of unlabeled insulins. The B/F ratio is listed for each insulin.

‡ Crude rabbit liver membranes (0.4 mg/ml) were incubated with ^{125}I-chicken, -pork, and -guinea pig insulins (0.1 nM) in Krebs-Ringer phosphate buffer, pH 7.0, with 1% bovine serum albumin. Aliquots were removed at various times up to 60 min and centrifuged in a Beckman microfuge (Beckman Instruments) for 1 min. The supernates were transferred to separate tubes containing 1 ml phosphate-buffered saline (pH 7.35) with 1 mg/ml bovine serum albumin. An equal volume of cold (4°C) TCA (10%) was added and the soluble and insoluble radioactivity were determined after centrifugation. Results presented (means of duplicate samples) are the percent insulin degraded after 15 and 60 min of incubation.

§ Crude rabbit liver membranes were incubated with labeled insulin for 60 min, as described above, in the presence of varying amounts of unlabeled pork insulin. The concentration of unlabeled pork insulin required to inhibit the degradation of each labeled insulin by 50% is listed for each ^{125}I-insulin.

These results suggest that although guinea pig, pork, and chicken insulins vary widely in their biopotencies and affinities for the insulin receptor, they are degraded in a similar fashion with respect to both time and inhibition of degradation by unlabeled pork insulin.

Experimental procedures. Rabbits were anesthetized with sodium pentobarbital (25–40 mg/kg) i.v. The right femoral artery was exposed and a polyethylene cannula (0.05 inch OD) was inserted after which animals were given sodium heparin (10 mg/kg) and potassium iodide (1 mg/kg). A mixture containing a high affinity insulin (chicken insulin or pork insulin) labeled with ^{125}I (or ^{131}I) and a low affinity insulin (guinea pig insulin or porcine proinsulin) labeled with ^{131}I (or ^{125}I) was

injected as a bolus (3–5 × 10^7 cpm of each isotope) into a marginal ear vein. Blood samples (0.5 ml) were collected from the femoral cannula into heparinized tubes and stored in ice until the termination of the experiment (60 min). Blood samples were centrifuged for 10 min at 2,000 rpm; 100 μl (0.1 ml) of each plasma sample were added to 0.9 ml phosphate-buffered saline pH 7.35. After mixing, 1 ml of cold (4°C) 10% trichloroacetic acid (TCA) was added to each tube. The samples were mixed and centrifuged at 2,000 rpm for 10 min. The supernates were removed, and the TCA insoluble material was counted on an Amersham/Searle Corp. gamma spectrometer with 89% efficiency for ^{125}I and 90% efficiency for ^{131}I. The spillover of ^{131}I radioactivity into the ^{125}I channel was 0.43. All samples were corrected for spillover and results were expressed as counts per minutes per milliliter plasma per total counts per minute injected for each insulin at each sampling time. In some experiments, plasma concentrations of ^{125}I-pork insulin after tracer injection were expressed as the percentage of ^{125}I-insulin in plasma which was capable of binding to excess antiporcine insulin antibody. Plasma samples (0.1 ml) containing ^{125}I-pork insulin were incubated in a total volume of 0.5 ml which contained guinea pig antiporcine insulin antiserum at a final concentration of 1:100. Nonspecific binding of ^{125}I-insulin was determined by incubating plasma samples in an identical fashion except that normal guinea pig serum (1:100) was substituted for the antipork insulin antiserum. Samples were incubated for 4 h at 4°C after which 0.5 ml of a 28% (wt/vol) solution of polyethylene glycol (PEG-6000, Fisher Scientific Co., Pittsburgh, Pa.) in water was added to precipitate the antibody-bound radioactivity (23). Samples were centrifuged for 15 min at 2,000 rpm, and the precipitates and supernates were counted for radioactivity.

RESULTS

Distribution of high and low affinity insulins in vivo. When anesthetized rabbits were injected with a mixture of ^{125}I-pork insulin (high affinity for receptors) and ^{131}I-guinea pig insulin (low affinity for receptors), there was a marked difference in the disappearance of the two insulins from plasma (Fig. 1A). During the first 10 min, the high affinity pork insulin was lost from the plasma at a much higher rate than the low affinity guinea pig insulin. After 10 min both high and low affinity insulins disappeared from the plasma at a slower rate and the disappearance curves were nearly parallel (Fig. 1A). By extrapolation of the linear portion of the decay curves (dotted lines in Fig. 1A), we calculated the apparent volume of distribution; the distribution space for the high affinity pork insulin was

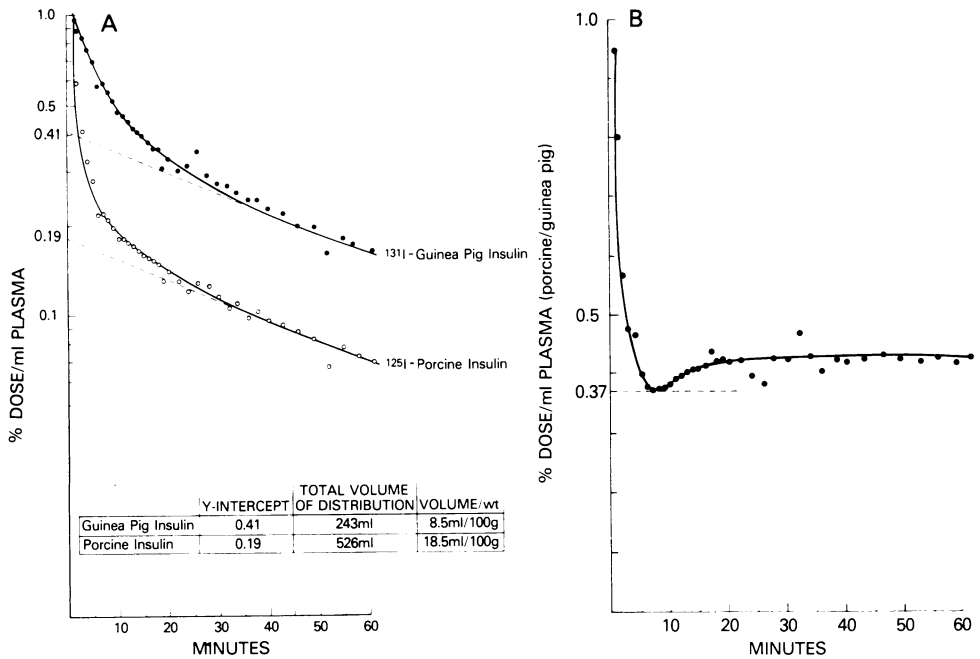

FIGURE 1 ^{125}I-pork and ^{131}I-guinea pig insulin (3–5 × 10^7 cpm) were injected as a bolus into the marginal ear vein of an anesthetized male rabbit, blood samples were drawn from the femoral artery, and the plasma samples were handled as described in Methods. (A) Shows the concentration in plasma of ^{125}I-pork and ^{131}I-guinea pig insulins. TCA insoluble radioactivity in plasma was expressed as a percent of the total TCA insoluble radioactivity injected. Tangent lines were drawn from the linear portion of each disappearance curve to the ordinate. The volume of distribution for each insulin equalled 100%/Y-intercept and was normalized to 100 g body weight as noted in the lower part of the graph. In the right panel (B) is the relative clearance of ^{125}I-pork insulin to that of ^{131}I-guinea pig insulin. The ordinate represents the ratio of the plasma radioactivity (percent dose injected per ml ^{125}I-pork insulin/percent dose injected per ml ^{131}I-guinea pig insulin). The dashed line denotes the minimum value; in Fig. 2C, we plotted the inverse of the minimum value.

approximately twice that of the low affinity guinea pig insulin (Fig. 1A). Fig. 2A shows seven experiments in which an insulin with high affinity for receptor and an oppositely labeled insulin of low affinity were injected simultaneously. In all cases the high affinity insulin (open bars) distributed into a space that was larger than the space of distribution of the low affinity insulin (crosshatched bars) with the difference being typically twofold. The absolute values for the volumes of distribution varied among different animals. For example, the distribution volumes for porcine insulin ranged from 21–65 ml/100 g body wt. This marked variability among animals disappeared when the ratio of the distribution volumes was calculated in each experiment (Fig. 2B). Thus, with pork and guinea pig insulins in four of five experiments the volume of distribution of pork insulin was slightly greater than twice that of guinea pig insulin whereas with chicken insulin and pork proinsulin the distribution volume showed an even higher ratio as expected for the difference in the affinities of these two insulins for the insulin receptor.

In these experiments we measured the radioactivity that was precipitable in TCA. Earlier studies have indicated that precipitation in TCA is less sensitive than the ability of the radioactive material to bind to anti-insulin antibodies; binding to antibody is only slightly less sensitive than the ability to bind to specific receptors (14). Table II shows that within the first few minutes after injection, estimates of degradation obtained by measuring the TCA precipitability closely approximated the estimates obtained with binding to antibodies. However, the difference between the two methods widened with time so that by 18–20 min after the injection of tracer, antibody binding was only 60% of that observed by precipitation in TCA. It can be seen in Fig. 1A that the linear extrapolation of the curves used to obtain the distribution volumes or ratios of distribution volumes depended largely on data obtained from 30 to 60 min after the

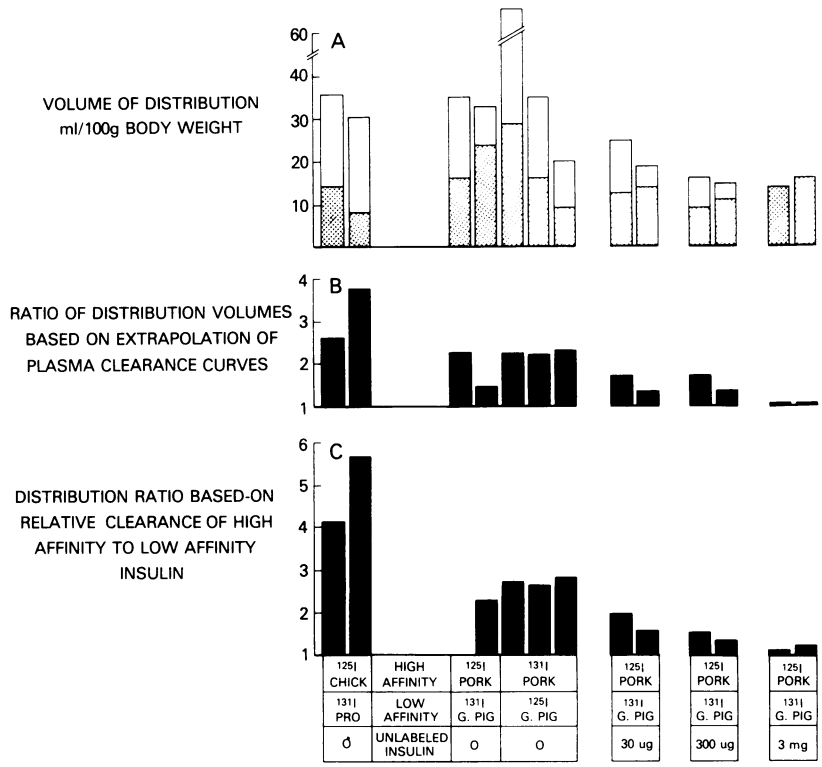

FIGURE 2 The experimental design is described in the legend to Fig. 1; in experiments using unlabeled insulin, the hormone was injected 2 min before the tracer. (A) Apparent volume of distribution of each iodinated insulin was determined by extrapolation of the plasma clearance curve as described in legend to Fig. 1A. The total height of the open bars represent the volumes of distribution of the high affinity insulin and cross-hatched bars represent the volume of distribution of the low affinity insulin. The abbreviation pro is used for pork proinsulin. Each set represents data from a single animal. (B) The same data are expressed as volume of distribution of high affinity insulin/volume of distribution of low affinity insulin. (C) The same data are expressed as the difference in distribution of high affinity insulin compared with the low affinity insulin calculated by dividing the minimum clearance ratio value (Fig. 1B) into 1.0.

TABLE II

Antibody-Bound and TCA Precipitated Radioactivity In Rabbit Plasma after Intravenous Injection of ^{125}I-Pork Insulin

	Minutes after injection								
	0.5	1	4	5	8	9	10	18	20
Exp. 1	$\frac{25,771}{27,645} = 0.93$	$\frac{10,782}{11,852} = 0.91$	$\frac{3019}{3674} = 0.82$	$\frac{2892}{3014} = 0.95$	$\frac{1832}{2428} = 0.75$	$\frac{1722}{2300} = 0.75$	$\frac{1708}{2289} = 0.76$	—	—
Exp. 2	$\frac{17,764}{21,310} = 0.83$	$\frac{13,239}{14,464} = 0.91$	$\frac{4433}{5223} = 0.85$	$\frac{3348}{3927} = 0.85$	$\frac{2262}{2969} = 0.76$	$\frac{2354}{2803} = 0.83$	$\frac{2031}{2969} = 0.68$	—	—
Exp. 3	$\frac{31,298}{34,213} = 0.92$	$\frac{17,270}{19,414} = 0.89$	$\frac{3931}{5042} = 0.78$	$\frac{3006}{3787} = 0.79$	—	$\frac{2024}{2962} = 0.68$	$\frac{1905}{2650} = 0.72$	$\frac{1264}{1993} = 0.63$	$\frac{1182}{1911} = 0.62$

Animals were injected with 3×10^7 cpm ^{125}I-pork insulin and plasma samples were analyzed for radioactivity bound in the presence of excess antipork insulin antiserum or precipitated in 5% TCA as described in Methods. Results are expressed as counts per minute bound to antibody/counts per minute precipitated in 5% TCA/0.1 ml serum aliquot. Ratios of antibody bound to acid precipitated radioactivity are presented next to the actual data. The data presented in exp. 1 and 2 from 0.5 to 10 min are from the same experiment presented in Fig. 6.

injection, a time interval during which there was a divergence between TCA insoluble and antibody-bindable radioactivity in plasma. Therefore we used an alternative approach to measure the difference in distribution between the high affinity and low affinity insulins that puts greater emphasis on data obtained at the earlier time points. This method is illustrated in Fig. 1B, where the concentration of pork insulin relative to that of guinea pig insulin was calculated by dividing the plasma concentration of pork insulin radioactivity by that of guinea pig insulin at each sampling time. The difference in distribution was expressed as the reciprocal of the minimum value of the curve; the minimum always occurred within 5 min after tracer injection, at a time when there was close agreement between TCA precipitability and antibody bindability. Relative distribution ratios obtained by this method of analysis are summarized in Fig. 2C and agree well with the extrapolated measurements.

Saturability. When unlabeled pork insulin was injected i.v. 2 min before the injection of labeled tracers, there was a decrease in the apparent volume of distribution of the high affinity pork insulin. The magnitude of the decrease was dependent on the amount of unlabeled insulin. Thus, injection of 30 μg insulin (5 nmol) decreased the apparent volume of distribution of pork insulin by 24% (Fig. 3A and B); 300 μg of unlabeled insulin decreased the volume of distribution by 50% (Fig. 3C). When 3 mg unlabeled insulin was injected before the tracers, the plasma disappearance curves for pork and guinea pig insulins were almost superimposable, indicating a saturation of the body compartment specific for high affinity insulins. In Fig. 3D, it would appear that unlabeled insulin also influenced the distribution space of the low affinity insulin. This was largely due to variation in animal weight. The actual volumes of distribution of both pork and guinea pig insulin after 3 mg unlabeled insulin were similar to the distribution volume of low affinity guinea pig insulin seen in previous experiments (Fig. 2A).

The mass of unlabeled insulin necessary to saturate the body compartment specific for high affinity insulins correlates well with the amount of insulin necessary to saturate insulin receptors in vitro. Thus, insulin receptors on cultured lymphocytes, hepatocytes, adipocytes, and human monocytes are saturated at insulin concentrations of 1 μM. If the exogenous insulin in the rabbits distributed into the distribution space of the low affinity insulin, the calculated concentration after 3 mg unlabeled insulin in vivo would have been $\cong 2$ μM.

Reversibility. The studies with high and low affinity insulins suggest that 50% of the injected high affinity insulin is sequestered in a body compartment additional to plasma and interstitial fluid. The sequestration of high affinity insulin was reversible; when unlabeled insulin was given 5 min after the tracers, there was an immediate increase in the plasma concentration of the high affinity ^{125}I-chicken insulin to the level of the low affinity guinea pig insulin (Fig. 4A), demonstrating the reversibility and saturability of the putative receptor compartment in vivo. As the interval between the injection of the labeled and the unlabeled insulin was prolonged, relatively less of the high affinity insulin was returned to the plasma compartment (Fig. 4B–D). Unlabeled insulin at high concentrations had only a slight effect on the labeled low affinity insulin (Fig. 4A–D). As a control, unlabeled bovine growth hormone, when injected 5 min after the tracer insulins, had little or no effect on the disappearance curves of either high or low affinity insulin (data not shown). Because of the limited availability of purified analogues with low affinity for receptors, experiments in which large amounts of unlabeled low affinity insulins were injected either before or after the tracers were not performed.

The experiment illustrated in Fig. 5 demonstrates

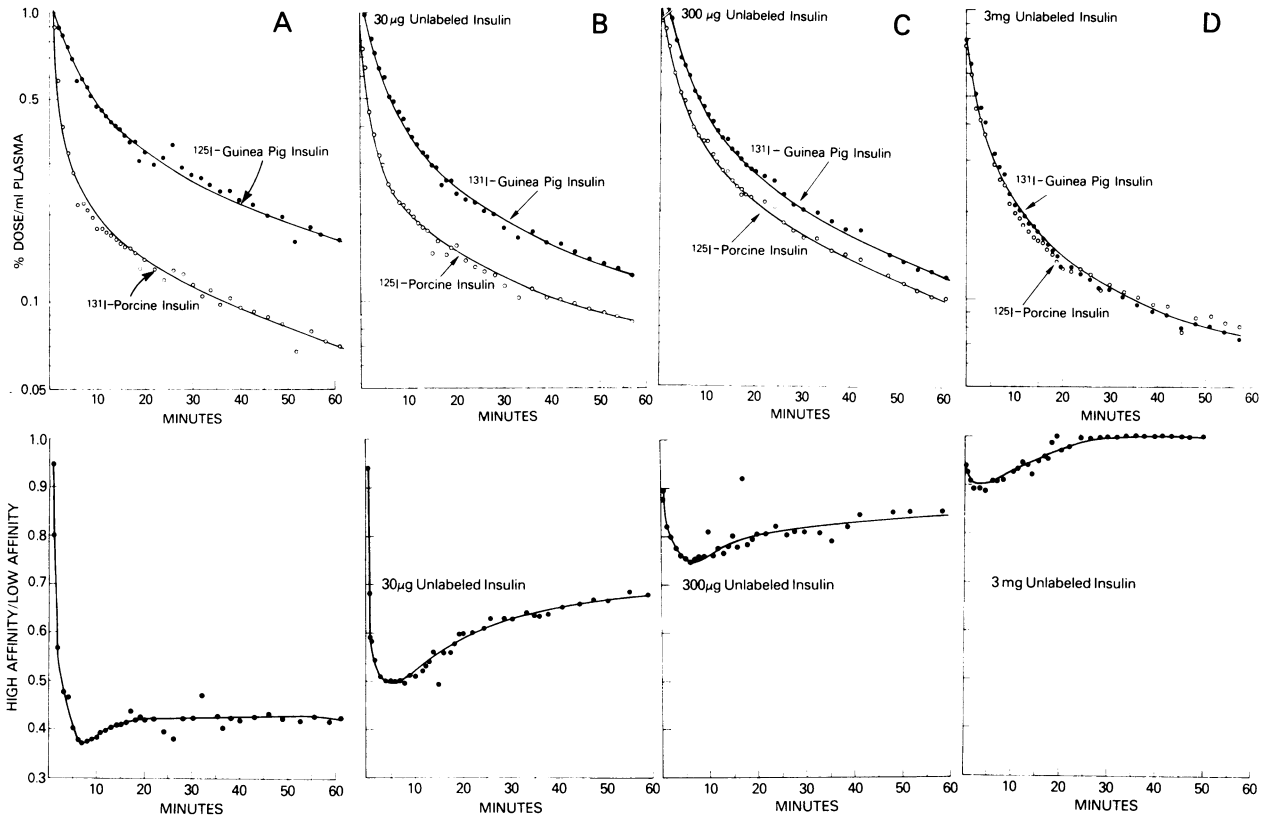

FIGURE 3 Anesthetized rabbits were injected with a mixture of ^{125}I-pork and ^{131}I-guinea pig insulins; the TCA precipitable radioactivity for each insulin is plotted as a function of time after injection (top), as in Fig. 1A. The same data are plotted as a ratio of high affinity/low affinity insulin (bottom) as described in legend to Fig. 1B. Unlabeled pork insulin (0 in experiment A, 30 μg in B, 300 μg in C, and 3 mg in D) was injected i.v. 2 min before the injection of the labeled insulins.

that the amount of insulin in the labeled hormone preparation was effectively at tracer concentrations and that the hormone itself at this concentration had little effect on the manner in which the hormone was metabolized in vivo. In these experiments injection of ^{131}I- and ^{125}I-pork insulin were administered 7.5 min apart. The disappearance curves for the two tracers were quite similar to one another, despite the fact that they had been administered 7.5 min apart. Unlabeled pork insulin (3 mg) was given 7.5 min after the second tracer. The unlabeled insulin produced an efflux of both labeled tracers. As might be expected from the earlier studies, the tracer that had been administered 15 min before the unlabeled insulin had a relatively smaller return to the plasma compartment than the tracer that had been administered 7.5 min before the unlabeled hormone.

Characterization of plasma radioactivity released. The fraction of plasma radioactivity that can bind to insulin antibodies falls at a faster rate than TCA precipitable radioactivity with the ratio of the two falling from 0.91 to 0.76 during the first 10 min (Table II and Fig. 6). The rise in plasma radioactivity produced by injection of unlabeled insulin was due largely to the return to the plasma compartment of relatively intact insulin because the ratio of antibody bindable to TCA precipitable radioactivity was either unchanged or rose. This was true even when the unlabeled insulin was injected 30 min after the injection of the labeled insulins. These data further support the idea that the rapid removal of the high affinity insulin from plasma is a sequestration in a reversible and saturable compartment. The antibody binding studies were done only with labeled pork insulin because of the unavailability of sufficient quantities of antisera to the other species of insulin.

Tissue localization. To identify the organs that were responsible for the selective uptake of high affinity labeled insulin, animals were injected with ^{125}I-chicken insulin and ^{131}I-guinea pig insulin. At 5 min after the injection, when the ratio of high affinity insulin to low affinity insulin in plasma was ≅0.25, organs were re-

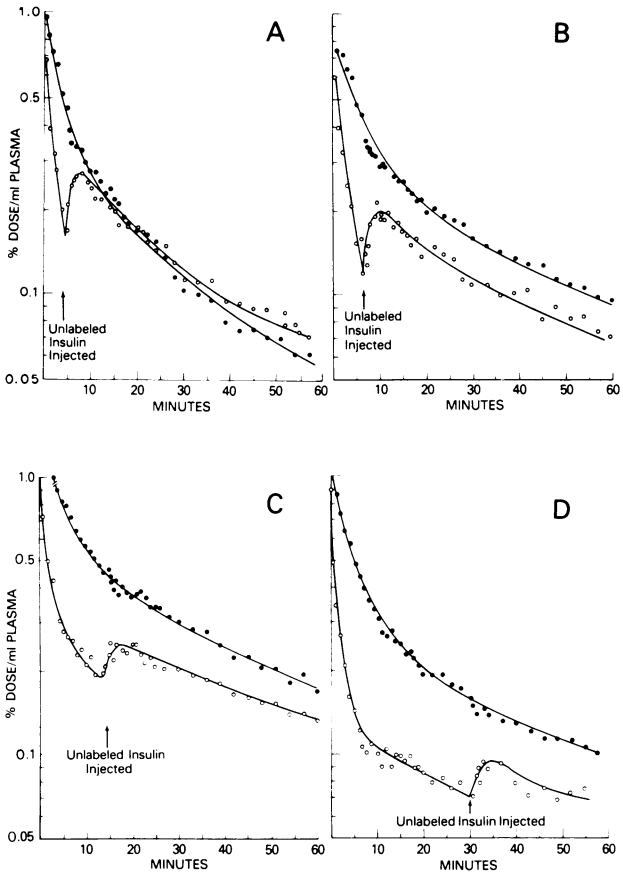

FIGURE 4 A mixture of ^{125}I-chicken and ^{131}I-guinea pig insulin was injected intravenously into anesthetized rabbits. At varying times after the injection of tracers, 3 mg unlabeled pork insulin was injected intravenously: (A) 5 min; (B) 7.5 min; (C) 15 min; (D) 30 min. In panels A and B it would appear that the ratio of ^{131}I-guinea pig to ^{125}I-chicken insulin in the plasma is not 1.0 at time 0 of the experiment. This can be accounted for by slight delays in obtaining a true initial blood sample immediately after tracer injection. Thus, by 15 s after tracer injection, a significant depletion of plasma high affinity insulins compared to low affinity insulins was observed in all experiments.

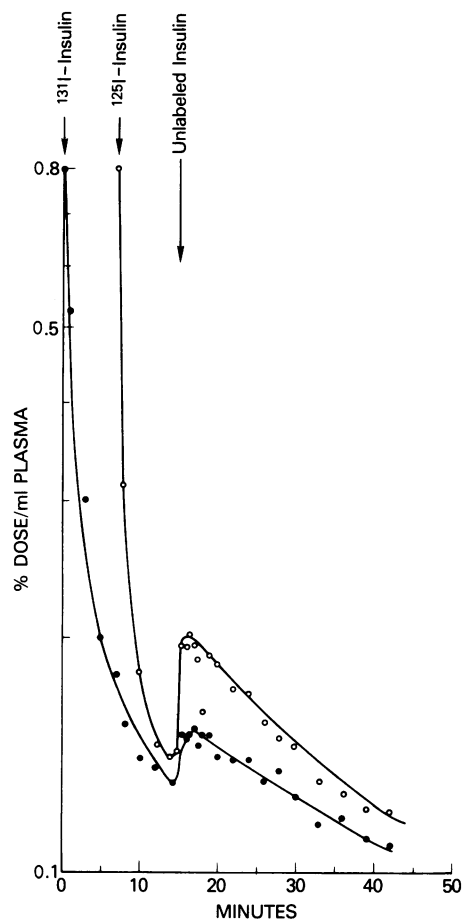

FIGURE 5 An anesthetized rabbit was injected i.v. with 5×10^7 cpm ^{131}I-pork insulin followed 7.5 min later by 5×10^7 cpm ^{125}I-pork insulin. After an additional 7.5 min, 3 mg unlabeled insulin was injected i.v. Arrows denote the timing of the injections.

moved and the TCA precipitable radioactivity determined. The liver and spleen and to a lesser extent the kidney and heart contained more highly affinity (chicken) insulin than the low affinity (guinea pig) insulin, indicated by a ratio in excess of 1.0. In another experiment, when 3 mg of unlabeled insulin were injected before the two labeled tracers, the preferential disappearance of the high affinity insulin from plasma was prevented, and the preferential uptake by liver, spleen, and heart was obliterated. The failure of the large dose of unlabeled insulin to affect the uptake of radioactivity by kidney and diaphragm suggests that the uptake of labeled insulin by these two organs was predominantly not related to the specific insulin receptor.

DISCUSSION

Demonstration of the receptor compartment in vivo. These studies demonstrate a body compartment in vivo that has all of the properties that we associate with specific cell surface receptors. Like hormone that is bound to receptors in vitro, hormone content of this compartment shows limited capacity (saturability) and reversibility, and the amount bound is predicted by the biological potency of the hormone, i.e., chicken insulin $\geqq$ pork insulin > pork proinsulin $\cong$ guinea pig insulin.

The experimental design introduced in this paper, although it has many advantages over previous studies, falls short of the ideal in many respects. The low affinity insulin used as a marker should be identical to the high affinity insulin in all respects, except that it should have no affinity for the receptor. Guinea pig insulin and, to an even greater extent, pork proinsulin fall short. Both have some, albeit low, affinity

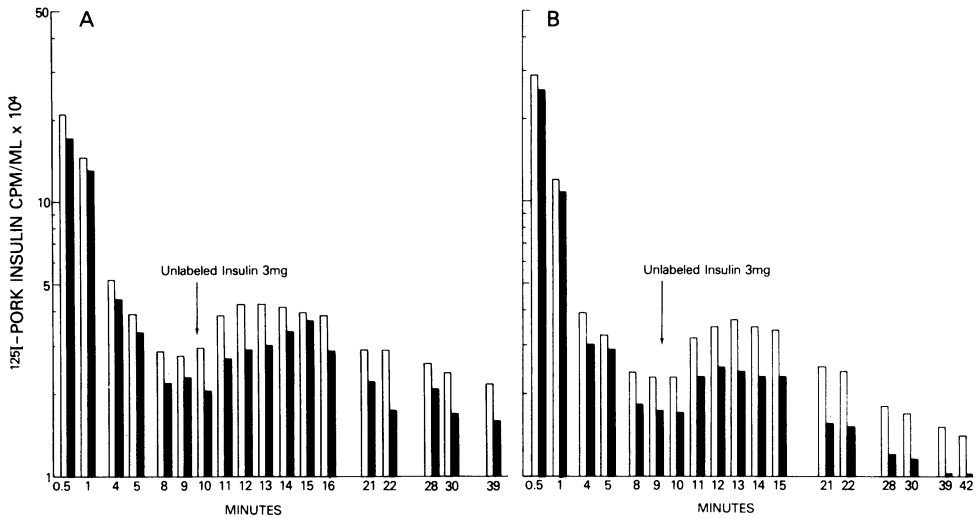

FIGURE 6 Rabbits were injected with 3×10^7 cpm ^{125}I-pork insulin followed 10 min later by 3 mg unlabeled pork insulin. Open bars represent plasma concentration of radioactivity precipitated in TCA and solid bars represent concentration of radioactivity bound to anti-insulin antibody as described in legend to Table II and Methods. Panels A and B illustrate data obtained from individual animals.

for receptor. Although in vitro the degradative system for insulin does not distinguish chicken, pork, guinea pig, and even desalanine-desasparagine insulin from one another, despite more than a 100-fold range of affinities for receptor, it is difficult to extrapolate from simple in vitro systems to the in vivo state with regard to hormone degradation. Pork proinsulin, in addition to its differences in structure and affinity for receptor, is also less well degraded by the insulin degrading systems, both in vivo and in vitro (24, 25). Thus, it remains uncertain as to how closely the low affinity insulins mimic the high affinity insulins in their physical spaces of distribution as well as in the rates of degradation and egress from the vascular space.

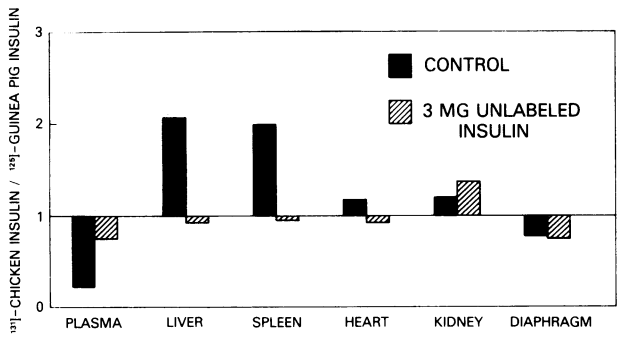

FIGURE 7 A mixture of ^{125}I-guinea pig and ^{131}I-chicken insulin ($3-5 \times 10^7$ cpm) was injected intravenously into anesthetized rabbits. 5 min after injection, the animals were sacrificed, and pieces of tissues were excised and homogenized in phosphate-buffered saline, pH 7.35, that contained 1 mg/ml bovine serum albumin and 1 nM *N*-ethylmaleimide (1:5 wt/vol) with a Polytron homogenizer for 10 s at a rheostat setting of five. Aliquots of tissue homogenates were analyzed for TCA insoluble radioactivity. Results are expressed as percent dose ^{131}I-chicken insulin per gram wet weight/percent dose ^{125}I-guinea pig insulin per gram wet weight tissue.

Despite these uncertainties, the data appear to give excellent approximations of hormone binding to its receptors in vivo. We are encouraged in this belief by the finding that measurements of the apparent volumes of distribution, which are obtained from measurements at 30–60 min after injection, where degradation of hormone has been substantial, give results that are extremely close to measurements of relative clearance which are based on measurements over the first 5–10 min after injection, at which time degradation is relatively much less of a factor. Likewise, chicken insulin and pork insulin gave remarkably similar results, despite the substantial differences in the two preparations; guinea pig insulin and pork proinsulin, which are probably degraded at substantially different rates, were quite similar to one another, and all four insulins behaved in vivo in accord with predictions made from the relative affinities for receptor based on in vitro studies. Interchange of the two radioactive isotopes had little effect and likewise, measurements of reactivity of the labeled pork insulin with antibody gave results that were quite close to those obtained by TCA precipitability especially at early times after injection. We conclude that, although the precise size of the receptor compartment is un-

certain, the rapid preferential disappearance of high affinity insulins from plasma represent rapid uptake by receptors which is rapidly reversible. Further support for our contention that little of this preferential disappearance can be accounted for by degradation comes from studies of TCA soluble radioactivity in plasma. If the early rapid disappearance had been due to preferential degradation of the high affinity insulin, we would expect that the TCA soluble radioactivity derived from the high affinity insulin should be greater than that of the TCA soluble radioactivity of the low affinity insulin. In experiments to be shown elsewhere, the opposite was observed. During the first 5 min after the injection of the labeled insulin, when the high affinity insulin was disappearing rapidly from the plasma, the TCA soluble component of the high affinity insulin in plasma was substantially less than that of the low affinity insulin. Only after ≅10 min did the TCA soluble radioactivity of the high affinity insulin equal that of the low affinity insulin and they remained approximately equal for the remainder of the experiment.

Our results are similar to those of previous investigators who studied the fate of labeled and unlabeled insulins in vivo. Berson et al. (26), in their pioneer studies of labeled insulin in humans recorded a volume of distribution for labeled beef insulin that was 37% of the body weight. We think that peptide hormones do not readily enter cells; therefore, it is likely that their measurements included a receptor compartment. Likewise, Madison and Kaplan (27) recorded that the liver removed ≅50% of the insulin during a single transhepatic passage. Other investigators, in similar studies with either intact animals or perfused organ preparations, noted significant extraction of insulin by the liver and other tissues. In addition, the insulin removal was decreased when the hormone concentration in the blood or perfusing medium was increased either by stimulation of endogenous insulin secretion or by exogenous insulin (28–40). For example, when labeled insulin was injected into rabbits, the inclusion of a high dose of unlabeled insulin (20 mg) slowed the disappearance of labeled insulin from plasma; the major difference in disappearance was noted early after the injection where saturation of receptors would predominate and little or no effect at later times when effects on the insulin degrading mechanisms would be most prominent (41). In most studies the investigators discussed "uptake". Though they did not always precisely indicate so, they generally treated this uptake as irreversible and related to hormone destruction rather than reversible binding to receptors. However, perusal of the data indicate that they were probably, in many cases, observing the same receptor compartment as we.

Sherwin et al. (42) in studying the fate of unlabeled insulin in vivo with an elegant and sophisticated analysis, demonstrated one compartment for insulin which had many of the properties of the receptor. Interestingly, the peak content of insulin in this compartment coincided with the maximum biological response to insulin in vivo. Although the size of their compartment was distinctly smaller than the one we observed in the present study, we suspect that the two compartments are similar or equivalent.

Some investigators found that insulin removal from plasma in vivo is reversible. Thus, Rasio (43) demonstrated in dogs that unlabeled insulin was capable of causing an increase in the plasma concentration of previously injected radioactive insulin. Because the labeled insulin appeared in the venous blood but not in lymph, Rasio suggested that the binding of insulin in vivo was predominantly located in vascular endothelium. Our studies were not designed to study vascular endothelium, but it should be noted that the liver showed preferential uptake of high affinity insulin whereas the kidney did not, although both organs have a very rich vascular supply. Sonksen et al. (44) also observed that some insulin bound to sites in the dog hind limb was capable of being released in an intact form after intravascular injection of unlabeled insulin.

Terris and Steiner (45) noted with noncyclically perfused rat livers that the uptake of labeled insulin, proinsulin, or desalanine-desasparagine insulin was in proportion to its affinity for the insulin receptor; unlabeled insulin competed for uptake. Binding showed both reversible and irreversible components; the latter was related to hormone degradation that was initiated by the hormone-receptor interaction. Their results and conclusions agree closely with our in vivo data.

Implications of the present study. Our studies show that circulating insulin is rapidly taken up by receptors. Classically, receptors subserve two major functions. They recognize the bioactive hormone by binding it and secondly the combination of hormone with receptor initiates the series of biochemical events that lead to hormone action. Often, in addition, hormone interacts with its receptors to regulate the concentration of the receptors and also to regulate by an independent process the affinity of the receptors (46, 47). The present study shows another function of the hormone interaction with receptor, namely that receptors act as a reservoir or capacitance in vivo. Receptors rapidly take up hormone at times when the concentration of hormone in plasma is rising and release hormone back into the plasma at times when the concentration of hormone in plasma is falling. In this way they are analogous to the binding proteins in the plasma that bind steroid hormones and iodothyronine

hormones which act as reservoirs for the free hormone in plasma (48, 49). Thus, even in the case of cells that lack a biological response to that hormone, the receptors on that cell may still have a function i.e., act as reservoirs for the plasma hormone.

Interestingly, the fraction of extrapancreatic insulin in the receptor compartment is quite close to that predicted from theoretical calculations based on in vitro measurements of receptor concentration on cell surfaces and receptor affinity measured in vitro. Our direct analyses of the data would indicate that between one-half and two-thirds of the extrapancreatic insulin at any time is bound to receptors. More sophisticated analyses of these data, presently underway in collaboration with Dr. Berman and Dr. McGuire, National Institutes of Health, Bethesda, Md., would indicate that the receptor-bound compartment accounts for ≅70% of the extrapancreatic insulin.

In the basal state in vivo, the plasma insulin concentration (H) ≅0.1 nM. From our data, in vivo the B/F of hormone ≧1 (and may be 2–3). We estimate from the in vitro studies that at 37°C in vivo the equilibrium constant (K_a) ≦ (2 nM)$^{-1}$. Thus, receptor concentration (R_0) ≧ 2 nM, and $H < 1/K_a \leqq R_0$ or more likely $H < 1/K_a < R_0$. The signal rate produced by a hormone at its target cell is probably dependent on the concentration of hormone-receptor complexes on the cell surface which in turn is a function of the product of $K_a \times H \times R_0$ where K_a = the equilibrium constant, H = the concentration of free hormone, and R_0 = the total concentration of receptor. Clearly, a given concentration of HR could be achieved by any combination of affinity, hormone concentration and receptor concentration. With insulin in vivo it is achieved with a low hormone concentration and a high receptor concentration. (In the other hormone systems where the concentrations of circulating hormone are far less than the reciprocal of the equilibrium constant, i.e., $H < 1/K_a$, we suspect that R_0 will be appropriately elevated to compensate). One advantage to the body of such a system where $H \ll R_0$. is that the hormone molecules, which are probably turned over in a period of a few minutes, are at much lower concentrations than the receptors which are turned over in a period of hours. In this situation it is also advantageous to have $R_0 > 1/K_a$. Thus, given a particular hormone concentration, the steady-state concentration of HR can be achieved either by adjusting the K_a or the R_0. But $K_a = \hat{k}/\check{k}$, i.e., it represents the quotient of the association and dissociation rate constants. Changes in K_a are almost all effected by changes in $\check{k}$, the dissociation rate constant. A substantial elevation of K_a could result in an intolerably slow dissociation rate, i.e., the increase in K_a would have a severe kinetic penalty. If R_0 is elevated instead, the same steady-state level of HR can be achieved without affecting the dissociation rate.

Hormone association in vivo, which depends on the association rate constant, hormone concentration and receptor concentration, was much faster in vivo than had been observed in vitro largely because the receptor concentration in vivo is multi-fold higher than the receptor concentration during studies in vitro. Likewise, the temperature in vivo is higher than that under the conditions studied in vitro.

Other implications. In many investigations concentrations of endogenous hormone in plasma were measured at intervals over a period of time and used as a measure of secretion (e.g., when converted to an area under the curve). Our study, which shows that a large fraction of the extrapancreatic hormone is bound to receptors, indicates that the plasma hormone reflects only a fraction of the total extrapancreatic hormone and the size of this fraction depends on the receptor concentration and affinity. Fluctuations in receptor concentration or receptor affinity, as has been demonstrated under numerous circumstances in vivo, will have a great effect on how closely the areas under the curves reflect secretion. For example, Karakash et al. (50) found that in the obese-hyperglycemic mouse (where presumably receptor concentrations are reduced to ≅⅓ of normal) the uptake of insulin across the liver was reduced substantially, presumably reflecting the decreased binding to receptor. Thus, identical curves of plasma hormone versus time will indicate identical secretory rates only when receptor concentration and receptor affinity are unchanged.

Likewise, when a tissue had a concentration of hormone which was greater than the concentration of hormone in the plasma at the same time (i.e. a tissue to plasma ratio in excess of one), it was often concluded that this indicated that the hormone was actually being manufactured (and stored) in that tissue (51). Our finding here of tissue to plasma ratios markedly in excess of one in tissues that contain the receptor would indicate that tissue to plasma ratios in excess of one more often reflect hormone on receptors rather than manufacture of hormone at that site.

Another of our findings that reflects on studies by others relates to insulin degradation in vivo. Freychet et al. (14) concluded from their studies that hormone binding to its receptor and hormone degradation were two independent processes. Later Terris and Steiner (45) concluded from their work that insulin binding to its receptors can lead to degradation of the hormone and that this process is an important one in vivo. In our studies where unlabeled insulin was injected shortly after the tracer insulins, the binding to receptor of the high affinity insulin was fully reversed but its level in plasma did not elevate above the level of the low affinity insulin suggesting that the hormone on the receptor compartment was not being protected from degradation. When the unlabeled insulin was

administered at later times, the concentration of high affinity insulin in plasma did not rise enough to reach the level of the low affinity insulin. If we assume that the low affinity insulin and the high affinity insulin were degraded at the same rate in the plasma compartment, then the failure of the high affinity insulin to reach the level of the low affinity insulin suggests that there is some preferential degradation of high affinity insulin from the receptor compartment (for a more comprehensive review of this relationship, the reader should consult reference 45). Further studies of the integrity of the low affinity and high affinity insulin and more sophisticated analytical procedures will be required to confirm this point, and to determine the relative role of receptor-mediated and nonreceptor pathways of degradation in vivo. If there is degradation of hormone via the receptor compartment, then surface receptors would still function as a reservoir for plasma hormone but would behave as a reservoir with a leak.

ACKNOWLEDGMENTS

We would like to thank Drs. R. S. Yalow, C. R. Kahn, M. Berman, and E. A. McGuire for their comments on the manuscript. Also, we thank Drs. F. C. Kosmakos, D. B. Jarrett, E. Van Obberghen, and H. Wachslicht for their assistance with the preparation of animals.

REFERENCES

1. Zeleznik, A. J., and J. Roth. 1976. Plasma membrane receptors for peptide hormones: *in vivo* role as reservoir for the circulating hormone. Program of the 58th Annual Meeting of the Endocrine Society. San Francisco, Calif. Abstract 27.
2. Stadie, W. C., N. Haugaard, and M. Vaughan. 1952. Studies of insulin binding with isotopically labeled insulin. *J. Biol. Chem.* **199:** 729–739.
3. Lee, N. D., and R. Williams. 1954. The intracellular localization of labeled thyroxine and labeled insulin in the mammalian liver. *Endocrinol.* **54:** 5–19.
4. Edelman, P. M., and I. L. Schwartz. 1966. Subcellular distribution of ^{131}I-insulin in striated muscle. *Am. J. Med.* **40:** 695–708.
5. Lunenfeld, B., and A. Eshkol. 1967. Immunology of human chorionic gonadotropin (hCG). *Vitam. Horm.* **25:**137–190.
6. Kammerman, S., and R. Canfield. 1972. The inhibition of binding of iodinated human chorionic gonadotropin to mouse ovary *in vivo*. *Endocrinology.* **90:** 384–389.
7. Han, S. S., H. J. Rajaneimi, M. I. Chu, A. N. Hirschfield, and A. R. Midgley, Jr. 1974. Gonadotropin receptors in rat ovarian tissues. II. Subcellular localization of LH binding sites by electron microscopic radioautography. *Endocrinology.* **95:** 589–598.
8. Roth, J. 1973. Peptide hormone binding to receptors: a review of direct studies *in vitro*. *Metab. Clin. Exp.* **22:** 1059–1073.
9. Cuatrecasas, P. 1974. Membrane receptors. *Ann. Rev. Biochem.* **43:** 169–214.
10. Kahn, C. R. 1976. Membrane receptors for peptide hormones and neurotransmitters. *J. Cell. Biol.* **70:** 261–286.
11. Kahn, C. R., D. M. Neville, Jr., and J. Roth. 1973. Insulin-receptor interaction in the obese-hyperglycemic mouse. *J. Biol. Chem.* **248:** 244–250.
12. Bar, R. S., P. Gorden, J. Roth, C. R. Kahn, and P. De Meyts. 1976. Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients. *J. Clin. Invest.* **58:** 1123–1135.
13. Ginsberg, B. H. 1977. The Insulin Receptor: properties and regulation. *In* Biochemical Actions of Hormones. G. Litwak, editor. Academic Press, Inc., New York. 313–349.
14. Freychet, P., C. R. Kahn, J. Roth, and D. M. Neville, Jr. 1972. Insulin interactions with liver membranes. Independence of binding of the hormone and its degradation. *J. Biol. Chem.* **247:** 3953–3961.
15. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.).* **194:** 495–496.
16. Berson, S. A., and R. S. Yalow. 1966. Iodoinsulin used to determine the specific activity of iodine-^{131}. *Science (Wash. D. C.).* **152:** 205–207.
17. Fahey, J. L., D. N. Buell, and H. C. Sox. 1971. Proliferation and differentiation of lymphoid cells: studies with human lymphoid cell lines and immunoglobulin synthesis. *Ann. N. Y. Acad. Sci.* **190:** 221–234.
18. Lesniak, M. A., J. Roth, P. Gorden, and J. R. Gavin, III. 1974. Binding of ^{125}I-human growth hormone to specific receptors in human cultured lymphocytes. *J. Biol. Chem.* **249:** 1661–1667.
19. Freychet, P., J. Roth, and D. M. Neville, Jr. 1971. Insulin receptors in the liver. Specific binding of ^{125}I-insulin to the plasma membrane and its relation to insulin bioactivity. *Proc. Natl. Acad. Sci. U. S. A.* **68:** 1833–1837.
20. Simon, J., P. Freychet, and G. Rosselin. 1974. Chicken insulin: radioimmunological characterization and enhanced activity in rat fat cells and liver plasma membranes. *Endocrinology.* **95:** 1439–1449.
21. Zimmerman, A. E., and C. C. Yip. 1974. Guinea pig insulin. II Biological activity. *J. Biol. Chem.* **249:** 4026–4029.
22. Neville, D. M., Jr. 1968. Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. *Biochem. Biophys. Acta.* **154:** 540–552.
23. Desbuquois, B., and G. D. Aurbach. 1971. Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassay. *J. Clin. Endocrinol. Metab.* **33:** 732–738.
24. Katz, A. I., and A. H. Rubenstein. 1973. Metabolism of proinsulin, insulin, and C-peptide in the rat. *J. Clin. Invest.* **52:** 1113–1121.
25. Rubenstein, A. H., L. A. Pottenger, M. Mako, G. S. Getz, and D. F. Steiner. 1972. The metabolism of proinsulin and insulin by the liver. *J. Clin. Invest.* **51:** 912–921.
26. Berson, S. A., R. S. Yalow, A. Bauman, M. A. Rothschild, and K. Newerly. 1956. Insulin-I^{131} metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin treated subjects. *J. Clin. Invest.* **35:** 170–190.
27. Madison, L. L., and N. Kaplan. 1958. The hepatic binding of I^{131}-labeled insulin in human subjects during a single transhepatic circulation. *J. Lab. Clin. Med.* **52:** 927. (Abstr.)
28. Mirsky, I. A., G. Perisutti, and F. J. Dixon. 1955. The destruction of I^{131}-labeled insulin by rat liver extracts. *J. Biol. Chem.* **214:** 397–408.

29. Wick, A. N., and D. R. Drury. 1958. Effects of superimposed native insulin on disposal of iodoinsulin in the body. *Proc. Soc. Exp. Biol. Med.* **97:** 514–516.
30. Mortimore, G. E., and F. Tietze. 1959. Studies on the mechanism of capture and degradation of insulin-I^{131} by the cyclically perfused rat liver. *Ann. N. Y. Acad. Sci.* **82:** 329–337.
31. Palmer, D. L., and R. E. Bolinger. 1961. Effect of nephrectomy and splanchnicectomy on plasma disappearance of labeled insulin in the rabbit. *Proc. Soc. Exp. Biol. Med.* **107:** 809–812.
32. Bürgi, H., K. Kopetz, K. Schwartz, and R. E. Froesch. 1963. Fate of rat insulin in rat-liver perfusion studied by adipose tissue assay. *Lancet.* **II:** 314–316.
33. Izzo, J. L., J. W. Bartlett, A. Roncone, M. J. Izzo, and W. F. Bale. 1967. Physiological processes and dynamics in the disposition of small and large doses of biologically active and inactive ^{131}I-insulins in the rat. *J. Biol. Chem.* **242:** 2342–2355.
34. Csorba, T. R., L. C. J. Hartley, K. L. Manchester, and N. Veall. 1969. The turnover and degradation of insulin analogues. *Diabetologia.* **5:** 422. (Abstr.)
35. Stoll, R. W., J. L. Touber, L. E. Menehan, and R. H. Williams. 1970. Clearance of porcine insulin, proinsulin and connecting peptide by the isolated rat liver. *Proc. Soc. Exp. Biol. Med.* **133:** 894–896.
36. Kaden, M., P. E. Harding, and J. B. Field. 1973. Effects of intraduodenal glucose administration on hepatic extraction of insulin in the anesthetized dog. *J. Clin. Invest.* **52:** 2016–2028.
37. McCarroll, A. M., and K. D. Buchanan. 1973. Insulin clearance by the isolated perfused livers of insulin deficient rats. *Diabetologia.* **9:** 457–460.
38. Mondon, C. E., J. M. Olefsky, C. B. Dolkas, and G. M. Reaven. 1975. Removal of insulin by perfused rat liver: effect of concentration. *Metab. Clin. Exp.* **24:** 153–160.
39. Misbin, R. I., T. J. Merimee, and J. M. Lowenstein. 1976. Insulin removal by isolated perfused rat liver. *Am. J. Physiol.* **230:** 171–177.
40. Navalesi, R., A. Pilo, and E. Ferrannini. 1976. Insulin kinetics after portal and peripheral injection of ^{125}I-insulin. II Experiments in the intact dog. *Am. J. Physiol.* **230:** 1630–1636.
41. Berson, S. A., R. S. Yalow, and B. W. Volk. 1957. *In vivo* and *in vitro* metabolism of insulin-I^{131} and glucagon-I^{131} in normal and cortisone-treated rabbits. *J. Lab. Clin. Med.* **49:** 331–342.
42. Sherwin, R. S., K. J. Kramer, J. D. Tobin, P. A. Insel, J. E. Liljenquist, M. Berman, and R. Andres. 1974. A model of insulin kinetics in man. *J. Clin. Invest.* **53:** 1481–1492.
43. Rasio, E. A. 1969. The displacement of insulin from blood capillaries. *Diabetelogia.* **5:** 416–419.
44. Sonksen, P. H., J. R. McCormick, R. H. Egdahl, and J. S. Soeldner. 1971. Distribution and binding of insulin in the dog hindlimb. *Am. J. Physiol.* **221:** 1672–1691.
45. Terris, S., and D. F. Steiner. 1976. Retention and degradation of ^{125}I-insulin by perfused livers from diabetic rats. *J. Clin. Invest.* **57:** 885–896.
46. Kahn, C. R., K. Megyesi, R. S. Bar, R. C. Eastman, and J. S. Flier. 1977. Receptors for polypeptide hormones: new insights into the pathophysiology of disease states in man. *Ann. Intern. Med.* **86:** 205–219.
47. De Meyts, P., A. R. Bianco, and J. Roth. 1976. Site-site interactions among insulin receptors. Characterization of the negative cooperativity. *J. Biol. Chem.* **251:** 1877–1888.
48. Sandberg, A. A., and W. R. Slaunwhite, Jr. 1963. Transcortin: A corticosteroid binding protein of plasma. V. *In vitro* inhibition of cortisol metabolism. *J. Clin. Invest.* **42:** 51–54.
49. Robbins, J., and J. E. Rall. 1960. Proteins associated with the thyroid hormones. *Physiol. Rev.* **40:** 415–489.
50. Karakash, C., F. Assimacopoulos-Jeannet and B. Jeanrenaud. 1976. An anomaly of insulin removal in perfused livers of obese-hyperglycemic (ob/ob) mice. *J. Clin. Invest.* **57:** 1117–1124.
51. Liddle, G. W., W. E. Nicholson, D. P. Island, D. N. Orth, K. Abe, and S. C. Lowder. 1969. Clinical and laboratory studies of ectopic hormone syndromes. *Recent Prog. Horm. Res.* **25:** 283–305.