Biochimica et Biophysica Acta, 443 (1976) 227–242

BBA 77399

THE INSULIN RECEPTOR OF THE TURKEY ERYTHROCYTE
CHARACTERIZATION OF THE MEMBRANE-BOUND RECEPTOR

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(Received November 18th, 1975)
(Revised manuscript received March 9th, 1976)

SUMMARY

The insulin receptor of the turkey erythrocyte has previously been shown to be very similar to that of the mammalian insulin receptors. As a first step in the isolation of this receptor a highly purified plasma membrane fraction has been prepared. The binding characteristics of the purified membrane-bound receptor were identical to those found with intact erythrocytes, but the membrane preparation had very little insulin-degrading activity. Isolation of the membrane by the methods described gave a 100-fold purification of the insulin receptor with 67% yield.

INTRODUCTION

The insulin receptor of the turkey erythrocyte is a good model system for studying insulin-receptor interactions [1]. The affinity of porcine insulin is identical for the turkey and mammalian receptors over the entire range of insulin concentrations, as is the affinity of each of three insulin analogues which differed 300-fold in biological potency. Insulin-induced acceleration of dissociation (i.e. the negatively cooperative site-site interaction) is indistinguishable over a 10^6 range of insulin concentrations. The sharp pH dependence of binding as well as the effects of temperature on association, dissociation and steady-state binding are also identical for the turkey and mammalian receptors. Thus, the mammalian and avian insulin receptors cannot be distinguished on the basis of their binding properties.

The avian erythrocyte is less complicated than mammalian lymphocytes, liver, fat or placental cells and therefore has some of the properties of an ideal cell from which to purify the insulin receptor. Avian erythrocytes are readily available in large quantities and are normally found in suspension, avoiding the necessity of enzymatic digestion or complex membrane purification. The cells although nucleated, do not synthesize macromolecules [2] and lack much of the cellular machinery of previously

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studied cells. Highly purified plasma membrane is thus simple to prepare in large quantities and further purification of the insulin receptor rendered less difficult.

Previous schemes for producing highly purified avian erythrocyte plasma membrane have been reported [3]. However, these methods yielded membranes which had lost most insulin-binding activity. In the present communication we report a scheme for the production of large quantities of highly purified plasma membrane of avian erythrocytes and a characterization of the binding properties of the insulin receptor of this membrane.

MATERIALS AND METHODS

Polypeptide hormones and ^{125}I-labeled insulin preparation were described previously [9]. Buffer A consisted of 85 mM Tris · HCl, pH 7.8, 30 mM NaCl, 10 mM glucose, 1 mg/ml bovine serum albumin. This buffer was also used diluted 1/10 (buffer A/10). Buffer B consisted of 150 mM Tris · HCl, pH 7.8, 125 mM NaCl, 100 mM glucose, and 2.5 mg/ml bovine serum albumin.

Turkey erythrocyte membranes were prepared by a modification of the method of Bilezikian and Aurbach [3]. 40 ml of buffer A/10 containing 2 mM $MgCl_2$ were added to 40 ml of washed, fresh turkey erythrocytes ($3 \cdot 10^9$/ml). The lysate was sedimented at $3000 \times g$ for 10 min, the hemoglobin-containing supernatant discarded, and the pellet resuspended in 40 ml of the same buffer. Sedimentation and resuspension were performed until the supernatant was clear, usually about eight times. The light pink pellet was then taken up in 40 ml of buffer A/10 containing 0.2 mM $MgCl_2$ and homogenized in a Dounce homogenizer with a tight pestle. This process was continued until 60–80 % of the nuclei were extruded, as determined by phase contrast microscopy. This homogenate was sedimented at $5000 \times g$ for 10 min, forming a two-layered pellet. The upper layer of the pellet, which contained membrane vesicles with a few nuclei, was resuspended by gently rocking the tube. The gelatinous lower layer of the pellet which contained nuclei and unbroken cells, was discarded. The membrane fraction was further purified by sedimentation at $15\,000 \times g$. The remaining nuclei formed a tight pellet which was not easily resuspended, while the membrane vesicles were readily resuspended in buffer A/10. This $15\,000 \times g$ sedimentation was repeated twice more, the pellet taken up in a final volume of 20 ml of buffer A/10, and frozen at −20 °C. Some membrane preparations showed an increase in binding after the first thaw, but thereafter insulin binding was stable for up to 5 months of storage.

Binding experiments were performed by adding ^{125}I-labeled insulin (25–50 pg) to 250 µl buffer B, then adding 25–750 µg of membrane protein in 10–50 µl of buffer A/10. Deionized water was added to bring the final volume to 500 µl. Non-specific binding control tubes also contained 1 µg per 0.5 ml of unlabeled insulin. After incubation for 150 min at 15 °C, 200-µl aliquots were added to 200 µl of cold buffer A in a microfuge tube (duplicate tubes for each sample), sedimented for 90 s in a Beckman microfuge and the radioactivity in the pellet determined as previously described [12].

Protein was determined by the method of Lowry et al. [4] and DNA by the method of Burton [5].

RESULTS

Characterization of membrane purity. The avian erythrocyte membranes were highly purified by a number of criteria. When viewed by phase contrast microscopy

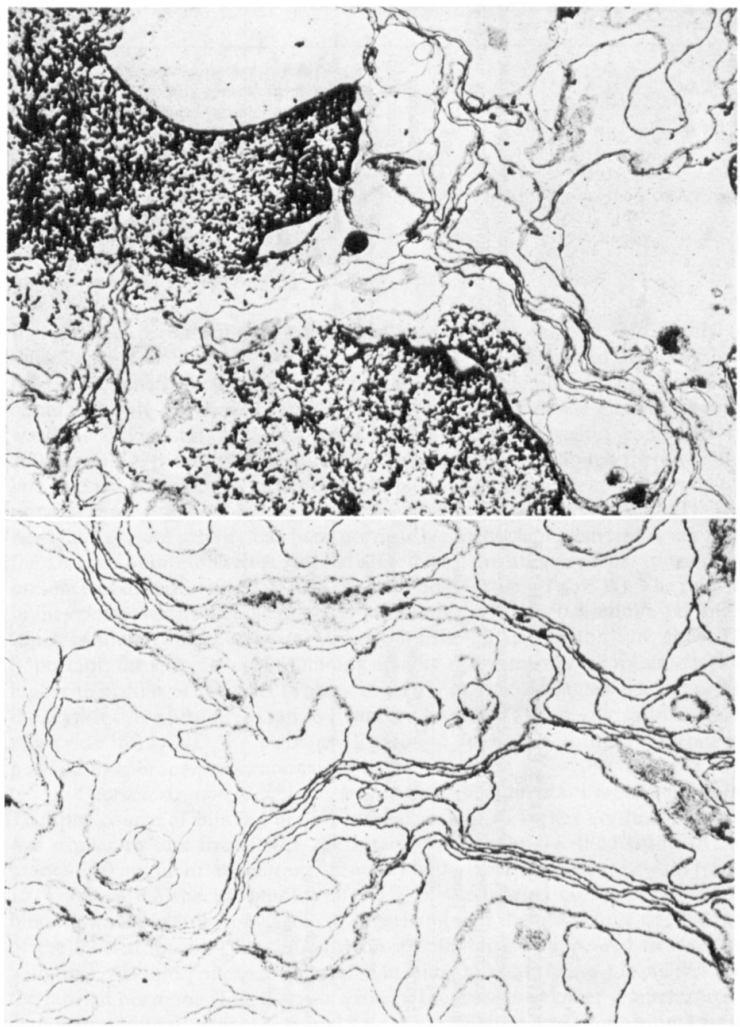

Fig. 1. Electron micrographs of turkey erythrocyte preparations. Turkey erythrocytes were lysed and washed, then homogenized and purified as described in Materials and Methods. Pellets of the washed lysate and purified membranes were fixed in glutaraldehyde. The top panel is the washed lysate and the bottom panel is the purified membranes. Original magnification × 10 000.

TABLE I

PURIFICATION OF TURKEY ERYTHROCYTE MEMBRANES

Erythrocytes, wash lysates and membranes were prepared as described in Materials and Methods. Total insulin-binding capacity was determined by Scatchard analysis [11], as described in Fig. 6. Specific binding activity was the insulin bound divided by the protein concentration. Yield (insulin bound times volume) was normalized to 100 % for the intact cells.

Preparation	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Insulin-binding capacity		Receptor purification (-fold)	Receptor yield (%)
				(pmol/ml)	(pmol/mg protein)		
Turkey erythrocytes	40	75	3000	4.5	0.06	1	100
Washed lysate	20	5.1	100	7.0	1.4	23	77
Membrane	20	1.0	20	6.0	6.0	100	67

TABLE II

CHARACTERISTICS OF TURKEY ERYTHROCYTE MEMBRANES

Parameter	Method of preparation	
	Present method	Aurbach method
Insulin binding (pmol/mg membrane protein)	6	0
Catecholamine binding (pmol/mg membrane protein)	0.3[a]	0.3[b]
Basal adenylate cyclase (pmol/10 min per mg)	0	12[b]
(Na^+, K^+)-ATPase (μmol P_i/h per mg membrane protein)	0.8[c]	0.7[d]
DNA (μg/pmol insulin-binding capacity)	1.4	–

[a] Kindly performed by Dr. E. Brown and Dr. G. Aurbach by the method of Aurbach and coworkers [3, 6].
[b] From Bilezikian and Aurbach [3]
[c] Kindly performed by Dr. J. Wolff [8].
[d] Value for human erythrocytes [7].

the purified preparation contained only membrane vesicles. No highly refractile nuclei were seen. Similarly electron micrographs (Fig. 1) showed that while the washed lysate contained nuclei enveloped by the plasma membrane, the purified fraction contained only membrane vesicles. The DNA content of the membrane preparation was low, about 1.4 μg DNA/pmol insulin-binding capacity; compared to about 1000 μg DNA/pmol insulin bound for the intact cells. Although there was no adenylate cyclase activity, the catecholamine binding of these membranes was about the same as those prepared by the method of Aurbach et al. (ref. 6, Table II). The loss of adenylate cyclase activity has been previously noted when membranes are prepared in the absence of dithiothreitol. On the other hand, membrane preparations made in the presence of dithiothreitol (20 mM) did not bind insulin (Table II). The purified membranes possess considerable ouabain-inhibitable ATPase, 0.8 μmol/h per mg protein, which is similar to the value obtained with the human erythrocyte ghost [7]. Most important, the specific insulin-binding activity of the membrane fraction was 100-fold higher than that of the cells (Tables I and II) and 5-fold higher per mg protein than the erythrocyte ghost preparation ("washed lysate"). The specific insulin-binding activity (vide infra) was the same as that found for other insulin receptors of highly purified membrane preparations.

Characterization of ^{125}I-labeled insulin binding to turkey erythrocyte membranes. The time course of binding of ^{125}I-labeled insulin to turkey erythrocyte membranes was similar to that found with the intact erythrocyte [1]. Incubation of the membranes (80 μg/ml of membrane protein) with labeled insulin (24 pM) for 2 h at 15 °C led to the specific binding of 2.5 % of the labeled hormone (Fig. 2). Specific binding was constant from 2 to 5 h, suggesting that there was little or no degradation of the labeled insulin (vide infra). Non-specific binding appeared to increase slowly with time. Addition of excess unlabeled insulin (340 nM) led to a rapid dissociation of the labeled hormone (Fig. 2, lower panel). Incubation at other temperatures (Fig. 3) demonstrated that, while the initial rate of association was proportional to temperature, the level of steady-state binding was higher at 4 and 15 °C than at 37 °C. The lower level of steady-state binding at 37 °C was also found with the whole cells. The difference in steady-state binding, i.e. increased binding at lower temperatures, was

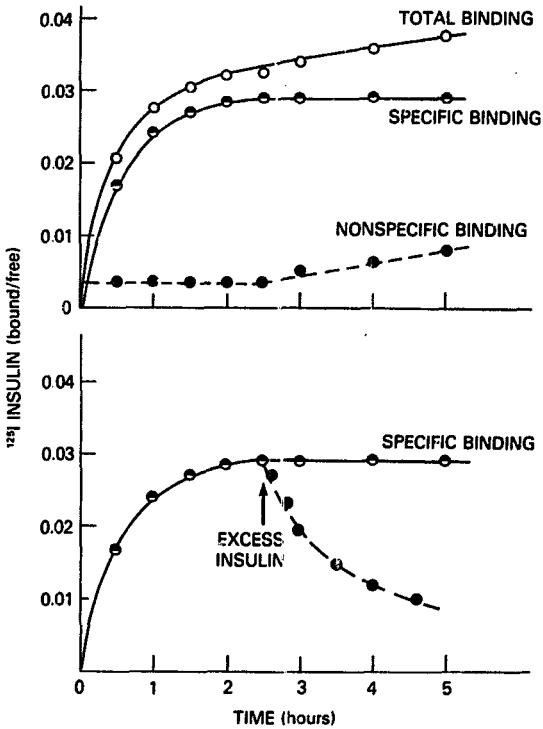

Fig. 2. Time course of binding of ^{125}I-labeled insulin to turkey erythrocyte membranes. Upper panel: ^{125}I-labeled insulin (18 pM) was incubated at 15 °C in 5 ml with 400 μg of membrane protein, with and without unlabeled insulin (340 nM). Duplicate 200-μl aliquots were withdrawn from each at the indicated times and sedimented, and the ^{125}I-labeled insulin bound to the pellet was determined (see Materials and Methods). Lower panel: ^{125}I-labeled insulin was incubated with membrane in the absence of unlabeled insulin, as described above. After incubation for 2.5 h at 15 °C, excess unlabeled insulin (340 nM) was added. Duplicate 200-μl aliquots were withdrawn and treated as described above. The specific binding decreased after addition of the unlabeled insulin (O—O).

related to the marked temperature dependence of dissociation (Fig. 4). At 37 °C the dissociation of ^{125}I-labeled insulin from membranes was almost complete in 15 min, while at 0 °C less than 25 % of the label dissociates after 2 h of incubation.

The amount of ^{125}I-labeled insulin bound at steady state was proportional to the membrane concentration. At membrane protein levels of 600 μg/ml, the bound/free ratio of insulin was 0.2 (Fig. 5) and this linear relationship of binding to membrane concentration has been shown up to a bound/free ratio of 0.8 with a membrane concentration of 2.4 mg/ml (data not shown).

The specificity and relative affinity of the insulin receptor of the erythrocyte membrane for polypeptide hormones was similar to that seen with the intact cells (Fig. 6). Half maximal displacement of ^{125}I-labeled insulin by porcine insulin

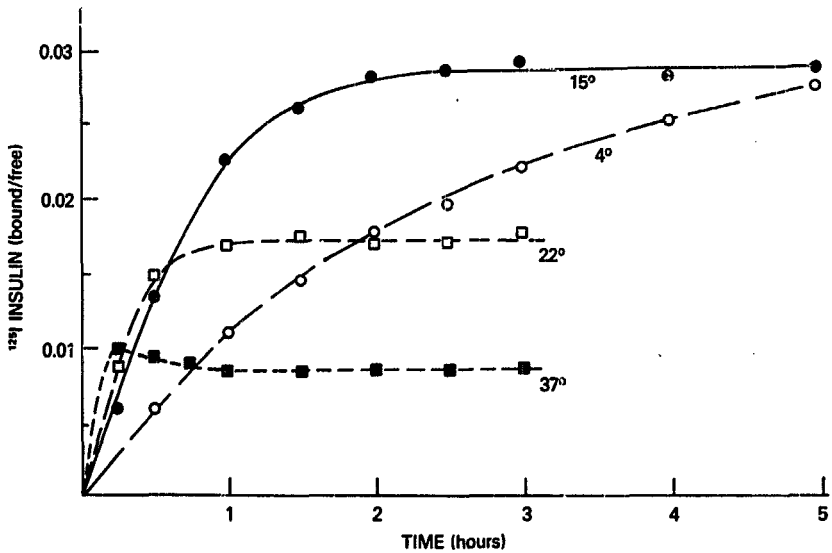

Fig. 3. Association of insulin with turkey erythrocyte membranes as a function of temperature. ^{125}I-labeled insulin (18 pM) was incubated with 400 μg of membrane protein with and without excess unlabeled insulin (340 nM), in a total volume of 5 ml at 4, 15, 22 and 37 °C. Duplicate 200-μl aliquots, withdrawn at the indicated times, were sedimented and specific binding was determined as described in Materials and Methods.

occurred at 4 ng/ml (0.7 nM), by proinsulin at 26 ng/ml (2.9 nM), by desalanine-desasparagine insulin at 300 ng/ml (55 nM) and by multiplication stimulating activity at 200 ng/ml (20 nM). In addition, chicken insulin, which has twice the biological activity of porcine insulin [10], displaced ^{125}I-labeled insulin with twice the potency of porcine insulin. Although all of these concentrations were about one half the amount required to get half maximal displacement of ^{125}I-labeled insulin on the intact erythrocytes, the relative potencies of 1 : 0.15 : 0.013 : 0.013 found with insulin, proinsulin, desalanine-desasparagine insulin and multiplication stimulating activity with membranes were almost identical to the values found with intact cells and bioassay in vitro [9]. Polypeptide hormones unrelated to insulin, such as adrenocorticotropin, somatotropin and glucagon did not compete with ^{125}I-labeled insulin for receptor binding.

Quantitative analysis and cooperativity. The Scatchard plot [11] of insulin binding to the turkey erythrocyte membrane, like that with the intact erythrocytes, was curvilinear (Fig. 7). In the turkey erythrocyte [1] and other cells [12, 13] this curvilinearity has been shown to be due to site-site interactions of the negative cooperative type. Experiments to directly demonstrate negative cooperativity by kinetic methods have been designed and discussed by De Meyts et al. [13]. In the present study, we find that the insulin receptor of the turkey erythrocyte membrane has negatively cooperative site-site interactions. Thus the dissociation rate of ^{125}I-labeled insulin from the membrane after dilution from steady state was increased by

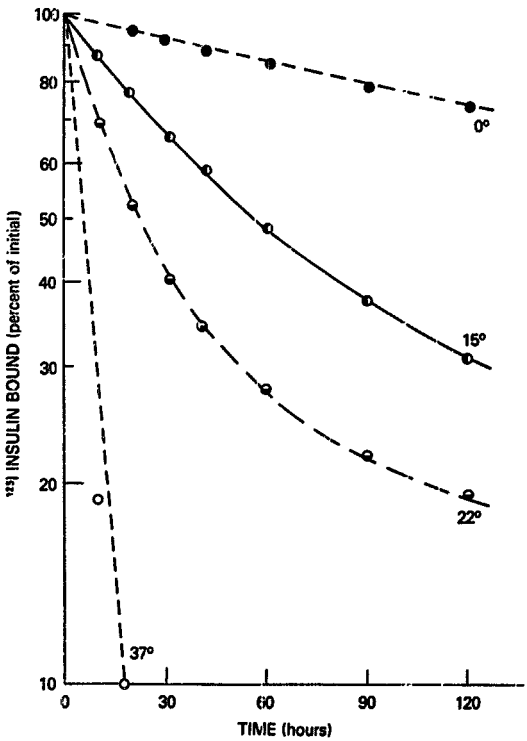

Fig. 4. Dissociation of insulin from the turkey erythrocyte membrane as a function of temperature. ^{125}I-labeled insulin (18 pM) was incubated for 2.5 h in 5 ml with 400 μg of membrane protein at 15 °C. Aliquots of the incubation mixture were then placed in water baths at 37, 22, 15 and 4 °C and unlabeled insulin was added to a concentration of 340 nM. Duplicate 200-μl aliquots were withdrawn at the indicated times and the ^{125}I-labeled insulin bound determined as described in Materials and Methods.

insulin over that caused by dilution alone (Fig. 3). The dependence of this cooperative phenomenon on the insulin concentration was identical for the membrane (Fig. 9) and the intact erythrocyte [1].

The specific binding capacity of the membrane obtained from the Scatchard plot (Fig. 7) was 300 fmol per 50 μg protein, or 6 pmol/mg membrane protein. This value is similar to that found with the highly purified rat and mouse liver membranes [12, 14] isolated by the procedure of Neville [15].

Influence of ionic environment on insulin binding. The dependence of insulin binding on ionic environment was similar for the turkey erythrocyte membrane and the intact cell [1]. The pH optimum of 7.8 (Fig. 10) was the same for both and both showed a marked decrease in binding at lower and higher pH. Cations also influenced

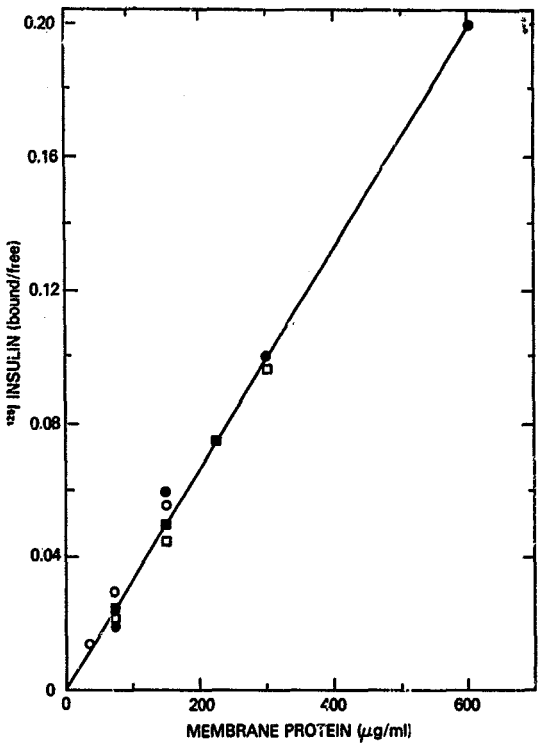

Fig. 5. Effect of membrane concentration on binding. ^{125}I-labeled insulin (15–25 pM) was incubated at 15 °C in 0.5 ml with the indicated amount of membrane protein, with and without unlabeled insulin (340 nM). After 2.5 h duplicate 200-μl aliquots were withdrawn and the ratio of specifically bound insulin/free insulin determined as described in Materials and Methods. Different preparations of membrane are represented by different symbols.

the amount of steady-state binding to the membrane, but to a lesser degree than was found with the intact cell [1]. Ca^{2+} and Mg^{2+} increased binding by 50 and 35 %, respectively. Maximal effects were seen at 8 mM. Mn^{2+} decreased binding to about 55 % of control at 20 mM (Fig. 11).

Degradation of insulin and loss of receptor. The purified turkey erythrocyte membrane demonstrated only a very small amount of insulin-degrading activity. After a 2 h incubation of ^{125}I-labeled insulin with membranes at 4, 15, 22 or 37 °C there was less than 3 % increase in trichloroacetic acid solubility and less than 4 % loss in the amount which could be bound by intact cells (Fig. 12B). In contrast, receptor loss did occur. Preincubation of membranes in the absence of insulin led to a decrease in the amount of ^{125}I-labeled insulin which could subsequently be bound to the membranes (Fig. 12A). After incubation of membranes for 2 h at 37 °C, about 35 % of the binding activity was lost; under the normal incubation conditions of 15 °C

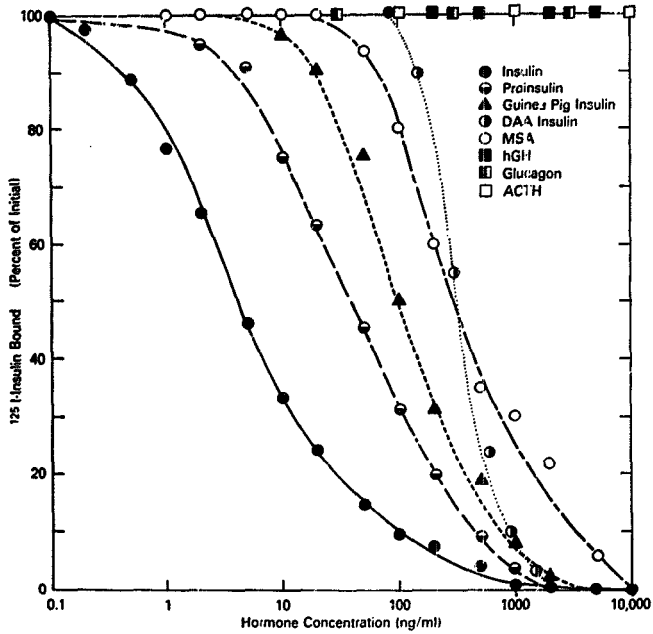

Fig. 6. For legend see opposite page.

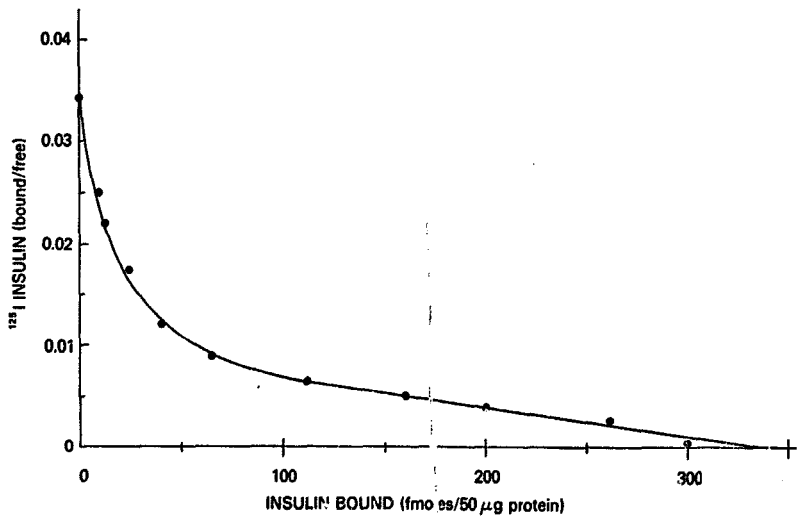

Fig. 7. For legend see opposite page.

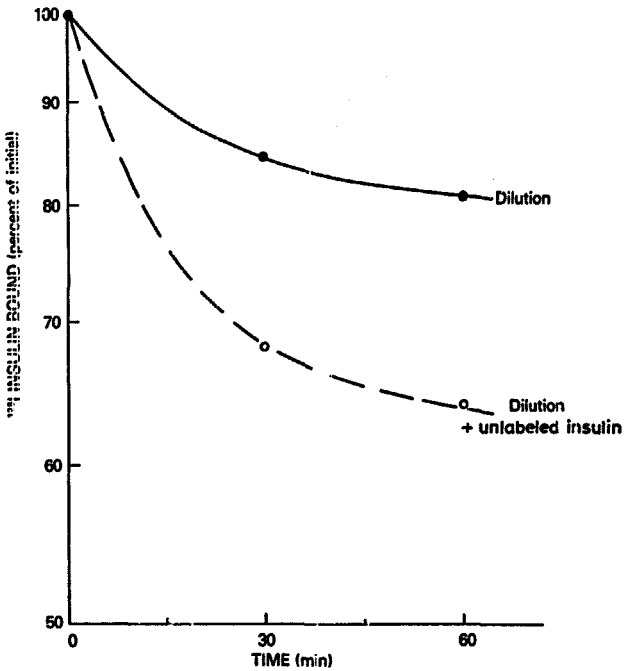

Fig. 8. Effect of insulin on rate of dissociation of ^{125}I-labeled insulin from membranes. ^{125}I-labeled insulin (20 pM) was incubated at 15 °C in 3 ml with 300 μg/ml of membrane protein. After 2.5 h, 100 μl were diluted to 10 ml with buffer or buffer plus 1 μg/ml of unlabeled insulin. This dilution was performed into capped SW 27 tubes (cellulose nitrate, Beckman). After the indicated incubation time at 15 °C the entire tube was centrifuged at 20 000 × g for 2 min in a Sorvall SS-2 at 4 °C. The supernatant was removed and the bottom of the tube, including the small pellet was cut off and counted. The residual radioactivity in the pellet was expressed as a percentage of that found immediately after dilution.

Fig. 6. Competition for ^{125}I-labeled insulin binding by insulin analogues and other polypeptide hormones. ^{125}I-labeled insulin (25 pM) was incubated at 15 °C in 0.5 ml with 150 μg of membrane protein and the indicated amount of each of the polypeptide hormones (Chicken insulin kindly supplied by Dr. J. Simon). After 2.5 h duplicate 200-μl aliquots were withdrawn and the specific bound/free ratio determined as described in Materials and Methods. Results are expressed as a percentage of the bound/free ratio found in the presence of labeled insulin alone (bound/free = 0.06). The steeper slope of displacement by desalanine-desasparagine (DAA) insulin was expected since this analogue does not induce site-site interactions.

Fig. 7. Scatchard analysis of insulin binding to turkey erythrocyte membranes. ^{125}I-labeled insulin (18 pM) was incubated at 15 °C in 0.5 ml with varying concentrations of unlabeled insulin. After 2.5 h duplicate 200-μl aliquots were withdrawn and the bound/free ratio determined as described in Materials and Methods. The bound insulin was determined by multiplying the fraction of ^{125}I-labeled insulin specifically bound by the total insulin concentration.

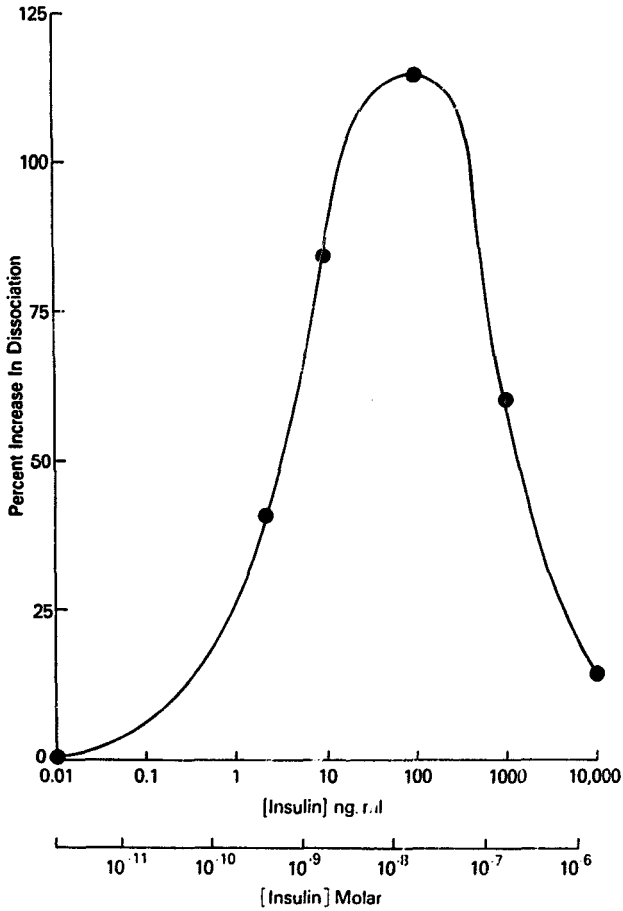

Fig. 9. Effect of insulin concentration on cooperativity. Experimental conditions were identical to those of Fig. 8 except that the amount of unlabeled insulin in the dilution medium was varied. The data are expressed as the percent increase in ^{125}I-labeled insulin dissociation at 30 min after dilution [13].

only 10 % of the binding activity was lost. Attempts to recover the lost activity in the supernatant were unsuccessful.

DISCUSSION

Purified plasma membrane of the avian erythrocyte binds insulin with a specific activity 100-fold higher than intact cells. The membrane purification scheme is

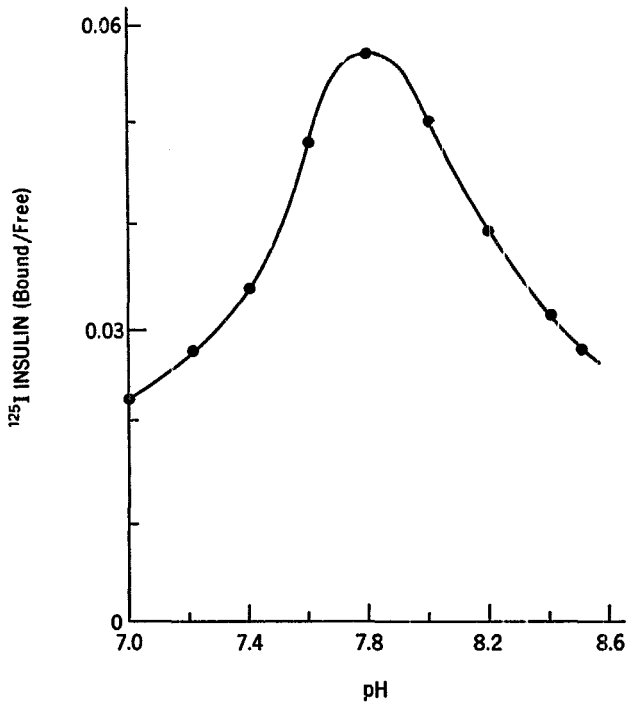

Fig. 10. Effect of pH on insulin binding. The buffer consisted of 50 mM Tris · HCl, 50 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid · HCl, 60 mM NaCl, 10 mM glucose, 1 mg/ml bovine serum albumin, with the pH adjusted as indicated. ^{125}I-labeled insulin (18 pM) was incubated at 15 °C with 150 μg membrane protein in 0.5 ml, with and without unlabeled insulin (340 mM). After 2.5 h duplicate 200-μl aliquots were withdrawn and the bound/free ratio determined as described in Materials and Methods.

technically easy and the yield of insulin receptor high. The simplicity of the isolation is possible because the plasma membrane need be separated from only two other major components: hemoglobin, which is removed by hypotonic lysis, and nuclei, which are extruded by Dounce homogenization. Magnesium at 2 mM is included in the hypotonic lysis buffer to prevent agglutination of the cellular ghosts, and the Mg^{2+} concentration lowered to allow better extrusion of the nuclei by the Dounce homogenizer. The membranes thus purified contain no nuclei in phase contrast or electron microscopy and only 0.1 % of the initial cellular DNA. These membranes also show high specific binding of insulin and catecholamines. This purification method is similar to that of Bilezikian and Aurbach [3]. However, the high dithiothreitol levels used in their method resulted in a loss of insulin binding.

The insulin receptor of the purified membranes appears to be identical to that of the whole cell in its affinity for insulin, specificity for insulin analogues, kinetics of

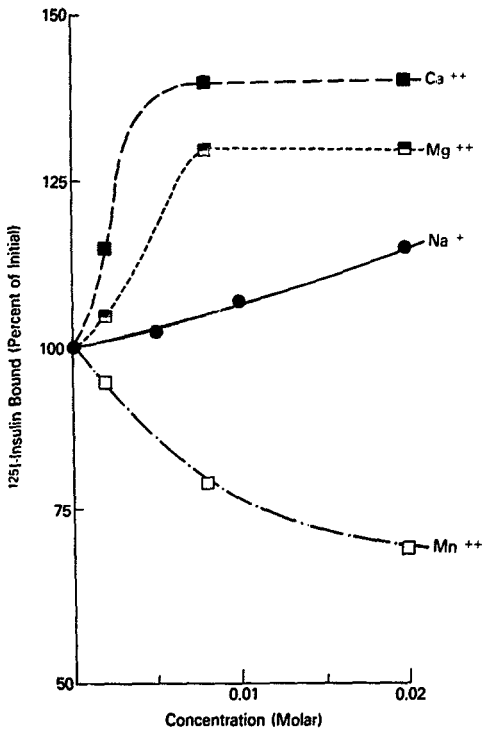

Fig. 11. Effect of cations on insulin binding. ^{125}I-labeled insulin (15 pM) was incubated for 2.5 h at 15 °C with 50 μl of membrane protein. The basic buffer consisted of 50 m*M* Tris · HCl, pH 7.8, 10 mM glucose and 1 mg/ml bovine serum albumin. The salt concentration of the mixture was varied by addition of the chloride salts of the cations. Sucrose was also added to maintain a constant osmolality of 260 mosM. The specific bound/free ratio was determined in the presence of the added cation and expressed as a percentage of the bound/free with buffer alone.

association and dissociation, cooperativity between receptor sites, and effects of pH on binding. These binding properties were also identical to those seen with other cells such as the cultured human lymphocyte [16], cultured human placental cell [21] and circulating human monocyte (Bar, R. S., personal communication) and with other membrane preparations, such as the rat and mouse liver membrane [11, 13]. Thus the avian insulin receptor found on erythrocyte plasma membranes retains the binding properties found in the whole cell and those common to all insulin receptors.

In the purification of polypeptide hormone receptors, membrane purification is a critical first step, since pure preparations with high specific binding activity facilitate further receptor purification. Various membrane preparations have been used as an initial step in receptor purification. Most workers, however, have used a simple "microsomal" fraction prepared by differential centrifugation. Such preparations

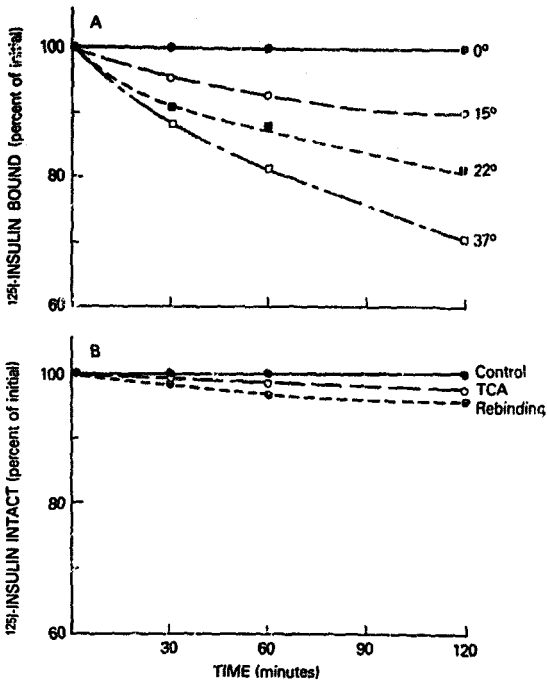

Fig. 12. (A) Loss of insulin receptors. Erythrocyte membranes (500 μg of protein) were preincubated in 4.5 ml at the indicated temperatures. At the indicated times two aliquots of 0.5 ml were withdrawn from each. ^{125}I-labeled insulin (18 pM) was added to both and unlabeled insulin (340 mM) to one. After an incubation of 2.5 h the bound/free ratio was determined and expressed as a percentage of the bound/free found after no preincubation. (B) Degradation of insulin by membranes. ^{125}I-labeled insulin (18 pM) was incubated at 37 °C in 3.5 ml, with and without 300 μg/ml of membrane protein. At the indicated times a 0.75 ml aliquot was withdrawn and sedimented at 10 000 × g for 5 min. The percent intact ^{125}I-labeled insulin in the supernatant was determined by trichloroacetic acid (TCA) precipitation and rebinding, as previously described [12].

have low specific binding activity. Thus, Cuatrecasas [17], attempting to purify the insulin receptor of rat liver, began with a particulate fraction which had a specific insulin binding capacity of only 0.15 pmol/mg protein; Dufau and co-workers [18], studying the gonadotropin receptor of rat ovaries used a particulate fraction which had a specific gonadotropin-binding capacity of 0.15 pmol/mg; and Shiu [19], studying the prolactin receptor of rabbit mammary tissue, used a particulate fraction which had a specific prolactin-binding capacity of only 0.05 pmol/mg. In contrast to these impure preparations, rat liver plasma membranes, purified by the Neville procedure [15] have a specific insulin-binding capacity of 3 pmol/mg and the turkey erythrocyte membrane also has a high specific binding activity for insulin of 6 pmol/mg. These values are 20–40-fold higher than that found by Cuatrecasas in the crude liver microsomes and are comparable to the level of purity that was found after Triton

X-100 solubilization, ammonium sulfate fractionation and DEAE-cellulose chromatography of the rat liver insulin receptor [17]. Similarly, the soluble glucagon receptor did not reach this level of specific binding activity until several steps of purification [20].

In summary, we have purified the plasma membrane of the turkey erythrocyte and have shown the membrane-bound insulin receptor to be identical to that found in the intact cells. This highly purified membrane preparation will now serve as a source of insulin receptors for further purification.

ACKNOWLEDGEMENTS

We wish to thank Dr. David Neville, Jr. for his helpful discussion and Mrs. Dorothy E. Beall for secretarial assistance.

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