Characterization of $^{125}$I-Glucagon Binding in a Solubilized Preparation of Cat Myocardial Adenylate Cyclase

FURTHER EVIDENCE FOR A DISSOCIABLE RECEPTOR SITE*

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SUMMARY

A solubilized preparation of cat myocardium, which contains adenylate cyclase, has been shown to specifically bind biologically active $^{125}$I-glucagon. $^{125}$I-glucagon binding was observed over the concentration range $1 \times 10^{-7}$ to $1 \times 10^{-5}$ M. Unlabeled glucagon displaced $^{125}$I-glucagon over a similar concentration range. The binding specificity of this preparation was also shown by the fact that parathyroid hormone did not bind to cardiac receptor sites in this preparation nor did it displace $^{125}$I-glucagon from its binding site. The binding of $^{125}$I-glucagon at 37 or 25°C is linear for approximately 30 min until maximum binding is reached. In contrast, the activation of adenylate cyclase is maximal within 5 min, indicating the presence of additional glucagon binding sites over and above those required for activation of the enzyme. The binding material was stable at 4°C for 4 days and indefinitely when stored in liquid nitrogen. Boiling the binding material for 15 min or incubating it with 1 mM HCl for 15 min destroyed most of its glucagon-binding ability. Optimal binding was observed over a broad pH range from 3.6 to 8.5, with a decline above pH 9.0. Preincubation of the binding material with trypsin decreased binding about two-thirds. Phospholipases A and C, DNase, RNase, neuraminidase, urea (1 M), GTP, and ATP were without effect on the binding.

Solubilized myocardial adenylate cyclase has been shown to have a molecular weight of about 100,000 to 200,000. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the crude solubilized preparation incubated with $^{125}$I-glucagon suggested a molecular weight for the binding fraction of approximately 26,000. Chromatography of the $^{125}$I-glucagon-receptor complex on either Sephadex G-100 or Bio-Gel P-30 produced a dissociation of the receptor from catalytic adenylate cyclase activity, which apparently represents a larger molecular weight component (> 100,000). The $^{125}$I-glucagon-receptor complex eluted from the gels at an elution volume consistent with the salt peak. However, this eluate was shown by hydrodynamic flow electrophoresis to be neither free glucagon or iodine. Moreover, when it was subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide gels, the complex had a molecular weight of about 26,000, identical with what was obtained with the crude material. It appeared, therefore, that the complex of glucagon to its receptor had a strong positive charge which resulted in adsorption of the complex to the gel. Following neutralization of the charge-gel interaction by Sephadex and Bio-Gel chromatography of the $^{125}$I-glucagon-receptor complex in 0.01 or 0.025 M NaOH, the binding fraction eluted in a more appropriate location for its apparent size. The property of adsorption to Sephadex G-100 and Bio-Gel P-30 may prove useful in purification of the glucagon receptor site.

The interaction of hormones with the membrane-bound enzyme adenylate cyclase resulting in increased intracellular levels of adenosine 3',5'-monophosphate has been the subject of intensive study in many tissues (1). Current evidence pertinent to the heart suggests that cyclic AMP mediates the inotropic and chronotropic effects of several hormones including the catecholamines (2), glucagon (3-5), histamine (6), and thyroxine and triiodothyronine (7). It is thought that the initial step in hormone-induced activation of adenylate cyclase is binding to a cell membrane receptor site (8, 9). Lefkowitz and his co-workers have demonstrated catecholamine binding to specific β adrenergic receptor sites in a preparation of microsomes from dog heart (10-12). In addition, the binding process has been shown to be separate and distinct from the activation of adenylate cyclase (13, 14). Using solubilized preparations of cat heart, we have demonstrated a critical requirement for acidic phospholipids in

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The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; SDS, sodium dodecyl sulfate.
the activation process following binding. Phosphatidylserine was shown to be required for the glucagon and histamine activation of the detergent-free myocardial adenylate cyclase (15, 16) and monophosphatidylinositol for the catecholamine activation (17). Recently we reported preliminary data concerning the binding of 125I-glucagon to myocardial receptors (18) and demonstrated a glucagon binding site (mol wt approximately 24,000 to 28,000) which was dissociable from the larger molecular weight (mol wt greater than 100,000) catalytic subunit of the adenylate cyclase (19). The purpose of the present investigation was to more fully characterize the binding of 125I-glucagon in this solvable preparation of cat heart and to provide further information relative to the dissociable receptor site.

EXPERIMENTAL PROCEDURES

Methods

Solubilization of Cardiac Muscle—Normal cats were anesthetized with pentobarbital, 20 to 35 mg per kg intraperitoneally, the heart was quickly excised, and the left ventricle was dissected free of endocardium and epicardium. About 500 mg of muscle were homogenized in 4.5 ml of a cold solution containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.7), 20 mM Lubrol-PX, and 1 mM EDTA-magnesium chloride (20, 21). The homogenate centrifuged at 12,000 x g for 10 min at 4° and the supernatant was used for the binding and chromatography experiments described below. Iodination of Glucagon—Glucagon binding to the solubilized myocardial receptor was determined using 125I-labeled glucagon. Glucagon was iodinated by a modification of the procedure of Hunter and Greenwood (22). The following solutions were added successively into a flint glass tube (10 X 75 mm): 20 μl of NaPO₄, 0.6 M, pH 7.4; 10 μl (55 μg) of crystalline glucagon; 3.5 mg per ml of 0.6 M HCl; 2.5 to 4.0 mCi of 125I; 10 μl of chlorpromazine; 3.5 mg per ml of 0.05 M NaPO₄, pH 7.0; and 25 μl of sodium metabisulfite; and 2.4 mg per ml of 0.05 M NaPO₄, pH 7.0. Five microliters of the iodination mixture are then added to a solution of 100 μl of Veronal buffer, 0.1 M, pH 8.6, and 15 μl of plasma containing bromphenol blue. This mixture is applied to Whatman No. 3MM paper and subjected to electrophoresis for 90 min at 25°, dried, and stripparried on a Nuclear Chicago Actigraph to determine the relative efficiency of glucagon iodination. The iodinations generally are 90 to 95% complete, the remainder being free iodine. 125I-glucagon was purified on a column of cellulose powder as described by Rodbell et al. (23). The 125I-glucagon was applied to a cellulose column (2.5-cm) prepared in a Pasteur pipette (0.6-cm diameter) and prewashed with 1% albumin in 10 mM sodium phosphate, pH 7.5. The iodinated glucagon is applied and the column is washed with 3.0 ml of a solution of 1% albumin in 10 mM sodium phosphate adjusted to pH 7.5 and then eluted with 0.6 ml of the same solution adjusted to pH 10.0 with concentrated ammonium hydroxide. The 125I-glucagon eluted in this manner was biologically active as determined by its ability to activate the particulate myocardial adenylate cyclase.

125I-Glucagon-binding Assay—The specific fractions of solubilized enzyme referred to in the text were incubated at 37° in a final volume of 100 μl containing 1.0% albumin in 10 mM Tris-HCl, pH 7.7, and 125I-glucagon (0.25 mCi per pmole). EDTA is not required for the binding of 125I-glucagon in this system. After the appropriate time interval, the incubation mixture was added to dry cellulose columns (2.5 cm) in disposable Pasteur pipettes with inside diameters of 0.6 cm and washes with 1.4 ml of 1% albumin in 10 mM Tris-HCl, pH 7.7. Bound 125I-glucagon did not adsorb to the column whereas free (unbound) 125I-glucagon did. The eluate was then counted in a Nuclear Chicago Auto-Gamma. This method removes more than 90% of the free (unbound) 125I-glucagon as determined by the number of counts found in the control samples of identical composition incubated simultaneously using 10 μl Tris-HCl, pH 7.7, in place of the enzyme fraction. Similar control samples are obtained from incubations in the absence of the solubilized binding fraction. Bovine serum albumin, recrystallized four times, was used at all steps in the iodination and binding experiments since it was observed that blank values were lower and stability with storage vastly superior to bovine serum albumin (Fraction V). Protein was measured by the method of Lowry et al. (24) using bovine serum albumin as a standard.

Materials

Carrier-free Na125I (in 0.1 N NaOH) was obtained from Union Carbide Co. [α-32P]ATP was purchased from International Chemical Nuclear Corp. Crystalline glucagon was a gift of Eli Lilly and Co. Lubrol-PX was a gift of I.C.I. America, Inc. DEAE-cellulose (DE52) was obtained from Reich-Angel. Sephadex G-100 was from Pharmacia. Bio-Gel P-30 was obtained from Bio-Rad. Bovine serum albumin, recrystallized four times, was purchased from Nutritional Biochemicals. Flint glass tubes (10 X 75 mm) were purchased from Kimble.

RESULTS

Biological Activity of 125I-Glucagon—Rodbell et al. showed that glucagon labeled with 125I retained biological activity as determined by its ability to activate particulate liver adenylate cyclase (23). 125I-Glucagon, prepared in a similar manner to that described by these workers activates the particulate myocardial adenylate cyclase. The solubilized myocardial adenylate cyclase, freed of detergent by DEAE-cellulose chromatography is unresponsive to glucagon unless phosphatidylserine is added to the incubation mixture (15, 16). Since the binding studies in this investigation utilized the solubilized heart preparation, it seemed important to demonstrate that the 125I-glucagon would activate the detergent-free solubilized adenylate cyclase. Fig. 1 shows that 125I-glucagon at 1 X 10⁻⁶ M activates the solubilized myocardial adenylate cyclase in the presence of phosphatidylserine and is, therefore, biologically active in the context of this particular system.

125I-Glucagon Binding to Solubilized Myocardial Preparation—125I-Glucagon binds to the solubilized preparation of myocardium both in the presence and absence of detergent (Fig. 2). The binding is concentration-related over the range 1 X 10⁻⁹ to 1 X 10⁻⁵ M, half-maximal binding occurring at about 5 X 10⁻⁷ M in both. The addition of unlabeled glucagon to the incubation produces a displacement in the amount of 125I-glucagon bound...
Binding of $^{125}$I-glucagon to solubilized myocardium in the presence and absence of detergent. Approximately 300 mg of cat heart muscle were solubilized in a solution containing 20 mM Lubrol-PX as described under "Methods." The homogenate was centrifuged at 12,000 × g for 10 min at 4°C. Approximately 1.3 ml of the supernatant fluid (protein concentration, 3.5 mg per ml) were applied to a DEAE-cellulose column (1.0 × 12.0 cm) equilibrated at 4°C with 10 mM Tris-HCl, pH 7.7. The flow rate was approximately 0.2 ml per min. The column was washed with 15 to 20 volumes of 10 mM Tris-HCl, pH 7.7. The protein fraction containing the glucagon-binding activity and adenylate cyclase was eluted with 1 M Tris-HCl, pH 7.7. This fraction has been shown to be free of detergent (21). The incubation conditions for $^{125}$I-glucagon binding and separation of bound from free $^{125}$I-glucagon have been described in the text. Each value represents the mean ± S.E. of five experiments from five cats.

In order to further demonstrate the specificity of glucagon binding in this system, we examined the effectiveness of another polypeptide hormone, parathyroid hormone, in displacing bound glucagon. Parathyroid hormone did not displace $^{125}$I-glucagon. Furthermore, specific binding of $^{125}$I-parathyroid hormone to the solubilized heart preparation could not be demonstrated.

Binding of $^{125}$I-Glucagon as Function of Time and Temperature—The binding of $^{125}$I-glucagon at 25 or 37°C is linear for approximately 30 min until maximal binding is achieved (Fig. 4). At lower temperatures, 18 and 0°C, binding also occurs although at slower rates, the maximum not being reached after 120 min of incubation.

Correlation of $^{125}$I-Glucagon Binding with Adenylate Cyclase Activation—As noted in Fig. 5, glucagon binding and activation of the particulate heart adenylate cyclase are closely correlated in terms of molar concentrations. However, maximal activation of the enzyme occurs within 5 min in both the particulate and solubilized (in the presence of phosphatidylserine) systems, whereas binding is only approximately 15 to 20% complete at this time point. This suggests the presence of an excess of binding sites over and above what is required for the activation of the adenylate cyclase.

Stability of $^{125}$I-Glucagon Binding Site—We determined the stability of the binding material in the solubilized preparation of myocardium (Table I). The ability of the material to bind glucagon appears to be unaffected by successive freezing and thawing and by storage at 4°C for 4 days. The material is stable.
FIG. 5. Correlation of 125I-glucagon binding with adenylate cyclase activation. The conditions for 125I-glucagon binding and assay of adenylate cyclase have been described in the text. Each adenylate cyclase incubation contained phosphatidylserine, 128 µg per ml. Phosphatidylserine was not added to the 125I-glucagon binding tubes since we had previously demonstrated that phosphatidylserine is not required for the binding of glucagon in this system (18). Labeled glucagon was present at a final concentration of 5 × 10⁻⁷ M. Each value represents the mean ± S.E. of three samples for the adenylate cyclase experiments and the mean of duplicate samples for the 125I-glucagon binding experiments.

Effect of pH—Optimal binding of 125I-glucagon was observed over a very broad pH range from 3.6 to 8.5 (Fig. 6). A decrease in binding occurred above pH 9.1.

Effect of Enzymes—The solubilized preparation of myocardium was incubated with a variety of enzymes in order to obtain information relative to the chemical nature of the binding material (Table II). Trypsin produced a 66% decrease in binding, indicating that the binding site was, at least partially, a protein. Neuraminidase, which cleaves sialic acid and hence is destructive of some glycoproteins was without effect. Phospholipase A and C, DNase, and RNase were also without effect.

Effect of Urea—Urea has been shown to decrease the binding of 125I-glucagon to liver membranes (23), 1 M urea producing approximately a 75% decrease in binding. Urea, 1 M, did not alter the binding of 125I-glucagon in the solubilized myocardial preparation (Table III).

TABLE I
Stability of glucagon binding site in solubilized preparation of myocardium

The solubilized extract, in the presence of detergent, was used for all incubations. Approximately 150 µg of extract protein were added to each incubation under the conditions described in the text. Each value represents the mean of duplicate samples.

| Solubilized extract | 125I-glucagon bound | pmoles/mg protein/60 min |
|---------------------|---------------------|--------------------------|
| Control             |                     | 4.0                      |
| 4°C, 96 hours       |                     | 3.6                      |
| Freezing and thawing four times | | 4.2 |
| 100°C, 15 min       |                     | 0.5                      |
| 1 N HCl, 37°C, 15 min |                 | 1.1                      |

indefinitely when stored in liquid nitrogen. Approximately 25% of the binding activity remains after preincubation with 1 N HCl for 15 min at 37°C and about 15% remains after boiling the preparation for 15 min.

Effect of Enzymes—The solubilized preparation of myocardium was incubated with a variety of enzymes in order to obtain information relative to the chemical nature of the binding material (Table II). Trypsin produced a 66% decrease in binding, indicating that the binding site was, at least partially, a protein. Neuraminidase, which cleaves sialic acid and hence is destructive of some glycoproteins was without effect. Phospholipase A and C, DNase, and RNase were also without effect.

Effect of Urea—Urea has been shown to decrease the binding of 125I-glucagon to liver membranes (23), 1 M urea producing approximately a 75% decrease in binding. Urea, 1 M, did not alter the binding of 125I-glucagon in the solubilized myocardial preparation (Table III).

Table II
Effect of enzymes on 125I-glucagon binding in solubilized preparation of myocardium

Aliquots of the solubilized myocardial extract containing approximately 300 µg of protein were incubated with 60 µg of trypsin for 30 min at 37°C followed by addition of 60 µg of trypsin inhibitor for 5 min at room temperature. Other aliquots containing 300 µg of protein were incubated with 25 units of neuraminidase. An aliquot of these mixtures containing 150 µg of protein was then incubated with 125I-glucagon for 60 min as described in the text. RNase and DNase were added directly to the incubations at final concentrations of 0.6 and 60 units per ml, respectively.

| Solubilized extract | 125I-glucagon bound | pmoles/mg protein/60 min |
|---------------------|---------------------|--------------------------|
| Control             |                     | 3.0                      |
| Trypsin             | + Trypsin           | 3.0                      |
| Neuraminidase       | + Neuraminidase     | 3.0                      |
| RNase               | + RNase             | 3.0                      |
| DNase               | + DNase             | 3.0                      |
| Phospholipase A     | + Phospholipase A   | 3.2                      |
| Phospholipase C     | + Phospholipase C   | 3.7                      |

TABLE III
Effect of urea on 125I-glucagon binding

Urea, in a final concentration of 1 M, was added to the incubation mixture immediately prior to the addition of 125I-glucagon. Each value represents the mean of duplicate samples.

| Solubilized extract | 125I-glucagon bound | pmoles/mg protein/60 min |
|---------------------|---------------------|--------------------------|
| Control             | + Urea (1 M)        | 5.5                      |

Effect of Nucleotides—Table IV shows that ATP had no effect on the binding of 125I-glucagon. In addition, GTP did not alter the binding of 125I-glucagon in contrast to what has been reported in liver membranes (20).

Effect of Uracil—Uracil has been shown to decrease the binding of 125I-glucagon to liver membranes (23), 1 M uracil producing approximately a 75% decrease in binding. Uracil, 1 M, did not alter the binding of 125I-glucagon in the solubilized myocardial preparation (Table III).

Dissociable Glucagon Binding Site: Sephadex G-100 and Bio-Gel P-30 Chromatography—We previously reported evidence for a glucagon receptor site in the solubilized preparation of cat myocardium which is dissociable from catalytic adenylate cyclase activity following binding of 125I-glucagon (19). The dissoci-
utilized small (2.8 ml) Sephadex G-100 columns to effect the separation of the receptor-125I-glucagon complex from the larger molecular weight fraction. This procedure has been modified to utilize larger Sephadex G-100 (86 ml) and Bio-Gel P-30 (86 ml) columns in order to more thoroughly study the elution pattern from the gel (Fig. 7A).

Solubilized myocardial adenylate cyclase (mol wt approximately 100,000 to 200,000) is excluded from the Sephadex G-100 gel and appears in the fraction 20 to 40 ml similar to that seen with dextran blue (mol wt approximately 2,000,000), which is also excluded from the gel. 125I-Glucagon binding activity is also found in this fraction (Fig. 7A). In the procedure used to demonstrate glucagon binding in this system, bound and free glucagon were first separated by cellulose chromatography. When 5 ml of the cellulose effluent containing the binding 125I-glucagon are chromatographed on the 86-ml Sephadex G-100 column (Fig. 7B), almost all of the labeled material appears in the salt volume (80 to 95 ml). This peak is clearly retarded behind the peak of two substances of similar molecular weight, growth hormone (approximately 32,000) and cytochrome c (approximately 12,800). A similar elution pattern was observed when the chromatography was performed on an 86-ml Bio-Gel P-30 column (Fig. 8A; compare with Fig. 7B). However, when the column eluate was subjected to electrophoresis on a sodium dodecyl sulfate polyacrylamide gel, the labeled fraction migrated in an area consistent with a molecular weight of about 26,000 as we previously reported (19).

To resolve this apparent paradox, we subjected the Sephadex eluate to hydrodynamic flow electrophoresis in 0.1 M Veronal buffer, pH 8.6, and demonstrated that the labeled material was distinct both from free glucagon and iodine. It seemed likely, therefore, that the binding site-glucagon complex was interacting with the gel itself. In order to investigate this point we altered the chromatographic conditions in several ways. Increasing the ionic strength of the elution buffer to 100 mM Tris pH 7.7, did not alter the elution profile nor did elution with 200 mM acetate buffer, pH 5.5. However, when elution was performed at strongly alkaline pH (0.01 N NaOH), a shift in the binding peak was observed toward the expected elution profile for a substance of its apparent molecular weight (Figs. 7C and 8D). This was particularly marked on the Bio-Gel P-30 column and suggested that the receptor-125I-glucagon complex interacted more strongly with the Sephadex gel. Increasing the ionic strength of the NaOH (0.025 N) produced a further definition and migration of the peak on Sephadex G-100. These data appear to be similar to the gel interactions described for iodothyronines and triiodothyronine and thyroxine on Sephadex (27, 28).

**Table IV**

| Solubilized extract | 125I-glucagon bound μg/mg protein/60 min |
|---------------------|-----------------------------------------|
| − ATP or GTP        | 5.1                                     |
| + ATP (1 X 10^-3 M) | 5.0                                     |
| + ATP, Mg^2+ (1 X 10^-3 M) | 5.0                                   |
| + GTP (1 X 10^-6 M) | 4.9                                     |
| + GTP (1 X 10^-3 M) | 5.1                                     |

**Effect of nucleotides on 125I-glucagon binding**

ATP or GTP at the final concentrations shown were added directly to the incubation mixtures. Each value represents the mean of duplicate samples.

**Discussion**

The action of glucagon on the heart, like most of the actions of this hormone on other tissues, appears to be mediated by the adenylate cyclase-cyclic AMP system. Glucagon activates adenylate cyclase in particulate preparations of rat (3), cat (4), and human heart (4) and increases the intracellular level of cAMP. The action of glucagon on the heart, like most of the actions of this hormone on other tissues, appears to be mediated by the adenylate cyclase-cyclic AMP system. Glucagon activates adenylate cyclase in particulate preparations of rat (3), cat (4), and human heart (4) and increases the intracellular level of cAMP.

**Effect of nucleotides on 125I-glucagon binding**

ATP or GTP at the final concentrations shown were added directly to the incubation mixtures. Each value represents the mean of duplicate samples.
FIG. 7 (left). Sephadex G-100 chromatography and $^{125}\text{I}$-glucagon binding. In the experiment shown in Panel A, 5 ml of the 12,000 $\times$ g supernatant of the solubilized myocardial homogenate prepared as described in the text were added to the Sephadex G-100 column of 80 ml volume equilibrated in 10 mM Tris, pH 7.7. The column was eluted with 10 mM Tris, pH 7.7, at a flow rate of approximately 1.2 ml per min at 25°C. Successive 2.5-ml fractions were assayed for $^{125}\text{I}$-glucagon-binding activity as described in the text. The appearance of standard molecular weight markers for this column are shown by the arrows. In the experiment shown in Panel B, approximately 150 $\mu$l of solubilized myocardium (protein concentration, 3.3 mg per ml) were incubated at 37°C for 60 min in a final volume of 600 $\mu$l containing 1.0% albumin in 10 mM Tris-HCl, pH 7.7, and $^{125}\text{I}$-glucagon at a final concentration of $5 \times 10^{-7}$ M. Upon completion of the incubation, the mixture was added to a dry 8.8-cm cellulose column in a 10-ml serological pipette with an inside diameter of 0.8 cm and washed with 8.4 ml of 1% albumin in 10 mM Tris-HCl, pH 7.7. Five milliliters of the eluate, containing the bound $^{125}\text{I}$-glucagon were then added to the Sephadex column. Successive 2.5-ml fractions were assayed for $^{125}\text{I}$-glucagon binding activity as described in the text. In Panel C, 0.2 ml of the 12,000 $\times$ g supernatant of solubilized cat liver prepared as described in the text were added to a 2.8-ml Sephadex G-100 column equilibrated in 10 mM Tris, pH 7.7. Successive 0.25-ml fractions were assayed for $^{125}\text{I}$-glucagon binding activity as described in the text.

FIG. 8 (center). Bio-Gel P-30 chromatography and $^{125}\text{I}$-glucagon binding. The conditions of the experiments in A and B were identical to those described in the legend to Fig. 7, B and C.

FIG. 9 (right). Dissociable glucagon binding site in a solubilized preparation of liver. In the experiment shown in Panel A, 0.2 ml of the 12,000 $\times$ g supernatant of solubilized cat liver prepared as described in the text was added to a 2.8-ml Sephadex G-100 column equilibrated in 10 mM Tris, pH 7.7. Successive 2.5-ml fractions were assayed for $^{125}\text{I}$-glucagon binding activity as described in the text. In Panel B, 30 $\mu$l of solubilized liver (4.0 mg per ml) prepared as described above were incubated at 37°C for 60 min in a final volume of 100 $\mu$l containing 1% albumin in 10 mM Tris-HCl, pH 7.7, and $^{125}\text{I}$-glucagon at a final concentration of $5 \times 10^{-7}$ M. Upon completion of the incubation, the mixture was added to a dry 1.4-cm cellulose column, bound from free glucagon separated as described in the text, and 0.25 ml of the cellulose effluent was applied to the Sephadex G-100 column. Successive 0.25-ml fractions were assayed for $^{125}\text{I}$-glucagon binding activity.

The complex interaction of glucagon with the adenylate cyclase in isolated liver plasma membrane has been the subject of intensive investigation in recent years by Rodbell et al. (23, 26, 36-41). These studies have provided critical information relative to the binding of $^{125}\text{I}$-glucagon to specific membrane receptor sites, the role of nucleotides in the binding and activation process, the role of phospholipids in the activation process, and the elucidation of the structural requirements in the glucagon molecule required for binding and activation. Over the past several years we have undertaken somewhat parallel investigations in order to understand the molecular interaction of glucagon with the adenylate cyclase in cardiac tissue. Studies from our laboratory have demonstrated that solubilized preparations of cat myocardial adenylate cyclase are unresponsive to stimulation by glucagon, catecholamines, and histamine (20, 21). When the enzyme is freed of detergent by DEAE-cellulose chromatography and the Sephadex column was equilibrated in either 0.01 N NaOH (O) or 0.025 N NaOH (O) prior to the application of 5 ml of the cellulose column eluate.

The inotropic effects of glucagon are potentiated by theophylline (34) which inhibits cyclic nucleotide phosphodiesterase, the enzyme catalyzing the breakdown of cyclic AMP to 5'-AMP. Furthermore dibutyryl adenosine 3',5'-monophosphate exerts an inotropic effect on heart muscle similar to that of glucagon (35).

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FIG. 10. SDS polyacrylamide gel electrophoresis. The gels were prepared according to a modification of the method of Weber and Osborn (29, 30) with a final acrylamide concentration of 10% and a ratio of acrylamide to bisacrylamide of 37:1 and 0.1% SDS. The electrophoresis buffer was 0.025 M sodium phosphate, pH 7.0, with 0.1% SDS. Prior to electrophoresis, samples were incubated at 37°C for 30 min in 50 μl of 0.01 M sodium phosphate (pH 7.0), 0.1% mercaptoethanol, and 0.1% SDS. Marker dye, 3 μl of bromophenol blue, 5 μl of mercaptoethanol, and 1 drop of glycerol were added to each sample and the samples were placed on the gel column and overlaid with electrophoresis buffer. After electrophoresis, the gels were sliced into horizontal segments and the radioactivity (125I) in each slice determined in an Auto-Gamma-counter. Panel A was performed with crude solubilized myocardial adenylate cyclase. Panel B utilized 125I-glucagon incubated in the absence of the solubilized material.

FIG. 11. Ferguson plots: the logarithm of the relative mobility of the 125I-glucagon binding fraction complex and three standard proteins is plotted versus gel concentration. The standard proteins are numbered: 1, fibrinogen (α chain); 2, fibrinogen (β chain); 3, fibrinogen (γ chain); 4, ovalbumin; 5, Bence-Jones protein (α type); 6, lysozyme.

The data in the present investigation more completely characterize the binding of 125I-glucagon to its receptor site(s) in a solubilized preparation of myocardium which contains, among other proteins, a solubilized adenylate cyclase. The preparation of 125I-glucagon utilized for these studies was shown to be biologically active in the context of this system, by its ability to activate both the membrane-bound adenylate cyclase and to activate the detergent-free, solubilized adenylate cyclase in the presence of phosphatidylserine. The binding of 125I-glucagon in the solubilized preparation appeared to be in large part specific. Increasing concentrations of unlabeled glucagon decreased the binding of the 125I-glucagon to the site. Half-maximal displacement occurred at a concentration of unlabeled glucagon of about 5 × 10⁻⁷ M. Approximately 80 to 90% of the bound 125I-glucagon is displaceable, the remainder presumably representing nonspecific glucagon binding. In addition to the displacement studies, specificity was demonstrated by the fact that another polypeptide hormone, parathyroid hormone, did not specifically bind to cardiac receptor sites (utilizing 125I-parathyroid hormone) and did not displace bound 125I-glucagon. Finally, the concentration range over which 125I-glucagon binds is quite similar to that observed for activation of adenylate cyclase in both the membrane-bound and soluble heart preparations. The latter data provide supporting evidence that the binding is related, at least in part, to the adenylate cyclase. However, as noted previously (18), there is additional binding of glucagon in this system over and above what is required to activate adenylate cyclase since the enzyme is maximally activated by 5 min, a point in time when binding is only about 25% complete.

In our early studies of the solubilized myocardial adenylate cyclase, we determined a molecular weight for the enzyme of approximately 100,000 to 200,000, using differential Sephadex chromatography. This estimate of the molecular weight of the cat enzyme is similar to that reported by Lefkowitz et al. (11) for the solubilized dog heart adenylate cyclase (160,000). During the course of our attempts to purify the glucagon binding site, we subjected an aliquot of the incubation mixture containing 125I-glucagon and the crude solubilized binding preparation to electrophoresis in an SDS polyacrylamide gel. Unexpectedly...
we found that the $^{125}$I-glucagon-binding fraction appeared in an area consistent with a molecular weight of 24,000 to 28,000. In a preliminary report we described that the process of glucagon binding resulted in a dissociation of the $^{125}$I-glucagon-receptor complex from the larger molecular weight catalytic moiety of the enzyme when the solubilized preparation was incubated with $^{125}$I-glucagon and then chromatographed on a Sephadex G-100 column (19). The labeled material from the Sephadex G-100 column also had an approximate molecular weight of 24,000 to 28,000 when electrophoresed on SDS polyacrylamide gels (19).

In the initial observations we utilized small, 2.8-ml Sephadex G-100 columns for separation, and this obscured the fact that the $^{125}$I-glucagon receptor complex was markedly retarded on Sephadex. However, when we utilized larger, 86-ml Sephadex G-100 columns, it became apparent that the labeled material was eluting at an elution volume consistent with the salt peak far behind two materials of similar molecular weight, growth hormone (mol wt approximately 32,000), and cytochrome c (mol wt approximately 12,800). Several lines of evidence suggested that this retardation represented a direct interaction of the $^{125}$I-glucagon-receptor complex with the Sephadex rather than a smaller molecular weight substance. (a) The iodinated material was repeatedly shown to have a molecular weight of about 26,000 on SDS polyacrylamide gel electrophoresis, and (b) hydrodynamic flow electrophoresis showed that the iodinated material was neither free glucagon (it did not remain at the origin) nor free iodine, as determined by the migration of a free iodine standard.

The interaction with the gel was not due to the low ionic strength of our eluting buffer (10 mM Tris-HCl, pH 7.7) since increasing the concentration of Tris-HCl to 100 mM did not alter the elution profile. From these data it seemed likely that a strongly positive charged material was adsorbing to the gel, a phenomenon well described with Sephadex and Bio-Gel, particularly with iodinated tyrosines (25, 28). Furthermore, this property of adsorption to Sephadex gels is used in the separation and purification of mixtures of iodine, monoiodotyrosine, diiodotyrosine, triiodothyronine, and tetraiodothyronine (thyroxine) (27, 28). When the Sephadex G-100 and Bio-Gel P-30 columns were equilibrated with 0.01 or 0.025 M NaOH, the charge interaction was for the most part neutralized and the elution peak of iodinated material occurred at the volume more appropriate for its molecular size.

This property of adsorption to Sephadex G-100 and Bio-Gel P-30 columns may ultimately prove extremely useful in the purification of the receptor site. The values for protein in the tubes containing maximum amounts of bound material as measured by the method of Lowry et al. (24) are quite low, ranging from 5 to 25 ng per ml. Thus, about 15-fold increases in specific activity for binding are noted when compared to the initial crude solubilized fraction. The major obstacle to further purification at this time is the relative inability to remove bound glucagon from its receptor site. While SDS blocks the binding almost completely when it is added prior to the addition of $^{125}$I-glucagon to the incubation mixture, when it is added after the binding occurs, as in SDS polyacrylamide gel electrophoresis, it does not dissociate the complex. Similar tight binding has been described for glucagon with liver membranes (43), lutemizing hormone and chorionic gonadotropin to testis and ovary (44), and noradrenephrine to cardiac receptors (12).

Based upon the information acquired over the past decade it became possible to define a reasonable working model of the hormone-sensitive adenylate cyclase as it exists in the cell membrane. The most current model was proposed by Rodbell et al. (36) which is a modification of an earlier model proposed by Robison et al. (45). Rodbell and his co-workers visualize the enzyme situated in the plasma membrane as consisting of at least three subunits. One, the receptor site (discriminator) is situated on the exterior of the cell and is the structural component of the enzyme which serves as the binding site for the hormone. This site seems to interact selectively with certain hormones and to account for the tissue specificity of hormone activation of adenylate cyclase. Two, the catalytic site (amplifier) is situated on the interior of the cell membrane, serves as the binding site for ATP and magnesium, and is responsible for catalyzing the conversion of ATP to cyclic AMP. The third subunit is the coupler (transducer) which in some manner connects the receptor subunit to the catalytic subunit, enabling the message of hormone binding to be translated into activation of the enzyme. The coupler subunit appears to be intimately related to certain membrane phospholipids. Substantiation for the presence of a coupler subunit comes from the observation that binding of hormone to the receptor site can be dissociated from activation of adenylate cyclase (13, 14, 18). We have shown that the addition of specific phospholipids, phosphatidylserine for glucagon and histamine (15, 16), and mono- or di-phosphatidylinositol for catecholamines (17) will restore the activation of solubilized preparations of myocardial adenylate cyclase under conditions in which the enzyme was not activated by, but could bind the hormone (18).

We propose a somewhat modified version of the model of Rodbell et al. (36) (Fig. 13). Hormone (in this case glucagon) binding is followed by dissociation of the receptor site. Dissociation is then rapidly followed by activation of the enzyme only when the critical phospholipid(s) is present to induce alteration in configuration of the catalytic site (36, 47). When glucagon binding occurs in the detergent-free solubilized preparation in the absence of added phospholipid, dissociation of the receptor site is also observed. However, in this situation

![Fig. 13. Model of hormone-sensitive adenylate cyclase.](http://www.jbc.org/Downloadedfrom/fig13.png)
glucagon does not activate the enzyme, presumably due to the absence of phospholipid and the failure to achieve the necessary conformational changes in the catalytic site required for activation. It is unclear whether this is analogous to the protein kinase system in which the binding of cyclic AMP to the receptor site results in a dissociation of the receptor site from the catalytic site, thus removing an inhibitory influence on the catalytic site (48–50). In addition, the model does not clarify the potential role of guanosine triphosphate in hormone binding and activation of the membrane-bound adenyl cyclase reported in several tissues including liver (20), platelets (51), thyroid (52), frog bladder (53), and pancreas (54). It remains to be determined whether or not these effects are of physiological significance.

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Characterization of $^{125}$I-Glucagon Binding in a Solubilized Preparation of Cat Myocardial Adenylate Cyclase: FURTHER EVIDENCE FOR A DISSOCIABLE RECEPTOR SITE

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