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Identification of the glucagon receptor in rat liver membranes by photoaffinity crosslinking

(hydroxysuccinimidyl-*p*-azidobenzoate/covalent attachment/hormones)

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ABSTRACT The photoaffinity crosslinker hydroxysuccinimidyl-*p*-azidobenzoate was used to attach ^{125}I-labeled glucagon covalently to a rat liver membrane protein of $M_r \approx 53{,}000$. Membranes that had been incubated with ^{125}I-labeled glucagon were treated in the dark with hydroxysuccinimidyl-*p*-azidobenzoate, and a covalent complex was then formed by irradiation with ultraviolet light. Characteristics of ^{125}I-labeled glucagon binding and covalent attachment to the M_r 53,000 peptide were consistent with this peptide being a component of the glucagon receptor involved in the activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. Binding and covalent attachment of ^{125}I-labeled glucagon to the M_r 53,000 peptide were inhibited by glucagon concentrations that were within the dose–response curve for adenylate cyclase activation, and GTP specifically decreased the photoaffinity crosslinking of ^{125}I-labeled glucagon to the M_r 53,000 peptides. Insulin did not compete for the photoaffinity crosslinking of ^{125}I-labeled glucagon. The same technique of photoaffinity crosslinking that covalently attached ^{125}I-labeled glucagon to the M_r 53,000 peptide with an efficiency of 1–2% can be used to attach ^{125}I-labeled insulin covalently to a M_r 125,000 peptide with an efficiency of approximately 10%. This peptide has been shown to be a subunit of the high-affinity insulin-binding site in rat liver membranes. The technique of photoaffinity crosslinking with agents like hydroxysuccinimidyl-*p*-azidobenzoate provides a rapid, simple method of covalently attaching ligands to their putative receptors. Photoaffinity crosslinking does not require chemical modification of the labeled ligand and has a less stringent requirement for specific reactive groups than the commonly used bifunctional crosslinking reagents.

Hormone activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] requires at least three membrane-associated proteins. These proteins have been identified functionally as hormone receptor, catalytic cyclase, and a guanine nucleotide-dependent regulatory component that couples hormone binding to cyclase activation. The regulatory component has been shown to interact physically with both hormone receptor (1) and catalytic cyclase (2). However, the molecular events resulting from these interactions that cause cyclase activation remain unknown. An understanding of these events requires the purification, physical characterization, and reconstitution of these proteins to assemble a functional hormone-sensitive adenylate cyclase. The ability to label components of the hormone-sensitive adenylate cyclase system specifically and covalently provides a useful approach to characterization of the properties of each of these components.

In this study, we have used a photoreactive heterobifunctional reagent hydroxysuccinimidyl-*p*-azidobenzoate (HSAB) to covalently link ^{125}I-labeled glucagon (^{125}I-glucagon) to a liver plasma membrane protein of M_r 53,000. This protein fulfills the criteria expected of the glucagon receptor that activates adenylate cyclase. The labeling technique of photoaffinity crosslinking by using HSAB after binding of the hormone to its membrane receptor does not require derivatization of the peptide common to most photoaffinity labeling procedures (3, 4) nor does it have the stringent chemical requirements for affinity crosslinking with bifunctional crosslinking reagents (5, 6). Thus, it has advantages over both of these techniques and is generally applicable for covalent attachment of ligands to their putative receptors.

MATERIALS AND METHODS

Preparation of Rat Liver Membranes. Livers were removed from Sprague–Dawley rats (125–200 g), and membranes were prepared by the procedure of Neville (7) through step 12. Membranes were frozen in small aliquots and stored at −80°C.

Adenylate Cyclase Assay. Adenylate cyclase was assayed at 30°C for 10 min in 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 mM creatine phosphate, 10 units of creatine phosphokinase per ml, 0.4 mM ATP, 2×10^6 cpm of [α-^{32}P]ATP, and rat liver membranes (5–50 μg) in a final volume of 100 μl. Effectors of adenylate cyclase, when used, were 100 μM GTP, 100 μM guanosine 5′-(3-thio)triphosphate, 10 mM NaF, and glucagon dissolved in 1 mM HCl at various concentrations. Typical rat liver membrane preparations had a basal adenylate cyclase activity (in the presence of GTP) of ≈25 pmol/min per mg of membrane protein and guanosine 5′-(3-thio)triphosphate-stimulated activities of 250–300 pmol/min per mg of membrane protein. Maximal glucagon activation was typically 4- to 6-fold larger than basal activity, with half-maximal activation in the presence of GTP occurring at 7 nM.

Preparation of ^{125}I-Glucagon. ^{125}I-Glucagon was purchased from New England Nuclear or prepared by either of two methods. Initially, modification of the method of Hunter and Greenwood (8) was used as described by Rodbell *et al.* (9). However, a gentler method which consistently gave lower nonspecific binding was developed during these studies by using immobilized preparations of lactoperoxidase and glucose oxidase (Enymobeads, Bio-Rad). The iodination mixture consisted of 75 mM sodium phosphate (pH 7.2), 0.2% β-D-glucose, 1.0 mCi of Na^{125}I (1 Ci = 3.7×10^{10} becquerels), 10 μg of glucagon, and 50 μl of Enzymobead reagent. The reaction was allowed to proceed for 4 min. The mixture was then immediately placed over a 1-cm column of cellulose powder, and ^{125}I-glucagon was purified as described (9).

Biological activity was determined by testing the ability of labeled glucagon to activate adenylate cyclase and its binding characteristics to rat liver membranes. Similar results were obtained with ^{125}I-glucagon prepared by either of the two methods

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Abbreviations: HSAB, hydroxysuccinimidyl-*p*-azidobenzoate; ^{125}I-glucagon, ^{125}I-labeled glucagon; ^{125}I-insulin, ^{125}I-labeled insulin.

or purchased from New England Nuclear. Labeled glucagon was stored in the presence of 1% bovine serum albumin at −20°C and prepared fresh every 2 weeks.

Preparation of ^{125}I-Labeled Insulin (^{125}I-Insulin). Insulin was iodinated by the Enzymobead technique similar to that for glucagon and was purified by gel filtration.

^{125}I-Glucagon Binding to Rat Liver Membranes. Labeled glucagon was bound to rat liver membranes by using 5–50 μg of membrane protein in 100 μl of a buffer that was essentially identical to that used for measurement of adenylate cyclase activity except that [α-^{32}P]ATP was omitted from the incubation and 1% bovine serum albumin was added. Incubations were for 10–30 min at 30°C, at which time samples were diluted with 5 ml of ice-cold 10 mM sodium phosphate (pH 7.4) and rapidly filtered on 0.45-μm Amicon-microporous filters that had been soaked in 10 mM sodium phosphate (pH 7.4) containing 1% bovine serum albumin. Filters were washed with an additional 10 ml of 10 mM sodium phosphate (pH 7.4), and radioactivity was measured in a gamma counter.

Typical membrane preparations gave 0.9–1.3 pmol of glucagon-binding sites per mg of membrane protein. The K_d for binding was generally 1–2 nM. Nonspecific binding was generally less than 10–15% of total binding.

Photoaffinity Crosslinking of ^{125}I-Glucagon to Rat Liver Membranes. After binding of ^{125}I-glucagon to rat liver membranes under various incubation conditions, the membranes were washed three times by dilution with 12 ml of ice-cold sodium phosphate and centrifuged at 43,000 × *g* for 10 min. The membranes were resuspended at 1–2 mg/ml in the same buffer, and a volume of HSAB in a dimethyl sulfoxide solution was added to give a 1:50 dilution of HSAB. Concentrations of HSAB tested ranged from 1 μM to 1 mM in the final reaction mixture, with optimal photoaffinity crosslinking occurring around 50 μM HSAB. The membranes were incubated with HSAB for 2 min in the cold in a darkened room and then photolyzed for 8 min with a Hanovia 140-W mercury arc lamp 15 cm from the membrane suspension. Tris·HCl (pH 7.4) was then added to a concentration of 20 mM to prevent further chemical reactions, and the membranes were washed twice by dilution and centrifugation.

NaDodSO$_4$/Acrylamide Gel Electrophoresis. Membranes in 20 mM Tris·HCl (pH 7.4) were prepared for NaDodSO$_4$ gel electrophoresis by adding NaDodSO$_4$ and 2-mercaptoethanol to final concentrations of 1% and 5% (wt/vol), respectively. The samples were heated in a boiling water bath for 5 min. Discontinuous NaDodSO$_4$ gel electrophoresis was performed by the method of Laemmli (10) with either 7.5% or 5–15% linear-gradient acrylamide gels. The slab gels were stained in 2% Coomassie blue/50% ethanol/10% acetic acid and destained in 10% ethanol/10% acetic acid, dried, and exposed to XR-2 Kodak x-ray film. Molecular weight standards were β-galactosidase (M_r 116,000), phosphorylase (M_r 94,000), bovine serum albumin (M_r 65,000), ovalbumin (M_r 45,000), and carbonic anhydrase (M_r 30,000).

Materials. HSAB was purchased from Pierce. [α-^{32}P]ATP and [^{125}I]iodine were obtained from New England Nuclear. Glucagon and insulin were generous gifts from W. Bromer and R. Chance (Lilly Research Laboratories).

RESULTS

Fig. 1 shows the Coomassie blue staining patterns and autoradiograph of a 5–15% gradient acrylamide/NaDodSO$_4$ gel of rat liver membrane proteins after binding of ^{125}I-glucagon and exposure to HSAB and ultraviolet light. HSAB in the absence of ultraviolet light (Fig. 1A, lanes 2 and 5) causes little or no change in the Coomassie blue staining pattern of the membrane proteins (compare with lane 3). When the membranes were exposed to HSAB and irradiated, there was a small decrease in the intensity of several bands (Fig. 1A, lanes 1 and 4 compared to lane 3). However, at the concentration of HSAB used, high molecular weight aggregates were generally not observed at the top of the gel. Higher concentrations of HSAB did not cause significantly more crosslinking of the high-abundance membrane proteins; this is at least in part due to its marked insolubility in aqueous solutions at concentrations much above 50 μM. With membranes treated with HSAB and photolyzed at higher temperatures, formation of higher molecular weight aggregates could be observed. This supports the idea that there are few preexisting complexes of major membrane proteins at low temperature that can be detected by crosslinking. Similar findings with low concentrations of the bifunctional crosslinking reagent disuccinimidyl suberate have been observed with adipocyte membranes (6).

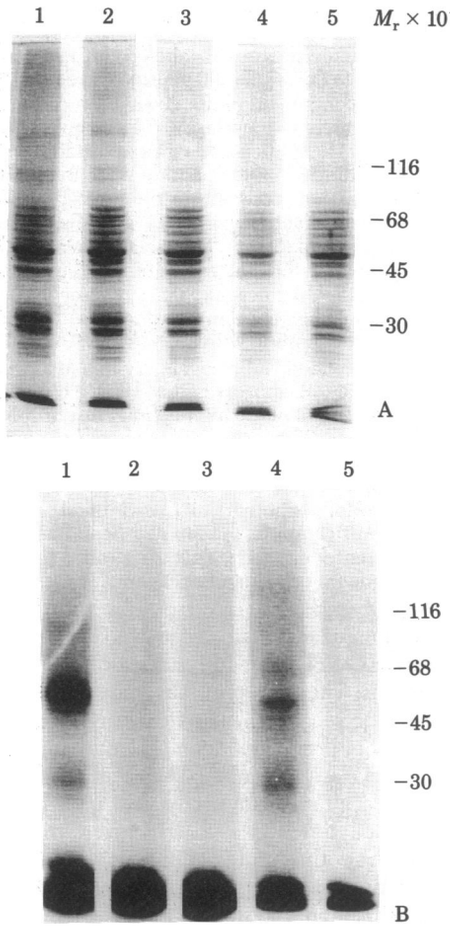

FIG. 1. NaDodSO$_4$/acrylamide gel electrophoresis of liver membrane proteins after binding of ^{125}I-glucagon and incubation of membranes with HSAB. (*A*) Coomassie blue staining patterns of major liver membrane proteins. (*B*) Autoradiograph showing the presence of photoaffinity-crosslinked ^{125}I-glucagon to membrane proteins. Membranes were incubated with 0.7 nM ^{125}I-glucagon in the absence (lanes 1–3) and presence (lanes 4 and 5) of 1 μM glucagon. Membranes (1–2 mg/ml) were then washed and incubated in the presence (lanes 1, 2, 4, and 5) or absence (lane 3) of 50 μM HSAB for 2 min on ice. Membranes were then irradiated (lanes 1 and 4) or incubated in the dark (lanes 2, 3, and 5) for an additional 8 min. After the entire 10-min incubation, Tris·HCl (pH 7.4) was added to a final concentration of 20 mM, and the membranes were washed by dilution and centrifugation. Membranes were solubilized and the proteins were reduced and subjected to electrophoresis on a linear 5–15% gradient acrylamide/NaDodSO$_4$ gel. Autoradiograph is a 4-day exposure of the gel.

Autoradiographs of this gel show that irradiation of the membranes in the presence of HSAB (Fig. 1*B*, lane 1) resulted in the appearance of a major band of $M_r \approx 53,000$. A few minor lower molecular weight bands are also evident. In the absence of ultraviolet light, these bands are not observed (lane 2), and the pattern is similar to that of the membranes that were not exposed to HSAB (Fig. 1*B*, lane 3). Significantly, the M_r 53,000 band was markedly decreased when ^{125}I-glucagon binding was performed in the presence of 1 μM glucagon (Fig. 1*B*, lane 4).

Fig. 2 demonstrates that native glucagon is capable of competing in a dose-dependent manner for the covalent binding of ^{125}I-glucagon to rat liver membrane proteins of M_r 53,000. The concentrations of glucagon used were within the dose–response curve for activation of adenylate cyclase, where 50% of maximal activation was achieved at ≈7 nM glucagon. This band was somewhat diffuse on 7.5% acrylamide/NaDodSO$_4$ gels (Fig. 2) and less diffuse on the 5–15% gradient acrylamide/NaDodSO$_4$ gels (Fig. 1*B*). This was not due to the gels themselves or to the HSAB because, as shown in Fig. 1*A*, Coomassie blue staining patterns gave sharp bands for high-abundance proteins. It is, therefore, likely that this diffuse migration pattern is a property of the protein or proteins covalently bound to ^{125}I-glucagon. One possibility is that this protein is a glycoprotein, many of which migrate as broad bands on NaDodSO$_4$/acrylamide gels (e.g., band 3 of the human erythrocyte membrane). Alternatively, the diffuse migration pattern may represent a heterogeneous group of similar polypeptides.

If the protein of M_r 53,000 that becomes covalently linked to ^{125}I-glucagon is the receptor that mediates glucagon's activation of adenylate cyclase, its affinity for glucagon should be decreased by GTP (11). When ^{125}I-glucagon was bound to rat liver membranes, the binding was very stable even after dilution and incubation at 30°C (data not shown). When GTP was added to the incubation, there was a rapid time-dependent dissociation of glucagon from the membrane receptor (11, 12). Rodbell and coworkers (11, 12) have demonstrated for the hepatic glucagon-sensitive adenylate cyclase system that this is the result of interaction of the glucagon receptor with the GTP-liganded guanine nucleotide regulatory component. Fig. 3 shows that GTP specifically decreased the photoaffinity crosslinking by HSAB of the M_r 53,000 membrane protein. The other areas of the gel did not appear to be affected by GTP. Slices of NaDodSO$_4$ gels containing the M_r 53,000 peptide were excised and radioactivity was measured to determine the actual amount of ^{125}I-glucagon covalently crosslinked. Approximately 1–2% of the specifically bound ^{125}I-glucagon was covalently crosslinked to the M_r 53,000 peptide. These findings strongly support the hypothesis that the M_r 53,000 protein is a component of the membrane receptor for glucagon that mediates adenylate cyclase activation.

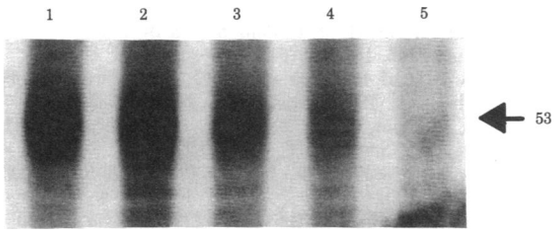

FIG. 2. Competitive inhibition by glucagon of covalent attachment of ^{125}I-glucagon to a M_r 53,000 membrane protein. ^{125}I-Glucagon (0.7 nM) was bound to rat liver membranes in the presence of the following concentrations of unlabeled glucagon: lane 1, 0; lane 2, 1 nM; lane 3, 10 nM; lane 4, 100 nM; and lane 5, 1 μM. Binding of ^{125}I-glucagon to membranes in the presence of 1 μM glucagon was ≈15% of total binding in the absence of unlabeled glucagon. Membranes were washed after binding and treated with 50 μM HSAB. Membranes were solubilized, and the proteins were reduced and subjected to electrophoresis on a 7.5% acrylamide/NaDodSO$_4$ gel. Autoradiograph is a 4-day exposure of the gel. The location of the M_r 53,000 protein is indicated.

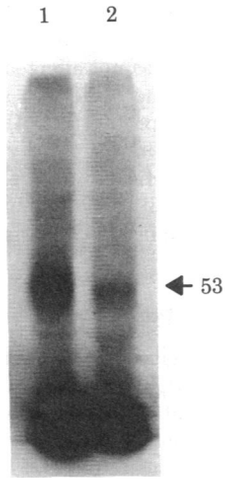

FIG. 3. GTP-mediated decrease in the covalent attachment of ^{125}I-glucagon to a M_r 53,000 peptide of liver membranes. Membranes were incubated with 0.7 nM ^{125}I-glucagon and then washed three times by dilution in 10 mM sodium phosphate (pH 7.4) and centrifugation. The membranes were then resuspended in the same buffer and incubated for 30 min at 30°C in the absence (lane 1) or presence (lane 2) of 100 μM GTP. The membranes were then washed and treated with 50 μM HSAB. Membranes were solubilized, and the proteins were reduced and subjected to electrophoresis on 7.5% acrylamide/NaDodSO$_4$ gels. Autoradiograph is a 4-day exposure. The location of the M_r 53,000 peptide is indicated.

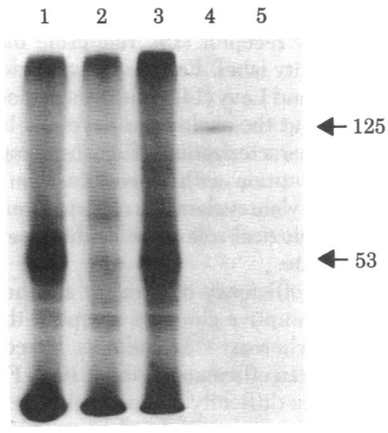

FIG. 4. Photoaffinity crosslinking of ^{125}I-glucagon and ^{125}I-insulin to specific liver membrane proteins. Membranes were incubated for 30 min at 30°C with 0.7 nM ^{125}I-glucagon (lanes 1–3) or 0.7 nM ^{125}I-insulin (lanes 4 and 5) in the presence of 1 μM glucagon (lane 2) or 1 μM insulin (lanes 3 and 5) or in the absence of unlabeled hormone (lanes 1 and 4). The membranes were then washed three times by dilution and centrifugation in ice-cold 10 mM sodium phosphate (pH 7.4). Membranes were resuspended in 10 mM sodium phosphate (pH 7.4) and treated with HSAB. The membranes were then solubilized in NaDodSO$_4$, and the proteins were reduced and subjected to electrophoresis on 7.5% acrylamide/NaDodSO$_4$ gels. Specifically bound radioactivity placed on the gel was ≈20,000 cpm of ^{125}I-glucagon in lanes 1 and 3 and 3000 cpm of ^{125}I-insulin in lane 4. One hundred micrograms of membrane protein was placed in each lane. The failure to observe free ^{125}I-insulin at the bottom of the gel is due to run-off of the dye front, which ran slightly faster in this region of the gel. The autoradiograph is a 5-day exposure. The locations of the M_r 53,000 and M_r 125,000 proteins are indicated.

Fig. 4 indicates that the competition for photoaffinity crosslinking of ^{125}I-glucagon to the M_r 53,000 protein was specific for glucagon (lane 2) and was not affected by insulin (lane 3). Furthermore, this technique could also specifically crosslink ^{125}I-insulin to a M_r 125,000 protein (lane 4) that has been shown to be a subunit of the insulin receptor by affinity crosslinking techniques (6) and by purification and photoaffinity labeling protocols (4, 13). Photoaffinity crosslinking of ^{125}I-insulin to the M_r 125,000 protein by use of HSAB was specifically inhibited by 1 μM insulin (lane 5).

DISCUSSION

We have crosslinked ^{125}I-glucagon to a rat liver membrane protein of $M_r \approx 53,000$. The M_r 53,000 protein has all the properties expected for a component of the glucagon receptor that mediates glucagon's activation of adenylate cyclase. (*i*) Photoaffinity crosslinking of ^{125}I-glucagon to the M_r 53,000 protein is inhibited by concentrations of native glucagon that are within the dose–response curve for cyclase activation; (*ii*) GTP specifically decreases the crosslinking, in agreement with the decrease in glucagon affinity for the receptor; and (*iii*) the crosslinking is not affected by hormones that interact with rat liver membrane receptors different from the glucagon receptor.

Bregman and Levy (14) have reported the labeling of a M_r 23,000 protein in rat hepatocyte membranes with ^{125}I-labeled azidoglucagon. In some preparations, we also see the labeling of a protein in this M_r range (see Fig. 1*B*), but the intensity of this band is not affected by GTP and native glucagon. It, therefore, seems unlikely that this band is a necessary component of the glucagon receptor involved in activation of adenylate cyclase.

The labeling of hormone receptors with radioactive photosensitive hormone derivatives has become a valuable tool for probing the structure of hormone receptors (3, 4). However, derivatization of hormones can severely reduce the binding affinity of hormone for receptor (15), rendering the derivative unsuitable as an affinity label. For the glucagon derivative described by Bregman and Levy (14), binding was identical to that of native hormone, but the analog was devoid of biological activity. No further characterization of azido-analog properties with regard to competition with native glucagon for binding and activation of adenylate cyclase was reported, making it difficult to assess the biological relevance of the labeling of a M_r 23,000–25,000 peptide.

Interestingly, the efficiency of covalent attachment of ^{125}I-glucagon to the presumptive glucagon receptor, the M_r 53,000 peptide, is 1–2%, whereas ^{125}I-insulin is linked to the M_r 125,000 peptide with an efficiency of about 10% (Fig. 4). These results underscore the difficulty in covalently labeling the glucagon receptor. In contrast, several techniques have been used to label the insulin receptor with a high degree of efficiency (4, 6).

Affinity crosslinking has proved to be a valuable approach to receptor characterization (5, 6) but requires the appropriate spacing and chemical accessibility of reactive groups on both receptor and hormone. These chemical criteria may be difficult or impossible to fulfill with specific systems. For example, we failed to crosslink ^{125}I-glucagon specifically to liver membrane proteins with the amino group-specific hydroxysuccinimide esters of succinic, adipic, and suberic acids (unpublished observation). Under identical conditions, ^{125}I-insulin is crosslinked to a specific liver membrane protein (6) that has been identified as a component of the insulin receptor (4, 6, 13).

It is, therefore, striking that photoaffinity crosslinking of bound hormone by using HSAB can be used to label the putative glucagon receptor when photoaffinity labeling using an azido-glucagon analog or affinity crosslinking using bifunctional hydroxysuccinimide esters did not work. In order for photoaffinity crosslinking to be effective with HSAB, an accessible amino group must be present on either hormone or receptor to initiate the labeling process. Once the chemical reaction of HSAB occurs, the aryl azide is activated by ultraviolet light to an aryl nitrene, which requires little or no chemical specificity (16) for further reaction. The covalent linkage of hormone and receptor can occur by reaction of the nitrene with both peptide and carbohydrate components of proteins. Therefore, the probability of successful crosslinking is substantially increased. It should also be possible to design photoaffinity crosslinking reagents with different chemical specificities. The technique is easy and rapid and should prove generally useful in the identification of putative receptors for a wide variety of ligands.

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1. Limbird, L. E., Gill, D. M. & Lefkowitz, R. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 775–779.
2. Pfeuffer, T. (1979) *FEBS Lett.* **101**, 85–89.
3. Das, M., Miyakawa, T., Fox, C. F., Pruss, R. M., Aharonow, A. & Herschman, H. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2790–2794.
4. Yip, C. C., Yeung, C. W. T. & Moule, M. L. (1978) *J. Biol. Chem.* **253**, 1743–1745.
5. Sahyoun, N., Hoch, R. A. & Hollenberg, M. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1675–1679.
6. Pilch, P. F. & Czech, M. P. (1980) *J. Biol. Chem.* **255**, 1722–1731.
7. Neville, D. M., Jr. (1968) *Biochim. Biophys. Acta* **154**, 540–552.
8. Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496.
9. Rodbell, M., Krans, H. M. J., Pohl, S. L. & Birnbaumer, L. (1971) *J. Biol. Chem.* **246**, 1861–1871.
10. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
11. Rodbell, M., Krans, H. M. J., Pohl, S. L. & Birnbaumer, L. (1971) *J. Biol. Chem.* **246**, 1872–1876.
12. Welton, A. F., Lad, P. M., Newby, A. C., Yamamura, H., Nicosia, S. & Rodbell, M. (1977) *J. Biol. Chem.* **252**, 5947–5950.
13. Jacobs, S., Hazum, E., Schechter, Y. & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4918–4921.
14. Bregman, M. D. & Levy, D. (1977) *Biochem. Biophys. Res. Commun.* **78**, 584–590.
15. Gliemann, J. & Gammeltoft, S. (1974) *Diabetologia* **10**, 105–113.
16. Bayley, H. & Knowles, J. R. (1977) *Methods Enzymol.* **46**, 69–114.