

# Insulin and epidermal growth factor-urogastrone: Affinity crosslinking to specific binding sites in rat liver membranes

(polypeptide hormone receptors/glutaraldehyde coupling/receptor solubilization and characterization/membrane labeling/affinity chromatography)

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**ABSTRACT** Both insulin and human epidermal growth factor-urogastrone (EGF/URO) can be covalently linked to specific rat liver membrane binding sites by glutaraldehyde coupling followed by sodium borohydride reduction to yield affinity-labeled membrane constituents sufficiently stable for solubilization and further analysis by various techniques. Solubilization of membranes covalently labeled with  $^{125}\text{I}$ -labeled insulin yields a component with chromatographic properties identical to those of a soluble insulin receptor characterized in previous studies. A second soluble insulin-binding component that is not revealed by the affinity-labeling method and that has not yet been reported can also be detected. Membranes similarly labeled with  $^{125}\text{I}$ -labeled EGF/URO yield one major and two minor ligand-specific soluble (Triton X-100) affinity-labeled components, as detected by chromatography on Sepharose 6B. Further analysis of the EGF/URO-labeled components by affinity chromatography on concanavalin A-Sepharose, by disc gel electrophoresis, and by enzymatic digestion suggests that the major specific binding component for EGF/URO in liver membranes is a glycoprotein subunit of approximately 100,000 daltons that possesses a 20,000-dalton portion inaccessible to proteolytic cleavage when the subunit is anchored in the membrane. The affinity labeling approach described should prove of use for the study of other polypeptide receptors that, like the EGF/URO receptor, lose their ligand recognition property subsequent to membrane solubilization.

Over the past decade, considerable progress has been made in the characterization of various membrane-localized hormone receptors (for review, see refs. 1-4). Particular success has been achieved with purification of the nicotinic cholinergic receptor (5, 6) and of the insulin receptor (7), in which cases the receptors, once solubilized with nonionic detergents, retain their specific ligand recognition properties. However, not infrequently, in the presence of detergent, the ligand-binding property of a particular membrane receptor is lost. One excellent example of this difficulty is illustrated by work with the muscarinic cholinergic receptor for which it was not possible to measure ligand binding in the presence of detergent and for which it was necessary to use affinity labeling of the receptor with a radioactive muscarinic antagonist alkylating reagent (8).

There is now considerable interest in epidermal growth factor-urogastrone (EGF/URO), a 6000-dalton polypeptide found both in the mouse and in man (9-13). The peptide is both a potent stimulant of cell proliferation and an inhibitor of gastric acid secretion. The specific binding of EGF/URO to membrane receptors has been documented in various tissues including rat liver (14-18). In our preliminary attempts to solubilize the receptor for EGF/URO it became apparent that specific ligand

binding could not be detected subsequent to detergent treatment and that, as for the muscarinic cholinergic receptor, an affinity labeling technique would be desirable for characterization of the soluble receptor. We therefore explored the use of glutaraldehyde coupling followed by sodium borohydride reduction as a means of covalently labeling specific polypeptide binding sites. We now report on the success of this approach for the affinity crosslinking of insulin and EGF/URO to specific binding sites in rat liver membranes. The method does not require the synthesis of specialized affinity-labeling reagents and should prove of use for the study of soluble receptors for other polypeptides.

## MATERIALS AND METHODS

Murine EGF/URO was purified from mouse submaxillary glands (19); human EGF/URO (also termed  $\beta$ -urogastrone) was generously supplied by Harold Gregory, ICI Ltd., Macclesfield, U.K. Concanavalin A (Con-A)-Sepharose (12 mg of protein/ml of Sepharose) was synthesized by J. M. Mauro III, C. W. Post College, NY. Porcine insulin and both mouse and human EGF/URO were labeled with carrier-free  $^{125}\text{I}^-$  (2-5 mCi per reaction with 5  $\mu\text{g}$  of polypeptide; final specific activity, 250-500 cpm/pg) by a modification of the Hunter-Greenwood method as described (3, 20, 21).

Liver plasma membranes were prepared from Sprague-Dawley rats (100-150 g) by differential centrifugation (22). The final membrane pellet was washed twice and resuspended in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.7, at a concentration of 8 mg of membrane protein per ml (23).

The affinity coupling of membrane with  $^{125}\text{I}$ -labeled ligand was performed as follows. Membranes were diluted 1:20 in 50 mM Hepes buffer (pH 7.7) containing 0.1 M NaCl and were incubated at 23° with radiolabeled ligand (2 nM). Nonspecific binding was measured in the presence of a 200-fold excess of unlabeled ligand. After 60 min, glutaraldehyde was added at an appropriate concentration (5 mM, unless otherwise indicated) and the suspension was incubated at room temperature for various times. The membranes were then pelleted at 40,000  $\times g$  for 10 min at 4°, resuspended, and incubated for 30 min in 50 mM sodium borohydride adjusted to pH 9.6. The membranes were centrifuged again (40,000  $\times g$ , 10 min), washed three times by resuspending the pellet in 10 ml of buffer with a Dounce homogenizer followed by recentrifugation, and finally dispersed (8 mg of protein per ml) in 50 mM Tris-HCl, pH 7.7 (Hepes interferes with protein determinations). Membranes

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Abbreviations: EGF/URO, human epidermal growth factor-urogastrone; Con A, concanavalin A; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.

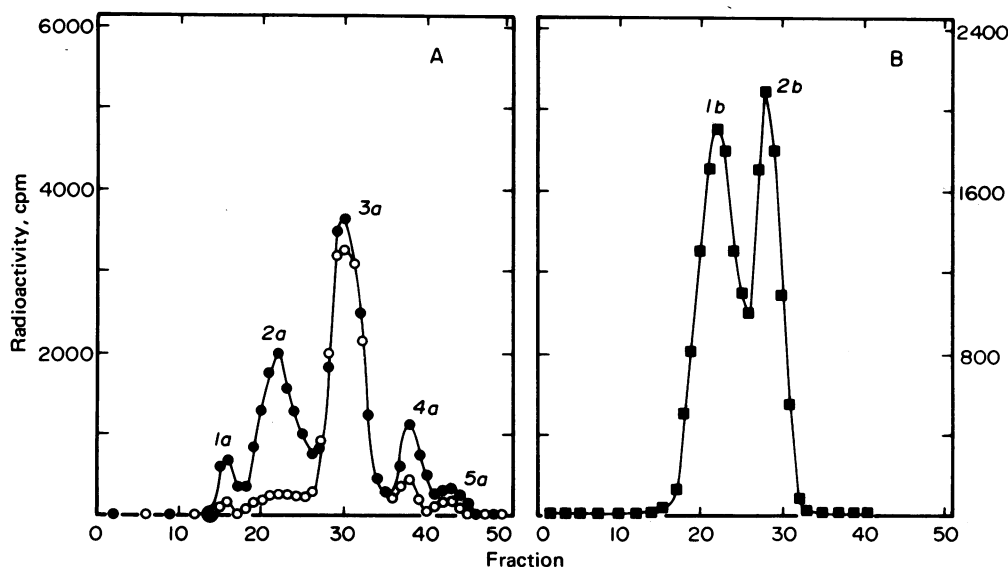


FIG. 1. Fractionation of insulin-binding material on Sepharose 6B. (A) Profile of  $^{125}\text{I}$ -labeled insulin radioactivity solubilized from liver membranes preincubated sequentially with  $^{125}\text{I}$ -labeled insulin, glutaraldehyde, and sodium borohydride, in the absence (●—●) and presence (○—○) of unlabeled insulin (2  $\mu\text{g}/\text{ml}$ ). Chromatography was performed on identical aliquots of solubilized protein. (B) Specific binding of  $^{125}\text{I}$ -labeled insulin to solubilized liver membranes after fractionation on Sepharose 6B. A 1.2  $\times$  60 cm column was eluted with 50 mM Tris-HCl, pH 7.7/0.1% (vol/vol) Triton X-100; 1.2-ml fractions were collected, and 100- $\mu\text{l}$  aliquots were assayed for binding of insulin.

were solubilized by incubation with 1% Triton X-100 for 20 min at 23° and then for 1 hr at 4° followed by centrifugation for 1 hr at 200,000  $\times g$  at 4°.

The specific binding of  $^{125}\text{I}$ -labeled insulin (20 nM) to a solubilized membrane preparation was performed by the polyethylene glycol assay method (24). This method was also used to evaluate the incorporation of radioactivity into solubilized affinity-labeled membrane components.

Polyacrylamide gels for electrophoretic analysis were prepared as described (25) with 5% acrylamide, 0.17% methyl bisacrylamide, and 0.1% sodium dodecyl sulfate ( $\text{NaDodSO}_4$ ). The  $\text{NaDodSO}_4$  disc gels as well as the Sepharose 6B columns were standardized by using the molecular weight markers apoferritin, gamma globulin, bovine serum albumin, chymotrypsinogen, and myoglobin. For the electrophoretic analysis of affinity-crosslinked solubilized protein, 2.5-mm unfixed gel slices were cut and measured for  $^{125}\text{I}$  content by crystal scintillation counting (efficiency, 85%). The precision of molecular weight determinations as determined by analyzing the same sample on four separate gels was  $\pm 6\%$ .

## RESULTS

The coupling procedure to be used for the EGF/URO binding site was first evaluated with insulin for two reasons. First, the specificity of binding of  $^{125}\text{I}$ -labeled insulin has been well documented (20, 26–28). Second, the binding of insulin to solubilized receptor is readily measured by the polyethylene glycol method, such that the chromatographic behavior of the affinity-labeled components derived from intact membranes can be compared with the behavior of the unlabeled solubilized insulin-binding components.

Subsequent to the covalent coupling of  $^{125}\text{I}$ -labeled insulin to liver membranes, treatment with Triton X-100 yielded a soluble preparation containing 20% of the radioactivity bound to the membranes. Over 80% of the solubilized radioactivity could be precipitated in the presence of polyethylene glycol [final concentration, 10.4% (wt/vol)] under conditions used for the routine assay of soluble insulin receptor. When the borohydride reduction step subsequent to glutaraldehyde coupling

was omitted, the proportion of the Triton-solubilized radioactivity that was precipitated by polyethylene glycol decreased markedly upon storage at 4° for 48 hr. In contrast, after the reduction step, the precipitability with polyethylene glycol remained constant for up to 1 week at 4°, indicating a stable bond between  $^{125}\text{I}$ -labeled insulin and the solubilized membrane macromolecule(s). The stability of the crosslinking of membranes was further confirmed by the prolonged retention (>12 hr) of radioactivity on Millipore filters (0.2  $\mu\text{m}$ ) under conditions such that a half-life of about 16 min for the insulin-receptor complex can be measured. It was concluded that reductive stabilization of the Schiff base of the glutaraldehyde-linked substituents was essential for the subsequent isolation of affinity-labeled membrane components.

Chromatography of solubilized insulin-labeled material on Sepharose 6B yielded two major peaks of radioactivity (peaks 2a and 3a in Fig. 1A) and two minor peaks (1a and 4a), one of which (4a) was eluted in the position of free insulin. The elution volumes of peaks 2a and 3a in Fig. 1 are expected for molecules of apparent Stokes radii of 72 Å and 38 Å, respectively. Importantly, the amount of radioactivity present in the first major peak (peak 2a) is markedly decreased when coupling is performed in the presence of unlabeled insulin and an identical amount of soluble membrane protein is analyzed. In contrast, the amount of radioactivity in the second major peak (peak 3a) is only slightly decreased when coupling is done in the presence of unlabeled insulin. The positions of peaks 2a and 3a of the affinity-labeled preparation correspond exactly to the elution volumes of two soluble insulin-binding components (peaks 1b and 2b in Fig. 1B) detected by the polyethylene glycol assay of effluent fractions after Sepharose 6B chromatography. Analysis of the binding isotherm of  $^{125}\text{I}$ -labeled insulin to Triton-solubilized material in peak 1b demonstrated specific insulin-binding properties consistent with data previously reported for a solubilized insulin receptor from liver membranes (24). Further study of the insulin binding component(s) present in peak 2b, not detected in previous work demonstrated the presence of nonreceptor constituents that can react with the insulin receptor and that may account for the "nonspecific"

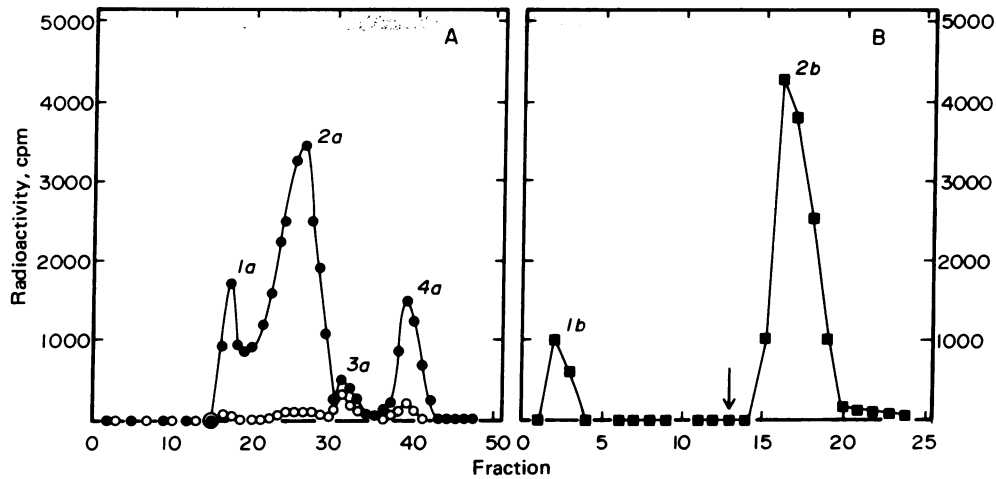


FIG. 2. Partial purification of EGF/URO-binding material. (A) Profile of <sup>125</sup>I-labeled EGF/URO radioactivity solubilized from liver membranes after glutaraldehyde/sodium borohydride crosslinking in the absence (●-●) or presence (○-○) of unlabeled EGF/URO (2 μg/ml). Identical aliquots of membrane protein were applied to a 1.2 × 60 cm Sepharose 6B column and 1.2-ml fractions were collected. (B) Peak 2a was placed on a 0.3 × 4 cm Con A-Sepharose column, and the column was washed with 14 ml of 0.1% (vol/vol) Triton X-100/50 mM Tris-HCl, pH 7.7, followed by elution (arrow) with the sugar/urea/detergent solution described in *Results*; 1-ml fractions were collected.

coupling observed for peak 3a (J. M. Maturo and M. D. Hollenberg, unpublished data).

The results with insulin indicated that, despite the somewhat low recovery of soluble radioactivity, the affinity-coupling procedure can yield a specifically labeled membrane constituent (peak 2a in Fig. 1) with chromatographic properties similar to those of a soluble insulin-binding component from non-crosslinked membranes. The nature of the material that resists solubilization remains an open question. It therefore appeared appropriate to apply the technique to the labeling of the EGF/URO binding site, for which assay of ligand binding in the soluble state is not possible.

Attempts to couple <sup>125</sup>I-labeled mouse EGF/URO irreversibly to membranes by the procedure used for insulin were unsuccessful. Although the coupling procedure substantially decreased the dissociation rate of <sup>125</sup>I-labeled mouse EGF/URO from intact membranes, analysis of solubilized material by Sepharose 6B chromatography and by NaDodSO<sub>4</sub> disc gel electrophoresis revealed that approximately 95% of the radioactivity migrated as free <sup>125</sup>I-labeled EGF/URO. In contrast, the use of <sup>125</sup>I-labeled human EGF/URO yielded affinity-labeled membranes suitable for subsequent analysis. After treatment with 5 mM glutaraldehyde (40 min, 24°) followed by borohydride reduction, 20% of the bound radioactivity could be solubilized in 1% Triton X-100; 80% of the solubilized radioactivity was precipitable in the presence of polyethylene glycol. These results are similar to those described above for insulin-labeled membranes. The success of the coupling procedure using human EGF/URO can be attributed to the presence of two lysines in the primary sequence, providing sites for glutaraldehyde crosslinking; lysine is absent from mouse EGF/URO. Attempts to crosslink human EGF/URO with the bifunctional reagent dimethyladipimidate were unsuccessful.

Chromatography of the solubilized EGF/URO-labeled material on Sepharose 6B yielded four peaks of radioactivity; for three of the four peaks (peaks 1a, 2a, and 4a in Fig. 2A), the amount of label was substantially decreased when coupling was performed in the presence of unlabeled EGF/URO, and when an identical amount of membrane protein was subjected to chromatographic analysis.

The major peak (peak 2a) was subjected to further fractionation on Con A-Sepharose (Fig. 2B). Most of the radioac-

tivity was adsorbed by the lectin column and could be eluted in 50 mM acetate, pH 6.2/0.1% (vol/vol) Triton X-100/1 M α-methyl-D-mannopyranoside/4 M urea; for the elution of radioactivity, the complete combination of detergent, sugar, and urea was required. Based on the ratio of radioactivity to protein in the material recovered from the Con A affinity column (peak 2b in Fig. 2B), a purification of about 30-fold was achieved, relative to the soluble material initially applied to the Sepharose 6B column.

Electrophoretic analysis of the purified fraction revealed the presence of four labeled components with mobilities corresponding to apparent molecular weights of 410,000, 200,000, 95,000, and 35,000 (Fig. 3).

Whereas the conditions of crosslinking chosen (5 mM glutaraldehyde, 30–40 min, 24°) were optimal for yielding sufficient soluble affinity-crosslinked protein for chromatographic

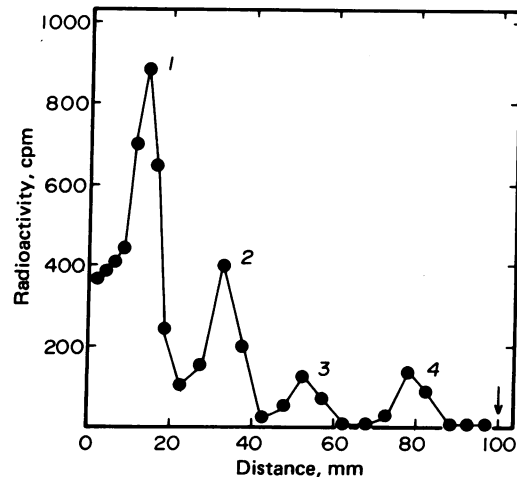


FIG. 3. Electrophoretic analysis of the soluble partially purified <sup>125</sup>I-labeled EGF/URO coupled components. Material in peak 2b from the Con A-Sepharose column (Fig. 2) was dialyzed overnight at 4° against 50 mM Tris-HCl (pH 7.7) and the volume was reduced to 1 ml under a stream of nitrogen at room temperature. To 100 μl of the concentrated sample was added, 10 μl of a 5% (wt/vol) solution of NaDodSO<sub>4</sub>. This mixture was heated at 80° for 5 min and subjected to electrophoresis in 5% crosslinked polyacrylamide gels. Radioactivity was measured in slices of unfixed gel. The arrow indicates the position of the tracking dye.

analysis, it was of interest to examine the products of cross-linking at lower glutaraldehyde concentrations (0.1, 0.5, 1.0, and 2.5 mM). At the low concentrations of glutaraldehyde, the precipitability by polyethylene glycol was substantially reduced (8–25%), indicating the presence of a large amount of uncoupled <sup>125</sup>I-labeled EGF/URO. Electrophoretic analysis (not shown) of the crude soluble material obtained at these lower concentrations of glutaraldehyde indicated the presence of peaks of radioactivity with mobilities corresponding to those of the three high molecular weight components detected after chromatographic purification (peaks 1–3, Fig. 3); the presence of the 35,000-dalton component could not be assessed because of the large amount of obscuring radioactivity (presumably free <sup>125</sup>I-labeled EGF/URO) migrating near the tracking dye. Upon increasing the concentration of glutaraldehyde, there was a shift to higher molecular weight in the proportion of radioactivity present in the three substituents detected (Table 1); the relative positions of the three peaks of radioactivity did not change. Analysis of soluble <sup>125</sup>I-labeled EGF/URO-crosslinked material coupled in the presence of an excess of unlabeled EGF/URO indicated that nondisplaceable crosslinked radioactivity accounted for less than 10% of the radioactivity detected in any portion of the analytical gel.

Because it is known that the particulate receptor for EGF/URO is sensitive to trypsin (15), it was of interest to evaluate the effects of both trypsin and chymotrypsin on intact membranes covalently labeled (5 mM glutaraldehyde, 40 min at 24° followed by borohydride) with <sup>125</sup>I-labeled EGF/URO. Brief treatment of labeled membranes (2 mg of protein per ml in 50 mM Tris-HCl, pH 7.6) with either trypsin or chymotrypsin (50 μg/ml, 10 min at 37°) yielded approximately 16% of the membrane-bound radioactivity in the supernatant (150,000 × g for 45 min). Both enzymes yielded three major components with similar electrophoretic mobilities in NaDodSO<sub>4</sub>/urea gels, corresponding to apparent molecular weights of 310,000, 170,000, and 75,000 and accounting for 70% of the radioactivity applied to the gel; approximately 20–30% of the radioactivity (possibly free <sup>125</sup>I-labeled EGF/URO) migrated with the tracking dye. All of the products of trypsin cleavage of labeled membranes with electrophoretic mobilities lower than that of the tracking dye appeared to be glycoproteins because they could be adsorbed by a Con A-Sepharose affinity column. When the membrane preparation was solubilized (1% Triton X-100) subsequent to affinity crosslinking with EGF/URO and then subjected to proteolysis with trypsin [500 μg/ml in 50 mM Tris-HCl, pH 7.6/1% (vol/vol) Triton X-100; 10 min at 37°, reaction terminated by addition of 1% (wt/vol) NaDodSO<sub>4</sub> and chilling to 4°], electrophoretic analysis indicated the presence of three major components with apparent molecular weights of 300,000, 115,000, and 60,000. Whereas all of the high molecular weight soluble components previously detected by electrophoretic analysis could adsorb to Con A-Sepharose (Fig. 3), the 60,000-dalton component resulting from trypsin treatment of soluble material was not adsorbed by Con A-Sepharose; in addition, several high molecular weight components were adsorbed by the Con A affinity column and could be detected by electrophoresis after desorption from the column with the sugar/urea/Triton X-100 mixture.

## DISCUSSION

The present work illustrates, with the receptor for EGF/URO, an approach to the characterization of membrane receptors that lose their specific ligand recognition property in the presence of detergent. Advantage is taken of the high affinity and selectivity of the particulate receptor to achieve “specific” cou-

Table 1. Relative distribution of radioactivity with increasing glutaraldehyde concentration

Glutaraldehyde, mM	% total cpm in each peak		
	Peak 1	Peak 2	Peak 3
2.5	45	45	10
1.0	8	81	11
0.5	0	70	30
0.1	0	30	70

Membranes were crosslinked in the presence of <sup>125</sup>I-labeled EGF/URO with increasing concentrations of glutaraldehyde for 30 min at 24° followed by sodium borohydride reduction. Crude solubilized membrane was analyzed electrophoretically as outlined in the legend to Fig. 3. The proportion of radioactivity in each of peaks 1–3 (Fig. 3) relative to the total radioactivity in these peaks was calculated.

pling with a bifunctional crosslinking reagent. Just as “specific” ligand binding in receptor studies with radioactive probes is defined as the binding for which unlabeled parent compound can successfully compete, so specific affinity crosslinking can be defined as that radioactivity coupled by the crosslinking reagent for which the unlabeled ligand can successfully compete. The experiments with both insulin and EGF/URO indicate that specific ligand coupling can be demonstrated for a number of membrane components subsequent to solubilization and chromatographic analysis. With insulin, although only a relatively modest proportion of the coupled radioactivity is solubilized, the chromatographic behavior of a major solubilized affinity-labeled membrane constituent corresponds to that of the unlabeled soluble receptor. It would appear that the crosslinking may occur in a restricted membrane environment such that the receptor does not become attached at random to various other membrane proteins but perhaps only to a select number of proteins in the immediate receptor vicinity. Indeed, as discussed below for EGF/URO, the multiple components detected may well represent crosslinked receptor oligomers.

Because the bifunctional reagent can link not only the ligand to the receptor but also potentially can link the receptor to many other macromolecules in the membrane as well as forming a polymer of the ligand itself, it was somewhat surprising that analysis of the solubilized EGF/URO-labeled material revealed the presence of only four major glycoprotein components of apparent molecular weights 410,000, 200,000, 95,000, and 35,000. Moreover, the results of the experiments using increasing concentrations of glutaraldehyde, in which there was a shift to discrete higher molecular weight components with increased crosslinking (Table 1), are reminiscent of work with the crosslinking of multi-subunit enzymes (29). The data can be interpreted in terms of the formation of multimers of a basic receptor subunit. Given the range of error (±6%) estimated for the electrophoretic measurement of molecular weights, and given the reservations concerning the estimates of glycoprotein molecular weights by NaDodSO<sub>4</sub>/urea electrophoresis (30), the data are consistent with the presence of an EGF/URO receptor with a “subunit” molecular weight of about 100,000 which can associate to form dimers and tetramers in the membrane; the relationship of the component of apparent molecular weight 35,000 to the larger components cannot be rationalized simply.

Brief proteolysis of EGF/URO-labeled membranes yielded three major glycoprotein components with apparent molecular weights 310,000, 170,000, and 75,000. When compared with the values obtained for solubilized material, the data suggest that only a relatively small portion of the receptor molecule may be anchored in the membrane. If, as suggested above, the sol-

uble components do represent aggregates of a 100,000-dalton receptor molecule, then the data for the tryptic fragments can be seen as consistent with the presence in the 100,000-dalton "monomer" of an anchoring portion of approximately 20,000 daltons; it is of interest that the combined transmembrane and intracellular portions of the major erythrocyte glycoprotein, glycophorin, is of comparable size (references summarized in ref. 30). Trypsin treatment of the solubilized EGF/URO-labeled material yields fragments distinctly different from intact membranes. Specifically, a major fragment of apparent molecular weight 60,000 is produced that does not adhere to Con A-Sepharose and probably represents one of the original components lacking a glycopeptide moiety; in contrast, all of the high molecular weight ( $\geq 30,000$  daltons) tryptic cleavage products from intact membranes retain an oligosaccharide portion which enables the fragments to adhere to Con A-Sepharose.

The results described in this work are indicative of the kind of information that can be gained by the affinity-crosslinking approach to receptor characterization. The initial picture of the EGF/URO binding site as a high molecular weight glycoprotein (approximately 100,000) anchored by a small portion (approximately 20,000) that can aggregate in the plane of the membrane, while yet speculative, is consistent with the data presented and is in keeping with information known about other peptide hormone receptors (3). It is important to note that the above interpretation can apply only to the material amenable to solubilization after glutaraldehyde crosslinking. It is anticipated that further work with this approach using sufficient amounts of receptor material for chemical analysis may yield the precise subunit structure of the EGF/URO receptor. It will be of interest to apply this approach to the study of other polypeptide receptors that, like the EGF/URO receptor, cannot be assayed in the soluble state. The information yielded by the affinity-crosslinking method should complement data yielded either by photoaffinity labeling methods\* or by the direct assay of soluble receptor characteristics.

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\* Since completion of this work, data have appeared (31) to demonstrate that a photoaffinity reagent prepared from mouse EGF can label a 190,000-dalton constituent in cultured 3T3 fibroblasts. The estimate of the subunit molecular weight of the EGF/URO receptor in the present work is in excellent accord with that value.

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