Artificial Hybrid Protein Containing a Toxic Protein Fragment and a Cell Membrane Receptor-binding Moiety in a Disulfide Conjugate

II. BIOCHEMICAL AND BIOLOGIC PROPERTIES OF DIPHTHERIA TOXIN FRAGMENT A-S-S-HUMAN PLACENTAL LACTOGEN

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The biochemical and biologic properties of a purified disulfide conjugate of diphtheria toxin fragment A and human placental lactogen (toxin A-hPL) have been studied by (a) assaying the ADP-ribosyltransferase activity of the intact conjugate, (b) assaying the binding of the intact conjugate to mammary gland plasma membrane lactogenic receptors, and (c) assaying the effect of the conjugate on the rate of protein synthesis in rabbit mammary gland explants maintained in organ culture. The toxin A-hPL conjugate retains one-third of the NAD⁺:EF-2 ADP-ribosyltransferase activity of toxin A, and 26% of the hPL-binding activity to lactogenic receptors. Binding activity was demonstrated by radioreceptor assay and by assaying toxin A activity bound to membranes which was competitively displaced by excess hPL. Since the toxin A-hPL conjugate retained activities of its separate subunits, it could be regarded as a structural analogue of nicked diphtheria toxin in which the B chain or binding chain has been replaced by another binding protein, albeit with different surface membrane receptor specificity (1).

The properties of the parent toxin have been well studied (2, 3). Nicked diphtheria toxin, a disulfide-linked two-chain molecule, binds via its B chain to receptors located on the surface membrane of sensitive cells with an affinity constant of $10^6 \text{M}^{-1}$ (4, 5). After a short lag period the toxin, or its active A chain, enters the cell cytosol through some as yet unknown process. Entry of the toxin A fragment is detected by observing the loss of functional elongation factor II or the loss of protein synthesis which has resulted from ADP-ribosylation of EF-2 catalyzed by the toxin A fragment.

We have synthesized the toxin A-hPL analogue of nicked diphtheria toxin in order to investigate the mechanism of receptor-mediated entry of protein into cells which the parent toxin exhibits. General questions such as the following are of interest: (a) do all surface membrane receptors mediate entry of bound protein molecules, and (b) is the entry of disulfide-linked two-chain toxins directed by only the properties of the binding chain?

In this report we present data showing that both toxin A enzymatic activity and hPL lactogenic receptor-binding activity are conserved in the toxin A-hPL conjugate. However, when toxin A-hPL conjugate is incubated with lactating mammary gland explants, no decrease in protein synthesis occurs, indicating that the lactogenic receptor is unable to mediate entry of the toxin A fragment of the toxin A-hPL conjugate to the toxin site of action within the cell cytosol. These results along with recent findings that lactogenic hormones do enter mammary gland cells (6, 7) are discussed in terms of several models of protein entry which are consistent with both sets of data.

A disulfide-linked conjugate of human placental lactogen and diphtheria toxin fragment A has been prepared and purified to 90% purity (1). In this paper we report on some of the biochemical and biologic properties of this artificial protein hybrid conjugate.

The toxin A-hPL conjugate may be thought of as a structural analogue of nicked diphtheria toxin in which the B chain or binding chain of the toxin has been replaced by another binding protein, albeit with different surface membrane receptor specificity (1).

The abbreviations used are: hPL, human placental lactogen; albumin, bovine serum albumin; EF-2, elongation factor II; SMVA-hPL, S-sulfomercaptovaleramidyl hPL; Nbs, 5,5'-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate.
Experimental Procedures

Materials

These were obtained from the following sources; ovine prolactin, Endocrinology Study Section, National Institute of Arthritis, Metabolic and Digestive Diseases; lactoperoxidase, grade B, Calbiochem; bovine serum albumin, Pentex; dihydroxyethyl disulfide treated EF-2 and N-Ethylmaleimide-treated albumin- Partially purified EF-2 (11 was a specified excess of a competitive analogue. Tracer-specific binding and nonspecific binding (11). Partially purified EF-2 (11 was a specified excess of a competitive analogue. Tracer-specific binding and nonspecific binding (11). Partially purified EF-2 (11 was a specified excess of a competitive analogue. Tracer-specific binding and nonspecific binding (11).

Methods

Preparation of Dihydroxyethyl Disulfide-treated EF-2 and N-Ethylmaleimide-treated Albumin- Partially purified EF-2 (11 was a specified excess of a competitive analogue. Tracer-specific binding and nonspecific binding (11). Partially purified EF-2 (11 was a specified excess of a competitive analogue. Tracer-specific binding and nonspecific binding (11).

Radioreceptor Assay for Determining Binding of Toxin A-hPL to Lactogenic Receptors- The radioreceptor assay reported by Shiu et al. (3) utilizing 125I-prolactin tracer and lactating rabbit mammary gland membranes was used to determine toxin A-hPL binding to lactogenic receptors. The activity of toxin A-hPL was compared with hPL and prolactin in their ability to compete with 125I-prolactin for receptor sites. Conditions were varied to the extent that our reaction mixtures were incubated at 25°C for 6 h in 0.14 M Beckman microcentrifuge tubes in a final volume of 150 μl in the same manner as previously described for the binding study of 125I-insulin to rat liver plasma membrane (10). Each reaction mixture usually contained 80 to 90 μg of rabbit mammary plasma membrane (Lowry protein equivalent to bovine serum albumin standard) and 30,000 cpm of 125I-prolactin diluted in 25 mM Tris/chloride, pH 7.6, containing 10 mM CaCl2 and 0.3% bovine serum albumin. Binding of 125I-prolactin to rabbit explant was studied under the same conditions except that the plasma membrane was replaced by three pieces of uniformly cut explants weighing totally 4.5 to 6.0 mg which were added to pre-mixed reaction mixtures and mixed with the solution by gentle stirring with a stainless steel rod. The explants were excised as described in the organ culture section. All of the above binding studies were run in triplicate, and the amount of radioactivity in each membrane or tissue pellet counted in a Beckman Biogamma counter.

In previous receptor studies, total binding is resolved into two additive components, specific binding and nonspecific binding (11).

Specific binding is that component which exhibits saturation and therefore is a good measure of the specific binding of the radiolabeled hormone to the receptor without exhibiting saturation or competition and is linearly related to free tracer concentration. Operationally, the nonspecific binding of a tracer is taken as the amount of binding occurring in the presence of a specified excess of a competitive analogue. Tracer-specific binding is equal to the total binding minus the nonspecific binding of the tracer.

Toxin A Activity Assay for Determining Binding of Toxin A-hPL to Lactogenic Receptors- Binding of toxin A-hPL conjugate to rabbit mammary plasma membrane was studied by incubating varied amounts of the conjugate (each in triplicate) with the plasma membrane in the absence and presence of 50 μg/ml of hPL under the same conditions as described in the radioreceptor assay, and assaying the amount of conjugate (via toxin A activity) remaining in the membrane pellet after centrifugation. These experiments were usually conducted on the same day by using the same amount of plasma membrane as the radioreceptor assay. After centrifugation and removal of the supernatant solution, the membrane pellet was dissolved in 100 μl of 0.1% sodium dodecyl sulfate in 50 mM Tris/chloride, pH 8.2, containing 1 mM EDTA and 3 mg/ml of albumin. A 50-μl aliquot of this solution was then assayed for ADP-ribosyltransferase activity in the presence of 40 mM diethiothreitol as described in the preceding paper (11). A series of standard toxin A-hPL conjugate solution (containing 0 to 5.0 ng) was also assayed in parallel to construct a standard curve for determining the amount of conjugate corresponding to the activity found in the pellet. Under these conditions no inhibition of toxin A-specific ADP-ribosyltransferase activity by sodium dodecyl sulfate or solubilized membrane was observed. The difference among triplicates of each experimental point was usually less than 10%. The difference in the amount of conjugate found in the membrane pellet between the reaction mixtures in the presence and absence of excess hPL was taken as specific binding of the conjugate (as reported in the fourth column of Table II) through hPL receptors on the membrane.

An expected value of specific binding of the conjugate is also calculated for comparison from the data of radioreceptor assay. This calculation is based on the assumptions that the binding activity of toxin A-hPL conjugate observed in the radioreceptor assay represents the binding activity of the intact conjugate and that the same receptor sites were involved in the binding of both bovine prolactin and toxin A-hPL conjugate, so that their binding activity was different only in the affinity to the receptor. Thus, according to the Scatchard formulation (12), a given number of molecules of prolactin specifically bound to each milligram of plasma membrane, Bn, is related to the concentration of free hormone, F, and the association constant, K, as below

$$B_n = \frac{nK_F}{1 + K_F F}$$

where n denotes total number of receptor site per milligram of plasma membrane. A similar equation for bound conjugate may be written as

$$B'_{10} = \frac{nK'_F F'}{1 + K'_F F'}$$

where $B'_{10}$, $K'_F$, and $F'$ are the binding parameters for the conjugate corresponding to $B_n$, $K_F$, and F, respectively. When $B_n = B_{10}$, i.e. under the conditions that both prolactin and toxin A-hPL conjugate occupy the same number of receptor sites per mg of plasma membrane, the relation

$$K_F F = K'_F F'$$

exists. It is evident that the condition to fulfill this relation is $K_F = K'_F$, or $F = (K_F/K'_F) \times F$. Thus, from this relation, the difference between the concentration of free conjugate and free prolactin to maintain occupancy of the same number of receptor sites is by a factor of $K_F/K'_F$, namely their relative affinity. The relative affinity of prolactin and toxin A hPL, however, may be closely approximated from the ratio of the concentration of prolactin and toxin A-hPL to exhibit 50% inhibition of maximum specific binding of 125I-prolactin tracer in the radioreceptor assay $C_{125}$ and $C_{125}'$, respectively as below

$$K_F = \frac{C_{125}}{C_{125}'}$$

and therefore

$$F' = \frac{C_{125}'}{C_{125}} \times F$$

Since the amount of prolactin specifically bound, $B_n$, for each free prolactin concentration $F$, can be calculated from the radioreceptor assay, the binding (or specific) binding of free conjugate, $F'$, at which the same $B_n$ is maintained can be calculated from Equation 5. Therefore, from $B_n$, and calculated $F'$ a standard curve of $B_n$ versus log $F'$ can be constructed. On the other hand, the concentration of free conjugate can be calculated from the experimental value of the amount of
conjugate that is not retained by the membrane pellet in the absence of excess hPL. An expected amount of the conjugate specifically bound to the plasma membrane can be obtained by interpolation of this experimental free conjugate concentration on the standard curve. If the experimentally determined amount of the conjugate bound to the membrane pellet is close to zero with the +15% variation, it may be considered that the binding activity of the conjugate observed in the radioreceptor assay represents the activity of intact toxin A-hPL conjugate.

**Assay of ADP-ribosyltransferase Activity of Intact Toxin A-hPL Conjugate** - ADP-ribosyltransferase activity of intact toxin A-hPL was assayed as described for toxin A in the preceding paper (1) with the following modifications. In these experiments dithiothreitol was omitted; dihydroxymethylenedilute TF was used as substrate; and N-ethylmaleimide-treated albumin was used to prepare dilution for toxin A-hPL and toxin standard solutions. Under this assay condition, a nonlinear increase in ADP-ribosylation of EF-2 was observed with increasing concentration of toxin A or toxin A-hPL. This result is not surprising, since the concentration of [14C]NAD+ (0.4 μM) and probably that of EF-2 both fall below their Km values (13). Since increasing these substrates (particularly EF-2) was prohibitive, the activities of the conjugate and toxin A were compared by the molar concentration of these proteins for catalyzing transfer of 10% of total radioactivity from [14C]NAD+ to the trichloroacetic acid precipitate of EF-2 after 15 min of reaction at 25 °C. The content of ADP-ribosyltransferase activity of intact toxin A-hPL and nicked Diphtheria Toxin - The content of toxin A in toxin A-hPL and nicked toxin was determined by assaying the toxin A-specific ADP-ribosyltransferase activity after complete reduction of these proteins. Nicked toxin or toxin A-hPL was treated with 40 mm dithiothreitol in 50 mm Tris·HCl pH 8.5, 1 mm EDTA for 1 h at room temperature and then diluted with the same buffer without dithiothreitol, but containing 0.3% albumin. An aliquot from each diluted solution (1 to 6 ng in nicked toxin or 1 to 5 ng in toxin A-hPL) was then assayed for ADP-ribosyltransferase activity in the presence of 20 mm dithiothreitol. The content of toxin A in each solution was then calculated by interpolating the observed activity on a standard activity curve of parallelly assayed toxin A (0.2 to 2 ng) in a range where only about 20% or less of total radioactivity of [14C]NAD+ was transferred to EF-2. The difference in toxin A content in the conjugate found between the two measurements by this method was within 10%. Similar results were obtained when the toxins A were measured directly without prior reduction.

**Mammary Gland Organ Culture** - Organ cultures were set up generally as described (14) on washed and siliconeized VWR cultured covered dishes (35 × 10 mm) containing 2 ml of medium 199, modified with Earle's salts plus glutamine (Microbiological Associates). Each 500 ml of medium contained in addition 300 μl of hydrocortisone, 8 mg/ml in ethanol, 1 ml of porcine insulin, 2.5 mg/ml in 5 mm NaCl, and 17.5 mg of bovine serum albumin. Following filtration, the medium was filtered through a 0.45-μm Nalgene filter unit 245. When hPL-containing medium was desired, hPL was pretreated by gel filtration on Sephacry G-50, medium, in 0.01 m sodium phosphate buffer, pH 7.4. Material from the peak tubes was added to a final concentration of 5 μg/ml. After filtration the hPL concentration by radioreceptor assay was 9.5 μg/ml. The pH of the medium was 7.3 to 7.4 and was maintained at this value during incubation at 37° by an atmosphere of 5% CO₂, 95% O₂. A lactating New Zealand rabbit was killed by cervical dislocation and at 0.77 mg/ml in 0.3% albumin recovery was 92%. Toxin A-hPL conjugate was filtered at 2.9 mg/ml and filtering loss was assumed to be negligible. Toxin A required carrier protein during Millipore filtration; the toxin A-hPL conjugate recovery was 92%. Toxin A-hPL conjugate was filtered at 0.1 mg/ml. Recovery was 40% assayed by hPL radioreceptor assay and 57% in the toxin A activity assay (1) as a value of 50% was used for calculations. Protein in organ-culture explants was assayed at 0 to 4 h and 16 to 20 h by adding 40 μl of 0.1 M 14C urea, 0.1 mg/ml, and reincubating for 4 h, at which time explants were blotted, quickly weighed, and homogenized in a small conical ground glass motor-driven homogenizer (Dual 20, Kontes Glass Co.) at 1500 rpm for four strokes in 1 ml of medium containing 0.7 mg/ml of casein as carrier (16). (Casein stock solutions were prepared by adding 10 mg of casein to 10 ml of H₂O and dialyzing with NaOH with frequent blending in a Vortex mixer to pH 8.5, over a 15-min period without allowing the pH to exceed 8.5. Approximately 90% of the casein becomes solubilized. Casein medium was cleared of insoluble casein by centrifugation at 40,000 × g for 30 min.) The homogenate and a 0.75-mg casein medium wash were pooled. An aliquot of 250 μl was mixed with 50 μl of 30% trichloroacetic acid and incubated at 70° for 30 min to hydrolyze RNA. The precipitate was pelleted by centrifugation (Eppendorf 3200, 2 min), and the supernatant was removed. The pellet was washed by resuspension and centrifugation with 0.25 ml of 5% trichloroacetic acid, followed by 0.5 ml of 2-propandiol/butanol (1:2, v/v) to extract lipid. The final pellet was solubilized in 0.5 ml of 0.045 m sodium EDTA, pH 8.5, and counted in 10 ml of toluene-TLA-Bisolve (Beckman) using a narrow 0°C window. Counts are normalized per mg wet weight of explant and per 4-h incubation time because of variations in incubation time (<20%).

**RESULTS**

**Binding of Toxin A-hPL Conjugates and Precursors to Lac-

togenic Receptors Determined by Radioreceptor Assay** - The specific binding activities of hPL, S-S-sulfomercaptovaleri-

dinated hPL, and toxin A-hPL conjugates were studied by comparing their ability to competitively inhibit binding of 125I-


trolactin to rabbit mammary plasma membrane. The results are presented by the competition curves shown in Fig. 1. The parallelism of these competition curves suggests that prolac-


tin, hPL, and hPL derivatives all interact with rabbit mam-


mary plasma membrane through the same receptor sites with different binding affinities. The curve for toxin A-hPL conju-


gate competition is drawn from points (□---□) obtained from five different preparations. These preparations are similar by this assay and show 26% of the binding activity of unmodified hPL. This activity is more than can be accounted for on the basis of an estimated contamination with hPL dimer of 5% (1, 16).

A gradual decrease in binding activity of S-S-sulfomercap-

tovaleminated hPL derivatives was observed when the ex-


tent of amidination was increased from about 1 to 4 mol/mol. On the other hand the binding activities of conjugates synthe-


sized from these S-sulfonates were virtually unrelated to the extent of amidination in their precursors. The concentration of total unlabeled hormone or hormone derivatives at which binding of 125I-prolactin was inhibited by 50% were, respectively, prolactin, 0.325 nm; hPL and SMVA-hPL (0.8), 1 nm; SMVA-hPL (1.8), 1.5 nm; SMVA-hPL (4.0), 2.1 nm; and toxin A-hPL conjugates, 3.8 nm.

When the mammary organ culture medium containing 3% albumin was substituted for the usual binding buffer, the hPL competition curve for 125I-labeled ovine prolactin was un-


changed. However, counts in the membrane pellet were re-


duced by 50% in this medium due to poor pelleting of the membranes, a consequence of the relative calcium deficiency of the culture medium.

The ability of rabbit mammary explant to bind hPL was also studied by radioreceptor assay using tracer 125I-prolactin. The results summarized in Table I clearly indicate that rabbit mammary explants are capable of specific binding increasing 125I-prolactin which can be progressively inhibited by increasing concentration of unlabeled hPL. This competition data for explants is comparable with that for membranes shown in Fig. 1. The lower tracer specific binding seen in the explant-binding ex-


periments, 3% as compared to 30% for the membrane experi-


ments.
Biochemical Properties of Diphtheria Toxin Fragment A-S-S-Human Placental Lactogen Determined by Toxin A Activity Assay—The ability of toxin tissue fragments and the presence of diffusion barriers in the membrane indicate that the conjugate is active in binding to prolactin receptors on the membrane. However, it may be conjectured that the binding activity observed in the above radioreceptor assay is due to hPL or an active derivative generated from the conjugate during incubation with the plasma membrane. In order to eliminate this interpretation of the data, it is necessary to measure directly the binding of intact conjugate to plasma membrane. This may be achieved by measuring the binding of toxin A moiety of the conjugate to plasma membrane in the absence and the presence of excess hPL under the same conditions of radioreceptor assay. If the toxin A moiety of the conjugate is bound to the plasma membrane via specific interaction between the hPL moiety of the conjugate and the lactogenic membrane receptors, toxin A binding should be competitively inhibited by excess hPL. The results of this type of experiment are shown in Table II. At each concentration of toxin A-hPL a certain fraction of toxin A-hPL bound to the membranes is competitively inhibited in the presence of 2.3 μg hPL. This amount of binding is considered to be specific binding and constitutes 40% of the binding at the lowest concentration of toxin A-hPL. The amount of conjugate bound in the presence of 2.3 μg hPL is regarded as nonspecific binding (see "Methods") and is ~3% of the total conjugate in each reaction mixture. When toxin A is incubated with membranes, binding also occurs but is unaffected by the presence or absence of excess hPL (not shown). When the data in Table II are plotted as specific bound conjugate against free conjugate, considerable scatter is evident and a binding constant cannot be calculated. In part this is due to the high value of nonspecific binding relative to the specific binding, which must be subtracted from the total binding. It should be emphasized that according to the definition of nonspecific binding (see "Methods" and Ref. 17), the observation of higher nonspecific binding than specific binding is not unusual for an experiment in which high concentration of a weak binding tracer (in the present case: toxin A-hPL) is used.

Table I

| hPL concentration (nM) | ¹²⁵I-Prolactin bound (cpm/mg) |
|------------------------|-----------------------------|
| 0                      | 356 ± 21                    |
| 0.23                   | 348 ± 29                    |
| 0.46                   | 248 ± 13                    |
| 0.63                   | 175 ± 23                    |
| 4.63                   | 169 ± 18                    |
| 458.0                  | 160 ± 22                    |

Table II

| Toxic A-hPL concentration (nM) | Total conjugate bound (pmol/mg) | Conjugate specifically bound (pmol/mg) |
|-------------------------------|---------------------------------|---------------------------------------|
|                               | hPL                             | +hPL                                  |
| Experiment 1                  |                                 |                                      |
| 0.33                          | 0.047                           | 0.024                                 |
| 0.99                          | 0.108                           | 0.071                                 |
| 3.29                          | 0.171                           | 0.127                                 |
| 9.87                          | 0.533                           | 0.416                                 |
| 19.70                         | 1.108                           | 0.988                                 |
| Experiment 2                  |                                 |                                      |
| 2.19                          | 0.187                           | 0.152                                 |
| 6.58                          | 0.442                           | 0.348                                 |
| 11.00                         | 0.572                           | 0.474                                 |
| 21.90                         | 1.157                           | 1.019                                 |

The conjugate used was toxin A-hPL (1.8) of Fig. 1. Similar data were obtained with other conjugates.

Thus, in Fig. 1, the ratio of tracer-specific binding (30%) to nonspecific binding (2%) at 0.122 nM ¹²⁵I-prolactin is 15. This ratio is expected to decrease to about 1.5 for a tracer that binds 10 times weaker (toxin A-hPL) if the experiment is carried out at the same tracer concentration (the receptor or plasma membrane concentration are the same for both experiments). This ratio is expected to decrease further with higher tracer concentrations which achieve significant fractional occupation of the
total sites. In order to compare the conjugate-binding experiments utilizing toxin A binding and radioreceptor assays, the expected binding for the Table II experimental conditions has been calculated from the radioreceptor binding curves in Fig. 1 as outlined under "Methods." The found value is about one-half the expected value at each concentration of conjugate. It appears that at least one half of the conjugate binding observed in the radioreceptor assay represents binding due to intact toxin A-hPL conjugate.

Susceptibility of Intercal Disulfide Bond of Toxin A-hPL and Nicked Diphtheria Toxin to Reduction - Toxin A-hPL is much more sensitive to intercal disulfide bond reduction than nicked diphtheria toxin. Exposure to 1 mM cysteine, pH 8.2, for 10 min results in about 50% reduction of toxin A-hPL into subunits (Fig. 2, Gel c), whereas identical conditions applied to nicked diphtheria toxin show no discernible reduction (Fig. 2, Gel f). Approximately 10% reduction of toxin A-hPL occurs at 0.1 mM cysteine for 10 min (Gel b).

The buffer and time conditions for these reduction experiments are identical with the assay conditions for toxin A used to assess the ADP-ribosyltransferase activity of toxin A-hPL using dihydroxyethyl disulfide-treated EF-2 and N-ethylmaleimide-treated albumin. The maximum -SH concentration in this assay is 4 µM (see "Methods"). On the basis of the above results no significant reduction of toxin A-hPL conjugate to free toxin A would be expected under this transferase assay.

Almost complete reduction of nicked diphereria toxin was achieved with 20 mM dithiothreitol (Fig. 2, Gel g). This agent was subsequently used to assess the toxin A content of conjugates by their ADP-ribosyltransferase activity following reduction.

Content of Toxin A in Toxin A-hPL Conjugates - The content of toxin A in all three toxin A-hPL conjugates measured in the presence of 40 mM dithiothreitol is summarized in Table III and is equal to about one-half the conjugate mass. These values agree with the values calculated for a 1:1 toxin A-hPL conjugate, considering the 10% error of the assay (see "Methods"). A control measurement of the toxin A content of nicked diphtheria toxin is also close to the expected value. These results are also in agreement with the previously reported results of SDS gel electrophoresis of partially reduced conjugate (1).

ADP-ribosyltransferase Activity of Toxin A-hPL - The toxin A moiety of toxin A-hPL conjugate retained part of its ADP-ribosyltransferase activity when the intact conjugate was assayed in the absence of dithiothreitol using dihydroxyethyl disulfide-treated EF-2. As summarized in Table IV, all but one of the assayed toxin A-hPL preparations were about one-third as active as free toxin A-SH in ADP-ribosyltransferase activity on an equimolar basis. The lower activity found in toxin A-hPL prepared from SMVA-hPL containing four introduced —SS03— groups may reflect the effect of this substituent (Table IV) and rises to 100% under reducing conditions (Table II).

Since the transferase activity of the conjugate relative to equimolar toxin A is one-third under nonreducing conditions (Table IV) and rises to 100% under reducing conditions (Table III).

### Table III

| Preparation % | Found | Calculated % |
|--------------|-------|--------------|
| Nicked toxin | 30    | 35<sup>a</sup> |
| Toxin A-hPL (0.7) | 51 | 48.8 |
| Toxin A-hPL (1.8) | 57 | 48.8 |
| Toxin A-hPL (4.0) | 40 | 48.8 |

<sup>a</sup> Calculated on assumption that toxin B has a M<sub>r</sub> of 39,000 (3).

### Table IV

| Concentration for 10% incorporation of total | Relative activity |
|--------------------------------------------|-------------------|
| 1,500 ng/100 µg | 100              |
| Toxin A-SH | 0.22 |
| Toxin A-hPL (0.7)* | 0.66 |
| Toxin A-hPL (1.8)* | 0.61 |
| Toxin A-hPL (4.0)* | 1.78 |

* The number in parentheses represents the number of S-sulfonate in moles per mole in hPL S-sulfonate from which the toxin A-hPL conjugate is prepared.
Effect of thiol pretreatment on ADP-ribosyltransferase activity of toxin

1.8 mol of -SSO,-lmol of hPL.

Pretreatment conditions are 50 mM Tris/HCl, 1 mM EDTA, pH 8.2, 25° for 10 min except in the case with dithiothreitol which is 30 min. Assay performed after a 1250-fold dilution of the pretreatment reaction using an EF-2 preparation freed of residual free thiol by extensive dialysis and filtration through Sephadex G-25. This preparation of toxin A-hPL was prepared from hPL-S-sulfonate containing 1.8 mol of -SSO,-mol of hPL.

| Thiol            | Concentration | ADP-ribose incorporated (pmol/35 min/mg) |
|------------------|---------------|----------------------------------------|
| Cysteine         | 0.05          | 3.99                                   |
|                  | 0.10          | 4.62                                   |
|                  | 0.50          | 5.02                                   |
|                  | 1.00          | 5.71                                   |
|                  | 5.00          | 7.66                                   |
|                  | 10.00         | 7.92                                   |
|                  | 20.00         | 7.82                                   |

| Dithiothreitol   | 50.00         | 8.41                                   |

III) it was of some interest to study the range of intermediate reducing conditions. This was done by preincubating conjugate or nicked diphtheria toxin with various concentrations of cysteine and then measuring ADP-ribosylation after a 1250-fold dilution (in order to minimize varying thiol effects on the assay). These results are shown in Table V and should be compared with the cysteine concentrations required for reduction of the interchain disulfide bond in Fig 2. Nicked toxin activity is correlated with interchain disulfide reduction, as has been previously reported (3). Conjugate activity is 46% of the maximum in the absence of added cysteine. The cysteine concentration at which one-half of the increase to maximum activity occurs is 1 mM, a concentration found by the gel data to give 50% reduction.

Mammary Gland Organ Culture Assay of Toxin A-hPL; Effect on Protein Synthesis —The effect of toxin A-hPL conjugate and its precursors on the rate of protein synthesis of lactating rabbit mammary gland explants are shown in Table VI. After 20 h of incubation the counts from L-[14C]leucine incorporation into protein are the same for explants exposed or not exposed to toxin A-hPL conjugate. The presence of hPL resulted in a 26% increase in incorporation over no addition, which may be significant. Intact diphtheria toxin causes a reduction of over 95% in incorporated counts while the counts in the toxin A addition is down by 80%. This latter effect is undoubtedly due to the intact or nicked toxin contamination of the toxin A which we estimated by SDS gel electrophoresis to be 0.3%. It appears that this assay at 20 h is sensitive to 15 ng/ml of intact toxin, a range of sensitivity equal to that of HeLa cells in tissue culture (18). After 4 h of incubation the effect of 5 µg/ml of diphtheria toxin is already seen with a 70% reduction in L-[14C]leucine incorporation; however, the lower concentration of toxin in the toxin A control has not had sufficient time to achieve its potential inhibition. At the end of both the 4- and 20-h incubations of toxin A-hPL conjugate with explants, the presence of intact conjugate was documented by both SDS gel electrophoresis and radioreceptor assay of the culture medium. No appreciable loss of conjugate was detected. These results show that toxin A-hPL conjugate is without detectable effects on mammary gland protein synthesis under conditions

**TABLE V**

| Effect of thiol pretreatment on ADP-ribosyltransferase activity of toxin A-hPL and nicked diphtheria toxin |
|--------------------------------------------------------------------------------------------------|
| Pretreatment conditions are 50 mM Tris/HCl, 1 mM EDTA, pH 8.2, 25° for 10 min except in the case with dithiothreitol which is 30 min. Assay performed after a 1250-fold dilution of the pretreatment reaction using an EF-2 preparation freed of residual free thiol by extensive dialysis and filtration through Sephadex G-25. This preparation of toxin A-hPL was prepared from hPL-S-sulfonate containing 1.8 mol of -SSO,-mol of hPL. |

**TABLE VI**

| Effect of toxin A-hPL conjugate and intact diphtheria toxin on protein synthesis of lactating mammary gland explants |
|---------------------------------------------------------------------------------------------------------------|
| Explants were maintained in organ culture at 37° as described under “Methods.” All additions were at time zero. Toxin A, hPL, and cycloheximide additions served as controls. Two pulses with L-[3H-]leucine were performed at zero time and 16 h and lasted approximately 4 h. Counts per min (CPM) refers to the average of trichloroacetic acid-insoluble counts which have been normalized to 4 h. Numbers in parentheses refer to number of assays. Standard deviation of the mean are given for triplicate assays. |

where (a) the gland is sensitive to the effects of toxin and (b) the gland contains hPL receptors.

**DISCUSSION**

The results presented in this report show that the toxin A-hPL disulfide conjugate which we have synthesized and purified retains both lactogenic receptor-binding activity of hPL and ADP-ribosyltransferase activity of toxin A. Transferase activity was assayed in the absence of reducing agent using an EF-2 preparation treated with dihydroxyethyl disulfide. The assayed activity was 33% of the activity found after reduction of the interchain disulfide bond. This level of activity most likely reflects the activity of the intact conjugate and cannot be accounted for by the 5% estimated toxin A dimer contamination of the conjugate (1). In contrast to the conjugate, nicked diphtheria toxin has only 1% of its full activity when assayed under nonreducing conditions. The masking effect is associated with the COOH-terminal portion of the B chain and is absent in the toxin mutant CRM45 which is missing this region (2).

Lactogenic binding activity of the conjugate was assayed utilizing lactating rabbit mammary gland membranes and [3H]-labeled ovine prolactin tracer. The competition curves for prolactin, hPL, and toxin A-hPL conjugate are parallel, but displaced toward higher concentrations, indicating that the same receptor is involved in each case but with altered affinities (Fig. 1). Toxin A-hPL conjugate was 26% as active as hPL on a molar basis. This level of activity could not be explained by the 5% estimated contamination of conjugate with hPL dimer (1). The reduced binding activity of toxin A-hPL is not unexpected, since the additional protein mass would more than likely interfere with the receptor-hPL conformational fit which has presumably been selected by evolutionary pressure. An example of extra protein mass reducing binding activity is proinsulin, where binding to insulin receptors is reduced by 80% as compared to insulin (19). To eliminate the possibility that the lactogenic binding activity observed in the radioreceptor assay of conjugate was due to free hPL, split from the conjugate during assay, the binding of conjugate was followed by a toxin A assay. Specific binding of conjugate via lactogenic receptors was considered to be the binding which was effectively blocked by excess of free hPL. When conjugate at 0.33 nm was incubated with membranes, 0.047 pmol of toxin A/mg of membrane protein was bound. When the incubation was performed in the presence of excess hPL, this value dropped to
0.023 pmol/mg giving a ratio of specific binding to total binding of 49% (Table II). The amount of conjugate bound at each conjugate concentration was about one-half the value calculated from the radioreceptor competition curves. However, because of scatter in the points of the radioreceptor assay the exact position of the toxin A-hPL conjugate competition curve is uncertain, and the observed and calculated values lie within this uncertainty.

Since the toxin A-hPL conjugate contained both toxin A and lactogenic receptor-binding activity it was of some interest to see whether this conjugate could act as a functional analogue of diphtheria toxin with altered receptor specificity. Could the lactogenic receptor mediate the entry of toxin A from bound toxin A-hPL conjugate to its site of action in the cytosol? This was tested by incubating lactating rabbit mammary gland explants with conjugates and assaying protein synthesis. No effect was observed under assay conditions which were sensitive to concentrations of intact diphtheria toxin 330-fold less than used for toxin A-hPL (Table VI). At the end of the incubation of explant with conjugate, the presence of conjugate was documented by assaying the organ culture medium by gel electrophoresis and radioreceptor assay. In addition, the activity of the lactating mammary gland explants used in this experiment to bind lactogenic hormones was documented by radioreceptor assay. These results indicate that the lactogenic receptor is unable to mediate the entry of toxin A or toxin A-hPL from the receptor-bound conjugate to the cell cytosol. Several explanations of these results are possible. The conjugate could differ in important properties from nicked diphtheria toxin and therefore not represent a functional analogue. The enhanced susceptibility of the conjugate to reduction compared to nicked toxin might result in interchain reduction and chain separation at the cell surface prior to entry. Another possibility is that the toxin A moiety, while not appreciably interfering with conjugate binding, might sterically interfere with entry. Experiments to test these possibilities are in progress.

Other explanations of these results are concerned with the properties of the lactogenic receptors. A simple explanation states that there are two classes of receptors, those specialized for protein entry into the cytosol and those lacking this characteristic, and lactogenic receptors fall into the latter category. This explanation, however, must be reconciled with recent data which indicate that lactogenic hormones enter the interior of mammary gland cells. Whitworth and Grosvenor (6) have reported that during an intravenous infusion of prolactin into lactating rats using histochemical techniques dependent on a reaction involving antiprolactin antibodies and a second chromogenic antibody. These two possibilities are in progress.

The entry of diphtheria toxin into sensitive cells can proceed by mechanisms which do not lead to loss of protein synthesis (21). Using biologically active "3-labeled diphtheria toxin, cellular uptake can be quantitated. Uptake is rapid, involves large quantities of toxin and is not detectably influenced by the presence of 4 mM ammonium ion. However, this concentration of NH4+ prevents toxin-mediated loss of protein synthesis. Ammonium ion is without effect on toxin binding to cells or on the ADP-ribosylation of EF-2 in vitro. The level of toxin uptake determined by labeled toxin is either in the presence or absence of NH4+, over 1000-fold more than that required to inactivate protein synthesis. Using the turnover number of toxin A, the intracellular EF-2 concentration and rate of synthesis of EF-2, it has been calculated that only a few toxin molecules per cell are needed to inactivate protein synthesis (2). One interpretation of these results, proposed by Bononcini et al. (21), which we find attractive is that there are two entry mechanisms which distribute toxin (or other proteins) to two separate sites. The mechanism observed with labeled toxin is a high capacity system and the entered toxin is bound within endocytotic vesicles or some other compartment from which it cannot escape. This mechanism may be similar to the mechanism for prolactin transport and maternal globulin transport into fetal circulations. The second mechanism is a low capacity system receptor-mediated, transporting at most only 100 to 1000 molecules/h to the soluble phase of the cell cytosol. This mechanism is responsible for action of diphtheria toxin and is blocked by NH4+. Toxin or conjugates containing toxin A transported by the first mechanism would be inactive, since they could not interact with EF-2 which cycles between the soluble phase of the cell cytosol and its ribosomal binding site.

In summary, a conjugate protein hybrid of diphtheria toxin Fragment A and human placental lactogen has been synthesized and purified. This conjugate retains the separate binding and enzymatic activities of its subunits. For use as a probe the conjugate can be regarded as a structural analogue of diphtheria toxin in which the receptor-binding chain of the toxin has been substituted by another binding protein, now made specific for lactogenic receptors. When assayed with mammary gland cells containing lactogenic receptors no intracellular toxin activity was detected, indicating that the toxin A-hPL conjugate did not behave as a functional analogue of diphtheria toxin. This occurred in spite of evidence indicating that lactogenic hormones can enter and be transported through mammary gland cells. Several explanations focusing on both the properties of the receptors involved and the properties of the toxin analogue which we have synthesized have been considered. Whether or not other analogues of diphtheria toxin can be synthesized which have altered receptor specificities and which can function to inhibit cellular protein synthesis will require further investigation.

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REFERENCES
1. Chang, T.-M., and Neville, D. M., Jr. (1977) J. Biol. Chem. 252, 1505-1514
2. Pappenehimer, A. M., Jr., and Gill, D. M. (1978) Science 182,
Biochemical Properties of Diphtheria Toxin Fragment A-S-S-Human Placental Lactogen

353–358
3. Collier, R. U. (1975) Bacteriol. Rev. 39, 54–85
4. Itelson, T. R., and Gill, D. M. (1973) Nature 242, 330–332
5. Uchida, T., Pappenheimer, A. M., Jr., and Harper, A. A. (1973) J. Biol. Chem. 248, 3845–3850
6. Whitworth, N. S., and Grosvenor, C. E. (1976) Fed. Proc. 35, 1366
7. Nolin, J. M., and Witorsch, R. J. (1976) Fed. Proc. 35, 1365
8. Shiu, R. P. C., Kelly, P. A., and Friesen, H. G. (1973) Science 180, 968–971
9. Thorell, J. I., and Johansson, B. G. (1971) Biochim. Biophys. Acta 251, 363–369
10. Soll, A. H., Kahn, C. R., and Neville, D. M., Jr. (1970) J. Biol. Chem. 250, 4702–4707
11. Neville, D. M., Jr. (1974) In Vitro 9, 445–454
12. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
13. Goor, R. S., and Maxwell, E. S. (1970) J. Biol. Chem. 245, 616–623
14. Topper, Y. J., Oka, T., and Vonderhaar, B. K. (1975) Methods Enzymol. 39, 443
15. Juergens, W. G., Stockdale, F. E., Topper, Y. J., and Elias, J. J. (1965) Proc. Natl. Acad. Sci. U. S. A. 54, 629–634
16. Li, C. H. (1971) in Symposium on Lactogenic Hormone, Ciba Foundation Symposium (Wolstenholme, G. E. W., and Knight, J., eds) pp. 7–26, Churchill (J. & A.) Ltd., London
17. Kahn, C. R. (1975) in Methods in Membrane Biology (Korn, E. D., ed) Vol. 3, p. 39, Plenum Press, N. Y.
18. Strauss, N., and Hendee, E. D. (1959) J. Exp. Med. 109, 145–163
19. Freychet, P., Roth, J., and Neville, D. M., Jr. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 1833–1835
20. Wild, A. E. (1973) Philos. Trans. R. Soc. Lond. Ser. B. Biol. Sci. 271, 395–410
21. Bonventre, P. F., Saelinger, C. H., Ivins, B., Wosenski, C., and Amorini, M. (1975) Infect. Immun. 11, 675–684
Artificial hybrid protein containing a toxic protein fragment and a cell membrane receptor-binding moiety in a disulfide conjugate. II. Biochemical and biologic properties of diphtheria toxin fragment A-S-S-human placental lactogen.

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J. Biol. Chem. 1977, 252:1515-1522.