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

I. SYNTHESIS OF DIPHTHERIA TOXIN FRAGMENT A-S-S-HUMAN PLACENTAL LACTOGEN WITH METHYL-5-BROMOVALERIMIDATE

(Ta-min Chang and David M. Neville, Jr)

From the Section on Biophysical Chemistry, Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20014

In order to study the mechanism of entry of plant seed and bacterial toxins into mammalian cells, methods have been developed to synthesize artificial protein hybrid conjugates containing a moiety which binds to a cell membrane receptor and an active fragment of a toxin protein. Utilizing methyl-5-bromovalerimidate, a disulfide cross-linked conjugate of human placental lactogen (hPL) and diphtheria toxin fragment A (toxin A) was synthesized. The reagent was prepared from 5-bromovaleryl nitrile by Pinner synthesis and then used to amidinate hPL. The bromo group thus introduced was converted to S-sulfonate by nucleophilic displacement with 1 M aqueous sodium thiosulfate at room temperature overnight. The S-sulfonated hPL reacted readily with the —SH group of reduced toxin A to form a 1 mol/mol of disulfide conjugate in high yield. Thus when reduced toxin A was incubated with a 4-fold excess of the hPL S-sulfonate at 4°C and pH 6.5 for 120 h, a conjugate yield of 50% relative to the toxin A input was obtained. Homopolymer formation was negligible and the product was purified by gel filtration on Sephadex G-150. Purity of the conjugate estimated by quantitative analysis of sodium dodecyl sulfate gels was 90%. The toxin A-hPL conjugate retained the activities of both toxin A and hPL, as reported in the accompanying paper. This method of preparing protein hybrid conjugates appeared to have advantages over previous methods utilizing bifunctional reagents with respect to both yield and freedom from homopolymer formation.

The mechanism of action of the toxins used as starting materials in these studies has been reviewed in detail (1-3). Each toxin consists of two polypeptide chains cross-linked by a disulfide bond. The A chain or active chain catalytically inhibits eukaryotic protein synthesis inside the cell. The B chain binds the toxin molecules to a specific cell surface receptor. A or B chains alone are nontoxic for cells; however, their separate activities can be measured. Purified A chains can catalytically inhibit protein synthesis in cell-free systems. Purified B chains can bind to their specific cell surface receptors. The toxic effect toward intact cells requires combination of the two chains. Recent studies with antitoxin antibodies and with molecules that can compete with the B chain for binding the cell surface receptor have provided evidence indicating that the action of the toxins involves at least three sequential steps: (a) binding of toxin through B chain, (b) entry of the A chain or the whole toxin molecule, and (c) inhibition of protein synthesis by the A chain. The mechanism by which the A chain or the whole toxin enter the cell is as yet unknown. At least three toxins, nicked diphtheria toxin, abrin, and ricin fit the above generalities. Abrin and ricin are similar toxins in that both their B chain and A chain specificities are closely related. The B chains of these toxins bind to galactose-containing residues. The binding specificity for diphtheria toxin has not been reported. The A chains of ricin and abrin inactivate ribosomes apparently by inhibiting the GTPase site on the 60 S ribosomal subunit (5). Diphtheria toxin A chain inactivates elongation factor II by catalyzing the transfer of ADP-ribose from NAD+ to the soluble enzyme (6).

The hybrid conjugates which we have constructed and report on in these papers may be regarded as structural analogues of toxin molecules in which the original binding chain has been substituted by another protein displaying different binding specificities. Our reasons for studying hybrid protein conjugates containing the active chain of these toxins are as follows. First, there is very little information available about the mechanism of entry of protein molecules which exhibit an obligatory first step of binding to specific cell surface receptors. We will refer to this process as receptor-mediated entry of proteins. We wish to know whether all surface membrane receptors participate.
in this phenomenon, or whether receptor-mediated entry is limited to a class of receptors displaying unique properties. Second, there is the question of whether the entry process is only dependent on the properties of the B chain or whether A chain properties are also involved. If entry requires only the B chain, then hybrid molecules with competent B chains will direct entry of any hybrid B chain. Such hybrids would have vast biologic potential. Since various cell types display different surface membrane receptors, it should be possible, by choosing the right B chain, to construct hybrid conjugates which are cell type-specific. Such hybrids would constitute an entirely new class of pharmacologic reagents. An obvious application would be cell type specific cancer chemotherapy. In the following paragraphs we discuss the problems in synthesizing artificial protein hybrid conjugates and the general methods which we have found to be successful.

Artificial protein hybrid conjugates can be produced by simply adding a cross-linking reagent to a mixture of the protein species. Only small amounts of conjugated hybrid are produced in this manner, most of the material consisting of homopolymers of varying sizes (7). For our purposes we believed it was necessary to make hybrid conjugates in such yields that the species could be purified, unequivocally identified by physical and biochemical methods, and the contamination with homopolymers reliably estimated. This required synthesis in the milligram range and suppression of homopolymer formation. Homopolymer formation is not a problem.

The back reaction can be eliminated with a sulfite trap. The natural polymeric form is thus thermodynamically stable than the homodimer, can be isolated. A search was therefore made for a method which would place different reactive groups on each protein species such that the rate of interspecies reaction would be substantially faster than the intraspecies reaction. Swan’s method for the synthesis of asymmetric disulfides fulfilled these needs (10).

\[
\text{RS} + \text{R'SSO}_2^- \rightleftharpoons \text{RSSR}' + \text{SO}_4^{2-} \quad (1)
\]

In the absence of oxygen the initial product is the heteroconjugate. The back reaction can be eliminated with a sulfite trap. As long as base-catalyzed hydrolysis and disulfide exchange can be minimized the heteroconjugate, even though less thermodynamically stable than the homodimer, can be isolated. A further advantage of this reaction is that the product is disulfide-linked, like the original toxins. Since one of our species, the A chain of diphtheria toxin, contained a single --SH group, which could be converted to --SSO$_2^-$ if desired, it became only necessary to introduce a single --SH or --SSO$_2^-$ group into our putative B chain. When putative B chains contained internal disulfide bonds these were left intact in order to minimize disturbance of the configuration of the binding protein which can lead to loss of binding activity. In part this was based on previous work with insulin (11) and prolactin.

Our attempt was then made to synthesize an imidoester S-sulfonate from a haloalkyl nitrile by first converting the halide into S-sulfonate by Bunte synthesis (12) followed by converting the nitrile into imidoester by Pinner’s method (13). This also failed due to a solubility problem encountered in the second step. However, by reversing these two procedures we have successfully developed a method to introduce an extrinsic S-sulfonate group into proteins according to the following reactions.

\[
\text{RS} + \text{CH}_2\text{C}-\text{CH}_2\text{C}-\text{N}(-\text{CN}) + \text{SO}_2^- \rightarrow \text{RSSR}' + \text{SO}_4^{2-} \quad (2)
\]

\[
\text{Protein-NH}_2 + \text{CH}_2\text{O}^- + \text{C(CH}_3)_2\text{-Br} + \text{Protein-NH-C(CH}_3)_2\text{-Br} + \text{CH}_2\text{OH} \quad (3)
\]

\[
\text{Protein-NH-C(CH}_3)_2\text{-Br} + \text{SO}_2^- + \text{Protein-NH-C(CH}_3)_2\text{-SSO}_2^- + \text{Br}^- \quad (4)
\]

\[
\text{NH}_4^+\text{Cl}^- + \text{Br}^- \rightarrow \text{CH}_3\text{CN} + \text{CH}_2\text{OH} + \text{HCl} \rightarrow \text{CH}_3\text{C-OCH}_3 + \text{Br}^- \quad (2)
\]

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\text{Protein-NH}_2 + \text{CH}_2\text{O}^- + \text{C(CH}_3)_2\text{-Br} + \text{Protein-NH-C(CH}_3)_2\text{-Br} + \text{CH}_2\text{OH} \quad (3)
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\]

When naturally occurring proteins with multiple polypeptide chains are reassembled from their subunits in the test tube (8). This is because the heterospecies are held together and preferentially oriented by strong noncovalent forces (8) which direct covalent cross-linking (9). The natural polymeric form is thus kinetically and thermodynamically the preferred species. In contrast, our early attempts to cross-link two polypeptide species which are not naturally associated showed that the preferred species was often the homodimer. A search was therefore made for a method which would place different reactive groups on each protein species such that the rate of interspecies reaction would be substantially faster than the intraspecies reaction. Swan’s method for the synthesis of asymmetric disulfides fulfilled these needs (10).

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\text{RS} + \text{R'SSO}_2^- \rightleftharpoons \text{RSSR}' + \text{SO}_4^{2-} \quad (1)
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The abbreviations used are: BVI, methyl-5-bromovalerimidate; BVA-protein, 5-bromovalerimidated protein (e.g. BVA-hPL); SMVA-protein, 5-S-sulfomercaptovalerimidated protein (SMVA-protein) in Reaction 4 by sodium thiosulfate via the Bunte reaction.

In this communication we wish to present this method of introducing an extrinsic S-sulfonate group into proteins and the ability of the S-sulfonate derivatives of hPL prepared by
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Preparation of Methyl-5-bromovalerimidate Hydrochloride—The compound was prepared by Pinner synthesis (13) according to the method of Rose et al. (16). In a hood, anhydrous methanol (0.154 mol, 6.2 ml) kept dry over molecular sieves was placed in an iced 25-ml three-neck round bottom flask and purged briefly with nitrogen. Dry hydrogen chloride, passed through concentrated sulfuric acid, was then bubbled through until the weight gain of the flask was 4 g (0.4 mol). The five-gram portion of methyl-5-bromovaleryl nitrile (0.154 mol) (caution, irritating on skin or to breathe) was added and quickly mixed. The flask, with greaseless glass stoppers was then transferred into a 4°C precooled desiccator containing calcium chloride desiccant. After 20 min at 4°C, when the reaction mixture became clear, the reaction mixture was transferred into a 4°C precooled desiccator containing calcium chloride desiccant. The reaction mixture was then allowed to dissolve completely. Freshly prepared 3 M sodium hydroxide (0.136 mol). Twenty-five grams of 5-bromovaleryl nitrile (0.154 mol) was added and allowed to dissolve completely. Freshly prepared 3 M sodium carbonate buffer, pH 8.2, and 6 M urea was added to a final concentration of 0.1 M and the solution was allowed to dissolve and stored at -70°C. EF-2 so prepared was freed of dimer when fresh, but gradually turned into dimer during storage, especially in the presence of both urea and oxygen.

Preparation of Peptide Elongation Factor 2—A partially purified EF-2 was prepared at 4°C from rat liver via "pH 5 enzymes" of Moldave (21) as outlined below. Protein in pH 5 enzymes fraction of rat liver extract from three adult animals was precipitated by adding solid ammonium sulfate to 70% saturation and collected by centrifugation. The pellet was redissolved in 20 ml of 20 mM Tris/HC1, 1 mM EDTA, 1 mM dithiothreitol, pH 8.2 (TE buffer) and dialyzed against 50 volumes of the same buffer overnight. The nondialyzable fraction was clarified by centrifugation and applied on a column of DEAE-Sephadex A-50 (5 x 4 cm) equilibrated in TED buffer. The column was first washed with 50 ml of TED buffer and then eluted with 400 ml each of TED buffer containing 0.07 M and 0.12 M NaSO4, respectively. Fractions of 20 ml were collected throughout. Protein concentration was monitored by absorbance at 280 nm and activity of EF-2 by toxin A-specific ADP-ribosyl acceptor activity. EF-2 was then concentrated by ammonium sulfate, with the peak eluted in the 50% saturation region. PE-2 peak fractions were pooled and adjusted to 70% saturation in ammonium sulfate. After cooling on ice for 15 min, the precipitate was collected by centrifugation and redissolved in 8 ml of TED buffer, dialyzed against 1 liter of the same buffer for 24 h with two changes of buffer. It was then centrifuged to remove a small amount of precipitate formed during dialysis and stored at -70°C. EF-2 so prepared was usually 2 to 4 μmol in toxin A-specific ADP-ribosyl acceptor and represented a 10-fold purification with 87% recovery of total acceptor activity over the extract. This preparation was also active in the EF-2 ribosyltransferase (22, 23) and stable for several months when assayed under our experimental conditions.

Amidination of Proteins with Methyl-5-bromovalerimidate—Amidination was usually carried out by adding protein solution of about 80 mg/ml in 20 mM Tris/HC1, 1 mM EDTA at pH 8.0 to a weighed amount of BVI. The reagent was dissolved by quick mixing and the pH of the solution was adjusted to the desired pH within 15 s with a few microliters of 1 N NaOH. The solution was then allowed to stand at room temperature for 30 min. At the end of the reaction, 1 N acetic acid was added to bring the pH of amidinated hPl to 6.5 and the sample was freed of buffer by dialysis (24 h) through a column of Sephadex G-25 medium (0.9 x 2.3 cm) in nitrogen-purged 20 mM Tris/HC1, 1 mM EDTA, 1 mM dithiothreitol, pH 8.2, or dialysis against 50 volumes of the same buffer for large scale preparation of amidinated protein. Reduced and alkylated RNase (RA-RNase) was amidinated in the same way except that the pH was kept at 6.5. The reaction mixture was incubated at room temperature for 4 h for samples less than 20 mg of total protein or overnight for large scale proteins. The reaction mixture was dialyzed against 400 ml each of EDTA buffer, pH 5 enzymes of Moldave (21) as outlined below. Protein in pH 5 enzymes fraction of rat liver extract from three adult animals was precipitated by adding solid ammonium sulfate to 70% saturation and collected by centrifugation. The pellet was redissolved in 20 ml of 20 mM Tris/HC1, 1 mM EDTA, 1 mM dithiothreitol, and 6 M urea at pH 9.5 to 10.0. Dithiothreitol was added to a final concentration of 0.1 M and the solution was allowed to stand at room temperature for 15 min. The reduced protein was freed of dithiothreitol by passing through a column of Sephadex G-25 medium (0.9 x 2.3 cm) in nitrogen-purged 20 mM Tris/HC1, 1 mM EDTA, either in presence or absence of 7 M urea at pH 6.5 and 4°C. The reduced toxin A so prepared was freed of dimer when fresh, but gradually turned into dimer during storage, especially in the presence of both urea and oxygen.

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without losing the ability to form conjugates with toxin A.

**Reaction of Reduced Toxic A-SH with S-Sulfocarboxamidinated Proteins**—Unless otherwise specified freshly reduced, dithiothreitol-free toxin A solution was usually mixed with SMYA-protein solution at the desired molar ratio in 20 mM Tes, 1 mM EDTA, pH 8.2 for 0.5 h or for a final concentration of the S-sulfonate with an aliquot of toxin A solution pretreated with 20 mM N-ethylmaleimide for 15 min at room temperature at the same molar ratio of the two protein reactants and incubated throughout. Whenever desired the reaction was stopped by adding fresh 0.1 M N-ethylmaleimide solution to a final concentration of 20 mM and incubated at room temperature for 15 min.

**SDS-Disc Acrylamide Gel Electrophoresis**—Unless otherwise described, SDS-disc gel electrophoresis was performed at pH 7.2 in gels containing 20% acrylamide and 0.05% diethyl bisacrylamide in System J3561 at 20° for 3 to 5.5 h and stained with heavy Coomassie blue staining solution as described (24). Samples were not reduced except where noted. The calculation of molecular weight of the reaction product was performed by log R, versus M, plots as described (24) using hPL monomer and dimer and toxin A monomer and dimer standards. Since these species have nearly identical monomer masses yet different sizes (see "Results") the calibrating line was chosen equidistant between the lines connecting the hPL monomer and dimer points and the toxin A monomer and dimer points.

**Determination of Toxic A Activity and EPF Concentration**—Concentration of EPF-2 and ADP-ribosyltransferase activity of toxin A were assayed according to the method of Collier and Kandel (19). For toxin A activity each reaction mixture of 0.25 ml contained 125 μmol of Tris/HC1, pH 8.2, 0.25 μmol of EDTA, 10 μmol of di-thiothreitol, 0 to 2.0 mg of toxin A, 200 pmol of EPF-2, and 100 pmol of [adenine-U-14C]NAD (specific activity 40,000 to 80,000 cpm). The reaction was started by addition of [14C]NAD* and stopped after 15 min of incubation at room temperature by addition of 0.25 ml of 16% trichloroacetic acid. The acid precipitate was then collected on a Whatman GF/C glass fiber filter, washed three times with 3 ml of 20% trichloroacetic acid, three times with 1 ml of 2-propanol/ether/chloroform, 2/2/1 (v/v). The dried filter was placed in a counting vial, digested in 1 ml of Nuclear Chicago solubilizer-contain ing internal disulfide linkage was first tested with reduced and alkylated ribonuclease (RA-RNase) by reacting the protein with 5-bromovalerimidate, followed by substitution of bromine with thiosulfate.

In the first column in Table I we observe the fall in RA-Nase-free amino groups from 11 to 5 mol/mol after reaction with the bromoalkyl imidate, indicating that extensive amidination has occurred. In the third column —SSO3− groups appear after incubation with thiosulfate. The sum of the remaining free amino groups plus the new —SSO3− groups is equal to the original free amino group content (last column).

**Amidination of Protein with Methyl-5-bromovalerimidate and Conversion of Product into 5-S-Sulfocarboxamidinated Protein**—The possibility of successful introduction of an extrinsic S-sulfonate group into a protein without disturbing internal disulfide linkage was first tested with reduced and alkylated ribonuclease (RA-RNase) by reacting the protein with 5-bromovalerimidate, followed by substitution of bromine with thiosulfate.

The extent of amidination of reduced and alkylated RNase by methyl 5-bromovalerimidate is followed by assaying the N,PhSO3− reactive amino groups (left-hand column). After incubation with sodium thiosulfate for the indicated times the sulfonate content is measured. To assess the extent of conversion of the introduced alkyl bromide to alkyl thiosulfate the sum of the reactive amino groups plus S-sulfonate groups is given in the last column.

**Results**

| Time with thiosulfate | Total —NH2 + —SSO3− | —SSO3− |
|-----------------------|---------------------|--------|
| RA-Nase | 10.9 | 0 | <0.04 |
| treated | 11.0 | 20 | <0.04 |
| RA-RNase | 5.1 | 2 | 0.8* |
| treated | 5.4 | 2 | 5.4 |
| BVI-treated | 4.8 | 2 | 5.7 |
| 4.9 | 20 | 5.7 |

* This number probably is a result of weak reaction of the alkyl bromide group with Nbs, since BVI-RA-RNase exhibited 0.3 —SH group/mol before treatment with dithiothreitol whereas untreated RA-RNase had an undetectable —SH content.
These data indicate that essentially complete substitution of —Br by —SSO₂⁻ has occurred after 2 h of incubation with thiosulfate.

These results show that the 5-bromovalerimidate is a useful reagent for amidination of proteins and the alkyl bromide groups thus introduced are readily converted by thiosulfate to S-sulfonate groups.

Introduction of S-Sulfonate Groups into Human Placental Lactogen Utilizing 5-Bromovalerimidate and Thiosulfate Substitution—Human placental lactogen was reacted with BVI at various molar ratios and then treated with thiosulfate. The extent of amidination was then determined by assaying the loss of N₂PhSO₂⁻-reactive amino groups (see Table II) which ranged between 0.8 and 4.0 mol of —NH₂/mol of protein. On the basis of Table I, the extent of amidination of the thiosulfate-treated protein is assumed to be equal to the extent of S-sulfonate incorporation.

The contrast between the high ratios of BVI to protein used and the low degrees of amidination obtained indicates that imidate hydrolysis is a major competing reaction. For this reason we have kept protein concentration at high values. It is likely, however, that multiple additions of BVI could serve just as well in cases where protein solubility is a limiting factor.

Reaction of Toxin A-SH with S-Sulfonated Human Placental Lactogen—The ability of SMVA-hPL to react with reduced toxin A to form disulfide conjugate was tested by incubation of toxin A and SMVA-hPL together at pH 6.5 and 4° as described under "Experimental Procedures."

In Fig. 1, Gel a is a reaction mixture inactivated with N-ethylmaleimide at zero time. Major bands (numbered at right) from the top down are: Band 3, hPL dimer; Band 4, toxin A; Band 5, SMVA-hPL. Table III lists the molecular weights of these species obtained from sequence data and their Rf values found on SDS gels. Although toxin A and hPL (or SMVA-hPL) have nearly identical masses, hPL and its dimer exhibit higher Rf values than toxin A and its dimer. Since SDS-gel electrophoresis separates primarily by size rather than mass (32) a more compact structure is indicated for hPL and its dimer compared to toxin A and toxin A dimer. When hPL or

| Table II |
|----------------------------------|
| Amidination of human placental lactogen by methyl-5-bromovalerimidate |

| Molar ratio (BVI)/protein | Free —NH₂ per mol | —NH₂ reacted per mol |
|---------------------------|-------------------|----------------------|
| 0                         | 10.0              | 0                    |
| 2.5                       | 9.2               | 0.8                  |
| 5.0                       | 8.2               | 1.8                  |
| 15.0                      | 6.0               | 4.0                  |
| 15.0*                     | 9.0               | 1.0                  |

Fig. 1 (left). Reaction of reduced diphtheria toxin fragment A with S-sulfomerocaptovaleramidinated derivatives of human placental lactogen. SDS-acrylamide gels of reaction mixture of toxin A-SH (0.57 mg/ml) with SMVA-hPL (5.33 mg/ml, 0.7 —SSO₂⁻/hPL = 0.7 mol/mol) at 4° for 48 h. The amount of protein analyzed in each gel was proportional to the initial reactants: a, 2.85 μg of N-ethylmaleimide-treated toxin A, 15.3 μg of SMVA-hPL; b, 2.85 μg of toxin A-SH, 26.7 μg of SMVA-hPL; c, toxin A plus dimer, 2.5 μg; d, hPL plus dimer, 3.5 μg. A slight distortion observed in the lower bands of Gel I was due to the effect of air bubbles between the gel and glass tube. Band numbering key is given in Table III.

Fig. 2 (right). Comparison of reaction mixtures of reduced diphtheria toxin fragment A with human placental lactogen and its derivatives. Toxin A-SH (0.44 mg/ml) and hPL or its derivatives (1.6 mg/ml) were incubated at room temperature for 24 h in the presence of 5 M urea and 100 mM strontium chloride. An aliquot of N-ethylmaleimide-stopped reaction mixture containing an amount of protein proportional to the initial toxin A-SH, 14.4 μg, and hPL, 16 μg, or its derivatives was analyzed on SDS gels. The gels presented are of the reaction mixtures of toxin A-SH with a, native hPL; b, BVA-hPL (the precursor of SMVA-hPL in c); c, SMVA-hPL (0.7 mol/mol of S-sulfonate). Gel d is a control reaction mixture of c with N-ethylmaleimide-treated toxin A.
SMVA-hPL is fully reduced its $R_f$ becomes identical with that of toxin A, indicating that the more compact structure of the native hPL is due to its internal disulfide bonds (30). Toxin A lacks internal disulfide bonds (20) and would be expected to have a more extended structure.

Gel b in Fig. 1 shows a reaction mixture incubated for 48 h before N-ethylmaleimide treatment. The new band, Band 2, has an $R_f$ in between that of toxin A dimer and hPL dimer, and has a calculated $M_r = 43,000$ as compared to the expected value of 43,372 for a toxin A-hPL conjugate. The individual reacting species are shown in Gels c and d. c contains toxin A monomer (Band 1) and toxin A monomer (Band 4) and d contains hPL monomer (Band 3) and hPL monomer (Band 5).

In these studies different preparations of SMVA-hPL contained varying amounts of dimer. This accounts for the varying amounts of the dimer seen in the zero time reaction mixtures. When the reaction was performed with starting materials of low dimer content, a small amount of the dimer was found to be generated.

In Gel a of Fig. 1 a faint band of $R_f = 0.342 \pm 0.002$ is noted. This is present in the SMVA-hPL and is listed as an unknown species in Table III. The $R_f$ of this band is clearly distinguishable from that of the toxin A-hPL conjugate ($R_f = 0.335 \pm 0.003$).

The prelimin ary identification of the reaction product in Fig. 1, Gel b, as toxin A-hPL rests on exclusion that the new band is neither hPL dimer nor toxin A dimer, yet has the expected $R_f$ value for a conjugate composed of a compact subunit the size of hPL and an extended subunit the size of toxin A.

The ability to react with toxin A-SH to form a disulfide conjugate appears to be a unique property of SMVA-hPL, as is shown by the gels shown in Fig. 2. Thus, when native hPL, BVA-hPL, and SMVA-hPL were independently incubated with toxin A-SH under the same conditions, only SMVA-hPL could react to form the conjugate (Fig. 2, Gel c). Both native hPL (Gel a) and BVA-hPL (Gel b) could only give rise to toxin A dimer as well as a small amount of unknown species, $R_f = 0.342$, which was also formed in the control reaction mixture of N-ethylmaleimide-treated toxin A with SMVA-hPL (Gel d). A similar negative result was also observed with thiosulfate-treated native hPL (not shown).

The reaction of toxin A-SH and SMVA-hPL was also carried out at 25°C. The yield of conjugate was somewhat less at this temperature while the yield of toxin A dimer was slightly greater compared to 4°C.

**Isolation and Characterization of Toxin A-hPL Conjugate**

Isolation of toxin A-hPL conjugate from the reaction mixture was achieved by passing the reaction mixture, without prior treatment with N-ethylmaleimide, through a Sephadex G-150, superfine column. As a reaction mixture was carried under "Experimental Procedures." The arrowed numbers represent the main band of each peak fraction observed in SDS gels which are in correspondence with the assigned bands shown in Fig. 1 and Table III. B, reaction mixtures of 13.2 mg of toxin A-SH with 24.8 mg of SMVA-hPL (−$\text{SSO}_2$/hPL = 1.8 mol/mol), dimers freed) in a final volume of 1.7 ml after 48 h of incubation under the same conditions as in A, but ADP-ribosyltransferase activity was not assayed. Shaded areas were pooled fractions for purified toxin A-hPL conjugates. Gels of reaction mixtures and pooled fractions are shown in Fig. 4.

**Table III**

| Band | Toxin A dimer | $R_f$ | $M_r$ found | $M_r$ expected |
|------|--------------|------|-------------|---------------|
| 1    | 0.304 ± 0.003 (18) | 42,000 | 42,200 |
| 2    | 0.335 ± 0.003 (15) | 43,372 | 43,000 |
| 3    | 0.379 ± 0.004 (22) | 44,454 |
| 4    | 0.579 ± 0.004 (22) | 21,145 |
| 5    | 0.629 ± 0.006 (22) | 22,227 |
| Unknown species | 0.342 ± 0.002 (12) | 42,000 |

* Figures in parentheses in $R_f$ column give number of gels used to calculate the mean deviation from the mean.
* The S-sulfonated derivatives of hPL and hPL dimer have the same $R_f$ values as the native species. Both forms have been used to calculate $R_f$. **
Synthesis of Diphtheria Toxin A-S-S-Human Placental Lactogen

Fig. 4 (left). A, SDS-acrylamide gels of the reaction mixture (a) and purified conjugate from pooled fractions (b) of the chromatogram in Fig. 3A. B, similar gels from reaction mixture (a) and purified toxin A-hPL conjugate (b) from chromatographic separation shown in Fig. 3B.

Fig. 5 (center). Reduction of toxin A-hPL conjugate into subunits by dithiothreitol. Toxin A-hPL (14.8 μg) from Fig. 3B, mixture of toxin A (21.0 μg) with SMVA-hPL (18.0 μg) or SMVA-hPL (18.0 μg) was incubated with or without 4 mM dithiothreitol in 20 mM Tris, 1 mM EDTA, pH 7.4, at room temperature for 15 min. N-Ethylmaleimide was then added to a final concentration of 10 mM. An aliquot from each reaction mixture corresponding to 7.4 μg of toxin A-hPL, or 7 μg of toxin A and 6 μg of SMVA-hPL, was analyzed in SDS gels. SMVA-hPL used was the one from which the conjugate was prepared. a, toxin A-hPL alone; b, toxin A-hPL + dithiothreitol; c, toxin A plus SMVA-hPL; d, same as c but plus dithiothreitol; e, SMVA-hPL + dithiothreitol.

Fig. 6 (right). SDS-acrylamide gels of reaction mixtures containing various initial molar ratios of toxin A-SH and SMVA-hPL incubated for 24 h at 4°C under the same conditions as given in Fig. 2. In Gels 1 to 4 toxin A-SH is constant at 0.53 mg/ml and the molar ratio of SMVA-hPL to toxin A is increased as follows: Gel 1, 1/1; Gel 2, 2/1; Gel 3, 4/1; Gel 4, 6/1; in Gels 5 to 8, SMVA-hPL is constant at 0.59 mg/ml and the ratio of toxin A to SMVA-hPL is increased as follows: Gel 5, 2/1; Gel 6, 4/1; Gel 7, 7/1. Gel 8 is a zero time control of Gel 4. The amount of protein electrophoresed is proportional for all reaction mixtures and consisted of 2.93 μg of toxin A-SH equivalents in Gel 1.

in Peak 2, rather than spilling over from the neighboring toxin A monomer and dimer peaks. In the accompanying paper we showed that Peak 2 also displays binding activity toward lactogenic receptors in radioreceptor assays (14).

Reduction of the isolated toxin A-hPL conjugate with a high dithiothreitol concentration gave a single band on SDS gels of \( R_f = 0.58 \), a value coinciding with the \( R_f \) of reduced toxin A and fully reduced hPL. When the conjugate was partially reduced under mild conditions, Fig. 5, Gel b, separation of approximately equal amounts of two subunits was observed. The hPL band in this case had a \( R_f \) value in between the value for native hPL and fully reduced hPL. An identical \( R_f \) was observed for SMVA-hPL partially reduced under identical conditions. The three different \( R_f \) values observed for hPL under varying reducing conditions probably represent reduction of 2, 1, and none of the two disulfide bonds of native hPL (30). Fig. 5 indicates that isolated toxin A-hPL contains approximately equal amounts of toxin A and hPL linked by a disulfide bond.

Estimation of Purity of Isolated Toxin A-hPL Conjugate—The conjugate isolated in Fig. 3B and run in Gel b, Fig. 4B, displays one single narrow band. However, at high loads discernable shoulders in the region of toxin A dimer and hPL dimer could be detected. No monomer contaminants could be detected. Table IV shows the estimated hPL dimer and toxin A dimer contamination determined by integrating scans in these dimer area and taking the ratio of these areas above the base- line to the total peak area above the base-line. The gels scanned contained between 2 and 9 μg of applied conjugate, and conjugate, hPL, and toxin A areas were linear with protein mass over this range. With the conjugate the linear range was determined as low as 0.7 μg/gel. In some experiments hPL gave one-third more area per mass on gel scan than toxin A, in others there was no difference. No correction has been made because we find differences of one-third between gel to gel just within the range of error of this method. The contamination of our isolated toxin A-hPL conjugate, Fig. 4, Gel b, appears to be about 5% with hPL dimer and 5% with toxin A dimer on a weight basis.

Effect of Different Molar Ratio of S-Sulfomercaptovaleramidated hPL to Reduced Toxin A on Yield of Toxin A-hPL Conjugate—In order to arrive at an optimum condition for preparing toxin A-hPL conjugate in high yield, the effects of varying the molar ratio of SMVA-hPL to toxin A-SH on the yield of the conjugate was studied. The experiments were carried out by incubating a number of reaction mixtures containing a fixed amount of toxin A-SH with varied amount of SMVA-hPL, or vice versa, in the same total final volume. After incubation an aliquot of the reaction mixture was stopped with N-ethylmaleimide and analyzed by SDS-gel electrophoresis. Typical gel patterns from these experiments are shown in Fig. 6. These data were also treated quantitatively by scanning the gels and integrating the area under each peak. The amount of toxin A-hPL conjugate formed in reaction mixtures of three different preparations of SMVA-hPL (with 0.7, 1.8, and 4.0 S-sulfonate per mol) after 24 h (A) and 120 h (B) of incubation are compared in Fig. 7. Thus, when other conditions were kept the same, the yield of toxin A-hPL after 24 h of incubation was always higher when a fixed amount of one reactant was incubated with excess amount of the other (Figs. 6 and 7A). However, when the results of the same set of
The per cent contamination of toxin A-hPL conjugate with toxin A dimer and the hPL dimer is shown in the last two columns for five different scanned acrylamide SDS gels of varying loads. This conjugate was isolated in Fig. 3B and is shown in Gel b of Fig. 4.

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**Table IV**

| Toxin A-hPL on gel | Total area above baseline | Area under toxin A-hPL peak | Per cent toxin A dimer | Per cent hPL dimer |
|------------------|---------------------------|-----------------------------|-----------------------|--------------------|
| µg²            | cm²                      |                                 |                      |                    |
| 2.3            | 3.63                     | 3.33                         | 5.0                   | 2.0                |
| 4.6            | 7.84                     | 7.46                         | 4.6                   | 0.51               |
| 5.6            | 10.2                     | 9.1                          | 5.4                   | 5.1                |
| 6.0            | 10.3                     | 9.2                          | 5.8                   | 5.3                |
| 9.2            | 14.7                     | 12.9                         | 5.2                   | 7.1                |

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The purity of toxin A-hPL conjugate from Fig. 3B was found in the reaction mixture corresponded to an overall yield of 45%. When the same amount of toxin A-SH was incubated with excess SMVA-hPL preparations with higher S-sulfonate content, the yield of the conjugate became further increased (Fig. 7B, Curves 3 and 4).

The overall yield of the conjugate with respect to toxin A was found to be 58 and 53%, respectively, for SMVA-hPL preparations with 1.8 and 4.0 mol of S-sulfonate/mol. Moreover, the rate of reaction of toxin A-SH with these two SMVA-hPL preparations also appeared to be greater than the one containing only 0.7 mol of S-sulfonate/mol (Fig. 7A). These results clearly suggest that for preparation of toxin A-hPL conjugate in high yield, the reaction should be carried out by reacting a fixed amount of toxin A-SH with excess SMVA-hPL containing about 2 mol of S-sulfonate/mol.

When the initial concentration of both reactants was increased 5-fold, the yield of conjugate relative to toxin A input was not significantly altered. Thus with toxin A-SH at 0.63 mg/ml and SMVA-hPL at 5.7 mg/ml the conjugate concentration after 48 h was 0.34 mg/ml, compared to 1.99 mg/ml, when the concentration was increased 5-fold.

**Effect of Urea and Strontium Chloride Formation of Toxin A-hPL Conjugate**—Strontium chloride was included in most reaction mixtures to function as a sulfite trap. Urea was also added to keep protein S-sulfonate in solution in the presence of strontium. These variables were studied. Both urea and strontium ion increased the yield by one-third and also appeared to accelerate the reaction rate. No effect of strontium ion was observed on gel patterns. This was in contrast to earlier studies where the reaction between prolactin S-sulfonate (prepared by sulfation of prolactin) and toxin A-SH was studied. In this study done at a 10-fold higher concentration of reactants, strontium ion was found to be necessary to prevent disproportioning of the prolactin-toxin A product to the homodimers.

**Time Course of Formation of Toxin A-hPL Conjugate**—The time course obtained from scanned gels is shown in Fig. 8. Utilizing 5.7 mg/ml of SMVA-hPL and 0.04 µg/ml of toxin A-SH as initial concentrations, the half-time of product formation was about 30 min, while 95% completion was achieved at 6 h. The yield of toxin A-hPL in this reaction was approximately 55% relative to the initial toxin A input.
D I S C U S S I O N

The data presented in the present report clearly indicate that amidination with methyl S-bromovalerimidate followed by nucleophilic substitution of bromine from BVA-protein with thiosulfate provides a convenient route for introducing an extrinsic S-sulfonate group into proteins. The product, S-sulfonercaptovaleramidino-protein, in turn reacts readily with a sulphydryl-bearing protein, such as reduced diphtheria toxin fragment A (toxin A) to form a cross-linked conjugate in high yield. Dimer and higher polymer formation is negligible. All of these reactions involve mild treatment that is likely to conserve the biologic activity of the proteins cross-linked. Thus amidination of proteins at pH 8.0 involves a mild reaction on primary amino groups with conservation of the positive charge. Retention of biologic activity is usually observed (7, 35, 36). In fact we have observed retention of biologic activity in BVI-treated hPL (14) with an average number of two amino groups substituted per mol. The subsequent nucleophile substitution of bromine with thiosulfate appears to be rapid and complete (Table I), as would be expected from the high carbon nucleophile constant, 6.36, of thiosulfate (37). The S-sulfonated hPL so prepared also retained its biologic activity when assayed in a radioreceptor-binding assay using rabbit mammary gland cell membranes (14).

Some aspects of our reaction of toxin A-SH with S-sulfonated hPL merit discussion. The reaction is actually a reverse of sulfitolysis of a disulfide, as shown by Reaction 1. The $K_{eq}$ of this reversible reaction generally lies considerably to the right in favor of formation of the disulfide. For example, the equilibrium constant for fully ionized cystine is 28 (38). The equilibrium constant for our reaction of toxin A-SH and hPL S-sulfonate is difficult to assess because of the lack of knowledge of the final SO$_3^-$ concentration and the probability of heterogeneity of the reactants. When equimolar mixtures of toxin A-SH and hPL S-sulfonate were reacted at 0.027 mM, 15 to 20% of the toxin A was found in the toxin A-hPL conjugate (Fig. 7). This figure could be increased to 53% by increasing the hPL S-sulfonate/toxin A ratio. However, little increase of product beyond a 4:1 reactant ratio was achieved, indicating that all of the toxin A might not be active. Since 15% of the toxin A was utilized in dimer formation, 30% appears relatively unreactive. Similar calculations from Fig. 7 with excess toxin A indicate that only 24% of the hPL S-sulfonate with 0.7 mol of S-sulfonate/mol is reactive. Because all of the hPL amino groups are potential sites for S-sulfonation introduction, it is likely that steric factors and local electrostatic factors vary the reactivity of the introduced S-sulfonate groups. In the case of toxin A, variable reactivity could result from the charge heterogeneity at the COOH terminus near the reactive cysteine residue. This heterogeneity results from the fact that the toxin A fragment is generated by random tryptic cleavage in a region containing a cluster of 3 arginine residues (2).

We have found no evidence that the toxin A-hPL product disproportions to the symmetric disulfides, as might be expected if a significant reverse attack by sulfite occurred. In Fig. 8 the product appears stable between 24 and 48 h. Although the reaction generates about 15% of toxin A dimer relative to toxin A monomer input, this dimer formation does not appear to result from disproportionalizing. The time course fails to show the initial lag characteristic of disproportional reactions (Fig. 8). Formation of toxin A dimer is independent of the presence of S-sulfonate groups, since toxin A dimer is produced equally well or better in reaction mixtures with native hPL or BVA-hPL (Fig. 2). When hPL or its derivatives are omitted from reaction mixtures, toxin A dimer formation falls to much lower levels. These facts indicate that the oxidizing power for toxin A dimer formation is associated with the hPL preparation. The source of this oxidizing power is unknown but could reside in the internal disulfide bonds of hPL and its derivatives.

Our failure to detect a significant reverse attack by sulfite in the absence of an added SO$_3^-$ trap (Table IV) could be explained in several ways. The backward reaction constant could be quite small. For symmetric disulfides this constant varies 1000-fold depending on the local electrostatic environment (38). The backward reaction could exist and be undetectable if it was asymmetric in character, preferentially generating hPL S-sulfonate rather than a mixture of hPL-SH and hPL-S-sulfonate. Such asymmetric nucleophilic attacks are known to occur (40, 41). A third possibility is that our reaction as performed includes a sulfite trap. It is unlikely that at pH 6.5 sulfite is lost as SO$_2$, during the evacuation of air from the reaction vessel. However, it is possible that the internal disulfide bonds in hPL S-sulfonate are more reactive towards sulfite than the S-S bond bridging the toxin A-hPL conjugate. Although the correct possibility is unknown it should be pointed out that we have observed the disproportional reaction with another conjugate, prolactin-S-S-toxin A, and this disproportionalizing is effectively reduced in the presence of 100 mM SrCl$_2$, presumably by virtue of the low solubility product of SrSO$_3$ (4 x 10$^{-7}$) calculated from its solubility (42) which leads to a low sulfite concentration (4 x 10$^{-7}$ M, calculated). The prolactin conjugate was generated by the reaction of prolactin S-sulfonate with toxin A-SH. However, the S-sulfonate groups were not extrinsic, but rather were formed from the internal disulfides by cyclic sulfitolysis and oxidation.

A number of attempts to make cross-linked protein conjugates either as probes for protein-membrane interactions (43-46) or as agents for selective killing of neoplastic cells (47-52), but as yet none have been recently reported. All these methods involved the mixing of a bifunctional reagent such as gluteraldehyde (43-46, 50, 51), toluene diisocyanate (49, 50), or p,p'-difluorom-m,m'-dinitrophenyl sulfone (48) with the proteins to be cross-linked. Owing to relatively nonspecific reaction of these reagents with amino groups in the proteins, both intramolecular and intermolecular cross-linkages would be formed and among intermolecularly cross-linked conjugates both homo- and heteropolymers would be produced. Purification of an equimolar conjugate, for example ferritin-insulin (46), resulted in very low overall yield (0.02%).

The method of preparing protein conjugates presented in this paper appears superior to methods utilizing bifunctional reagents, since intramolecular cross-linking is prevented and formation of homopolymers is limited. This results leads to products of high quality that are diisocyanate-hPL conjugates and among intermolecularly cross-linked conjugates both homo- and heteropolymers would be produced. Purification of an equimolar conjugate, for example ferritin-insulin (46), resulted in very low overall yield (0.02%).

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REFERENCES

1. Pappenheimer, A. M., Jr., and Gill, D. M. (1973) Science 182, 353-358
2. Collier, J. R. (1975) Bacteriol. Rev. 39, 54-85
3. Olsnes, S., Olsnes, S., and Pihl, A. (1974) J. Biol. Chem. 249, 3557-3562
4. Olsnes, S., and Pihl, A. (1973) Biochemistry 12, 3121-3126
5. Benson, S., Olsnes, S., Pihl, A., Skorve, J., and Abraham, K. (1970) Eur. J. Biochem. 8, 513-519
6. Honjo, T., Nishizuka, Y., Kato, I., and Hayaishi, 0. (1971) J. Biol. Chem. 246, 4251-4260
7. Dutton, A., Adams, M., and Singer, S. J. (1966) Biochemistry 6, 2439-2488
8. Pappenheimer, A. M., Uchida, T., and Harper, A. A. (1972) Immunochemistry 9, 891-908
9. Collier, R. J., and Kandel, R. J. (1971) J. Biol. Chem. 246, 1496-1500
10. Drazin, R., Kandel, J., and Collier, R. J. (1971) J. Biol. Chem. 246, 1504-1510
11. Drazin, R., Kandel, J., and Collier, R. J. (1971) J. Biol. Chem. 246, 1504-1510
12. Moldave, K. (1963) Methods Enzymol. 6, 757-761
13. Nakazawa, K., Ueda, K., Horio, T., Yoshihara, K., Nishizuka, Y., and Hayashi, 0. (1967) Biochem. Biophys. Res. Commun. 33, 143-149
14. Gill, D. M. (1972) J. Biol. Chem. 247, 5984-5971
15. Neville, D. M., Jr., and Glossmann, H. (1974) Methods Enzymol. 32B, 92-102
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
17. Michel, A., and Dirks, J. (1974) Biochim. Biophys. Acta 365, 15-27
18. Li, C. H. (1971) in Symposium on Lactogenic Hormones, Ciba Foundation Symposium (Wolstenholne, G. E. W., and Knight, J., eds) pp. 7-26, Churchill (J. & A.) Ltd., London
19. Haynes, K., Osuga, D. T., and Feeney, R. (1967) Biochemistry 6, 541-547
20. Janataova, J., Fuller, J. K., and Hunter, M. J. (1968) J. Biol. Chem. 243, 3612-3622
21. Niall, H. D., Hogan, M. L., Tregear, G. W., Segre, G. V., Huang, P., and Friessen, H. (1973) in Recent Progress in Hormone Research (R. O. Greep, ed) Vol. 29, pp. 387-404, Academic Press, New York
22. Neville, D. M., Jr. (1971) J. Biol. Chem. 246, 6328-6334
23. Tanford, C. (1963) in Physical Chemistry of Macromolecules, pp. 622-639, John Wiley and Sons, N. Y.
24. Reynolds, J. A., and Tanford, C. (1970) J. Biol. Chem. 245, 5161-5165
25. Hartman, F. C., and Wold, F. (1967) Biochemistry 6, 2439-2488
26. Zolock, D. T., and Niehaus, W. G., Jr. (1975) J. Biol. Chem. 250, 3171-3180
27. Swain, C. G., and Scott, C. B. (1953) J. Biol. Chem. 201, 4-17
28. Cecil, R., and McPhee, J. R. (1955) Biochem. J. 60, 496-500
29. McPhee, J. R. (1961) Biochim. Biophys. Acta 365, 143-149
30. Traut, R. R., Bollin, A., Sun, T. T., Hershey, J. W. B., Sundberg, J., and Price, L. K. (1973) Biochemistry 12, 3266-3273
31. Anfinsen, C. B., and Haber, E. (1961) J. Biol. Chem. 236, 1361-1365
32. Neville, D. M., Jr. (1971) J. Biol. Chem. 246, 6328-6334
33. Tanford, C. (1963) in Physical Chemistry of Macromolecules, pp. 625-639, John Wiley and Sons, N. Y.
34. Reynolds, J. A., and Tanford, C. (1970) J. Biol. Chem. 245, 5161-5165
35. Hodgkin, C. D., ed (1959-1968) Handbook of Chemistry and Physics, pp. 664-665, Chemical Rubber Publishing Co., Cleveland, O.
36. Nicolson, G. L., and Singer, S. J. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 942-945
37. Nicolson, G. L., and Singer, S. J. (1974) J. Cell Biol. 60, 225-29
38. Jarett, L., and Smith, R. M. (1974) J. Biol. Chem. 249, 7024-7031
39. Moolten, F. L., and Cooperband, S. R. (1970) Science 169, 68-70
40. Swanepoel, R., and Swanepoel, O. A. (1967) Arch. Biochem. Biophys. 121, 729-731
41. Parker, A. J., and Kharasch, N. (1960) J. Am. Chem. Soc. 82, 3071-3075
42. Hodgkin, C. D., ed (1959-1968) Handbook of Chemistry and Physics, pp. 664-665, Chemical Rubber Publishing Co., Cleveland, O.
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T M Chang and D M Neville, Jr

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